Journal of Industrial Microbiology (1996) 16, 29–35
© 1996 Society for Industrial Microbiology 0169-4146/96/$12.00

Internal substrate concentrations during biotransformation of octanoic acid into 2-heptanone by spores of *Penicillium roquefortii*

C Larroche, I Besson and J-B Gros

Laboratoire de Génie Chimique Biologique, Université Blaise Pascal, F-63177 Aubière Cedex, France

Internal substrate concentrations were monitored during biotransformation of octanoic acid into 2-heptanone by spores of *Penicillium roquefortii*. The fatty acid rapidly enters the spores in its undissociated form, and a Collander-type relation shows that it strongly accumulates in the spore wall and membrane; this accumulation is reversible. The reaction takes place with cytoplasmic substrate concentrations that quickly fall to zero, and the process is limited by octanoic acid penetration into the cells. This entry is accompanied by proton efflux and involves an active transport process with a H^+-ATPase system that exhibits Michaelian behavior. The driving force is postulated to be ΔpH, which takes a value set by the initial substrate concentration through the stoichiometry of the H^+/octanoic acid exchange.

Keywords: spores; fatty acid; internal concentrations; transport; biotransformation; *Penicillium roquefortii*

Introduction

Biotransformation is a useful tool for the production of various chemical compounds, and represents an area of biotechnology in which advances in basic biochemistry, microbiology, chemistry and biochemical engineering are rapidly being commercialized successfully [4]. Its most promising domain is the production of flavors and fragrances as it affords 'naturally' derived compounds [37].

Fungal spores can be used as biocatalysts since they are known generally to exhibit high catalytic activity in terms of dry matter [36]. However, they are less widely used than other forms of microorganisms, probably due to the need for unusual cultivation conditions, ie solid substrate fermentation, for their recovery in large amounts [26].

The spores of *Penicillium roquefortii* are known to convert fatty acids into methyl ketones [10,23]. These odd-numbered compounds are primarily recognized for their contribution to the flavor of blue-veined cheeses, and are generated during cheese ripening from lipid metabolism by the filamentous fungus *P. roquefortii*. They may be used in the manufacture of salad dressings, soups, crackers and cakes [14]. The need to produce 'natural' C5 to C11 2-alkanones has led to processes using fatty acid breakdown by spores of this filamentous fungus [6,11,12,27].

In previous papers, we reported results obtained for the synthesis of methyl ketones from fatty acids by spores of *P. roquefortii* in an aqueous synthetic medium by batch [17,22], continuous [20] and fed-batch [7,19] processes involving free or immobilized conidia.

Results obtained with this last system suggested that the reaction rates did not depend on the residual substrate concentration in the medium. These data led us to consider that the reaction could be limited either by internal substrate concentration or by its penetration rate in the spores, or both. In this paper, we report results achieved during the monitoring of the internal concentration of octanoic acid during batch biotransformations.

Correspondence: C Larroche, Laboratoire de Génie Chimique Biologique, Université Blaise Pascal, F-63177 Aubière Cedex, France
Received 18 April 1995; accepted 26 September 1995

Materials and methods

Microorganism

The strain of *Penicillium roquefortii* used was isolated from French blue cheese and retained after a strain screening [22]. It was deposited with ATCC under the accession number 64383, and is conserved by periodic transfers on Czapek agar.

Spore production, storage and recovery

Spores were produced on buckwheat seeds [21] in 1-L bottles containing a layer of cotton wool at the bottom to prevent free water accumulation in the medium, and fitted with polyurethane foam plugs (Caubère, Yèbles, France). The medium harvested after cultivation was stored at −20°C for subsequent use without any treatment.

This spore-supporting material was thawed for 2 h and poured into a sterile 0.05% (v/v) Tween 80 solution. The suspension was then gently stirred for 1 h, and filtered through gauze. The spore suspension was recovered by allowing the filtrate to remain for 12 h at +4°C, and the pellets obtained after centrifugation ($2600 \times g$, 10 min) were added to the bioconversion medium. The final biocatalyst content was determined using a hematocyte counter (Malassez cell).

Biotransformation medium

The medium contained 0.2 ml L^{-1} ethanol [22] and 350 mg L^{-1} chloramphenicol to prevent bacterial contamination [23]. It was either potassium- or sodium phosphate (0.1 mol L^{-1})-buffered (pH = 5.5 or 6.5) or made up with

distilled water. With the latter, pH was continuously adjusted to the desired value with a 1 mol L^{-1} sodium of potassium hydroxide or solution. The substrate was sodium octanoate for buffered media and free octanoic acid (Unichema International, Gouda, The Netherlands) for the others; it was added after inoculation with spores at levels ranging from 1.5 to 6×10^8 spores ml^{-1}.

Experimental design

Reactions were carried out at 27°C either in conical flasks or in a fermentor. Conical 250-ml flasks were filled with 150 ml buffered medium, plugged with polyurethane foam (see above) and placed on a rotating table operating at 200 rev min^{-1}.

A stirred reactor of 750 ml working volume (New Brunswick Scientific, Edison, NY, USA; Bioflo C30) was also used with non-buffered media. The stirring speed was 300 rev min^{-1}, and the aeration (0.06 vvm) was started 15–30 min after substrate addition.

Assay methods

Both internal and external substrate concentrations of octanoic acid were determined. Each sample (10 ml) was poured into a 15-ml Corex tube and centrifuged ($5000 \times g$, 5 min) at 10°C. The supernatant phase was recovered and 4 ml of it were added to 1 ml of a 4 mol L^{-1} HCl solution. After vortexing the tube for 15 s, 5 ml 1,2-dichloroethane was added and the resulting suspension was vortexed again for 1 min. The organic layer was recovered as the bottom phase for assay of octanoic acid in the extracellular medium.

A solution containing 0.8 mol L^{-1} HCl (4 ml) was added to the pellet, followed by 5 ml 1,2-dichloroethane after stirring for 1 min. The resulting suspension was further mixed for 1.5 min and stored at −20°C overnight. The solution was agitated again for 1.5 min after it had thawed and the organic layer was recovered after two successive centrifugations ($5000 \times g$, 5 min and $3000 \times g$, 5 min). Assay of this organic solution provided octanoic acid content in the internal medium.

Octanoic acid was derivatized to its methyl ester by a boron trifluoride-methanol complex [1] and injected into a gas chromatograph fitted with a Supelcowax (Supelco Inc, Bellefonte, PA, USA) capillary column (30 m × 0.32 mm ID, film thickness 0.5 μm), a split injector and a flame ionization detector. The carrier gas was nitrogen, and the oven temperature was kept constant at 160°C for 7 min, then raised to 200°C (25°C min^{-1}), while the temperature of both injector and detector was 250°C. The split ratio was 1/100 and the internal standard, methyl decanoate, was added before sample treatment.

Calculation of internal concentrations

Assay of the organic extract of a pellet (see above) afforded the total amount of substrate in the spore aggregate. This system contained both intracellular and interstitial medium, and the intracellular concentration C_i was derived from Equation 1:

$$C_i = \frac{Q - V_e C_e}{V_i} \quad (1)$$

where Q was the amount of metabolite in the pellet, C_i and C_e the internal and external metabolite concentrations, respectively; V_i and V_e were the internal and external volumes in the pellet.

Calculation of C_i needed V_i, V_e and C_e. C_e was obtained from analysis of the supernatant fluid; V_i was previously determined as 666×10^{-6} L g DW $spores^{-1}$ [18], DW being the dry weight of the cells (2.07×10^{-8} mg $spore^{-1}$). V_e was calculated from Equation 2, established from data in Larroche *et al* [18]:

$$V_e = 1.88 \times 10^{-3}\, DW + 3.58 \times 10^{-3}\, V + 29 \times 10^{-6} \quad (2)$$

where DW was the dry weight of spores in g, V the volume centrifuged in L; V_e was in L. The term $3.58 \times 10^{-3} V$ accounted for the liquid remaining on the tube wall after the supernatant fluid was discarded, while 29×10^{-6} corresponded to the volume in L remaining above the pellet.

Measurement of the spore volume and diameter

This was carried out via determination of the perimeter of spores using a Leitz ASM 68K image analyzer. A sample drop was placed under a microscope fitted with a camera, and the image was displayed on a monitor attached to a computer system and an electronic digitizer. The digitizing table determined the x and y coordinates that were touched with a special pen, and a program calculated the length covered by the pen outlining a spore. Calibration by means of a standard millimeter placed under the microscope provided absolute values of spore perimeter [24].

Visual observations showed that the spores were spherical. The volume of a single spore could be calculated as:

$$V_c = \frac{Pr^3}{6\,\pi^2} = \frac{\pi\, D_c^3}{6} \quad (3)$$

where V_c was the cell volume, Pr the spore perimeter and D_c the cell diameter ($Pr = \pi D$).

Estimation of wet spore density

The density of wet conidia was assessed by measuring the free-settling velocity of spores suspended in water. A spore suspension (2×10^8 spores ml^{-1}) was homogenized and quickly poured into a 50-ml burette. The sedimentation front was readily followed due to the green color of the cells.

Results

Kinetic behavior of spores during batch reactions

The time-course of octanoic acid breakdown by spores of *P. roquefortii* was quite complex, exhibiting a lag phase followed by a short transient period of increased reaction rate. The batch process proceeded with a constant and maximal reaction rate until the substrate was exhausted (Figure 1), consistent with data already reported [17,20]. The value of this maximal reaction rate varied with initial substrate concentration as a Michaelian function (Figure 2). The Michaelis constant was found close to 1.72 mmol L^{-1}

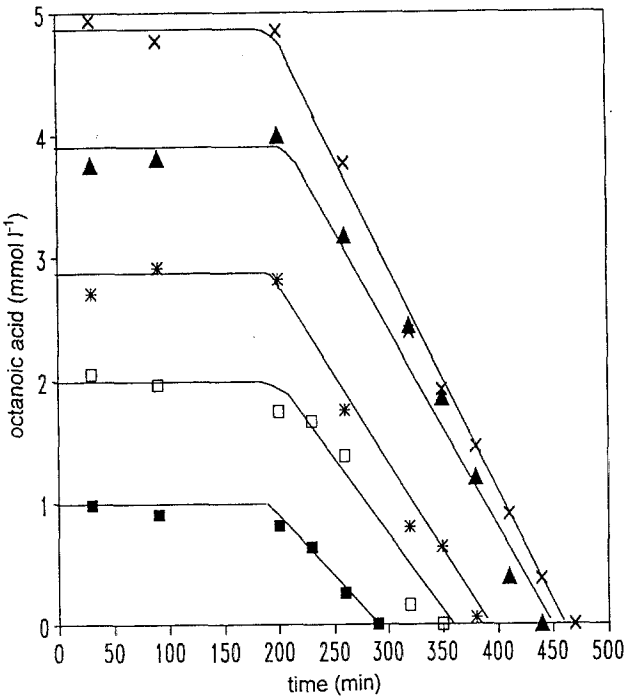

Figure 1 Time-course of residual octanoic acid concentration during batch reactions carried out with several initial substrate contents. Experiments performed in Erlenmeyer flasks containing 0.1 mol L^{-1} potassium phosphate-buffered medium at pH = 5.5, T = 27°C, biocatalyst content 1.8×10^8 spores ml^{-1}, 200 rev min^{-1}, substrate added in the form of sodium octanoate

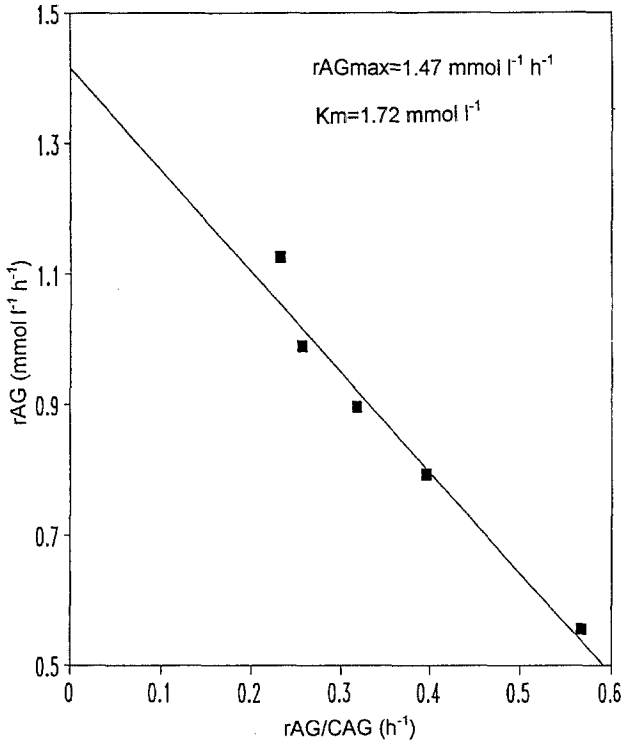

Figure 2 Eadie–Hofstee plot for batch processes carried out at pH = 5.5, from data in Figure 1

at pH 5.5. This pH corresponded to the best value for batch reactions [22,23].

These results confirmed that the residual octanoic acid concentration did not affect the reaction kinetics and they prompted us to study the behavior of internal substrate concentrations.

Initial internal concentration of octanoic acid

General behavior: A fast and intensive penetration of octanoic acid into the spores was evidenced under all the experimental conditions used. The maximal internal concentrations were obtained in less than 10 min, and they remained constant throughout the lag phase. The external pH was constant even with non-buffered media, because this parameter was continuously controlled (see Materials and Methods).

A marked accumulation of substrate took place inside the spores (Table 1). The internal concentrations were found to depend on the external pH and on the octanoic acid content in the medium, while spore loading had no measurable influence in the range 1.5 to 6.1×10^8 spores ml^{-1}. These findings agreed with data obtained with other systems such as formic acid with methylotrophic bacteria [5], butyric acid with *Neurospora crassa* [32] or various fatty acids with yeasts [15].

The internal/external ratio increased as the pH decreased and remained constant at a given pH when the external concentration varied (Table 1). In fact, total internal fatty acid concentrations varied linearly with extracellular levels of undissociated octanoic acid (Figure 3), ie, as generally considered [3,29], the acid entered the spores in its protonated form.

Heterogeneity in internal concentrations: These high internal concentrations suggested an heterogenous distribution in the conidia. Octanoic acid accumulates strongly in cell membranes [28] according to a Collander-type relation, suggesting that the fatty acid concentration could be different in the membrane and in the internal solute of spores of *P. roquefortii*. Osborne *et al* [28] gave a correlation for the accumulation of hydrophobic compounds in the membrane of *Rhizopus nigricans* cells which could be written, for an organic acid:

$$C_{AGm} = 0.19\ P^{0.84}\ C_{AHe} \qquad (4)$$

where C_{AGm} was the membrane concentration of octanoic acid, expressed in mmol kg dry membrane^{-1}, C_{AHe} the external undissociated acid concentration and P its partition coefficient in a standard octanol/aqueous buffer system. P was taken as $10^{-2.93}$ for octanoic acid [16].

Fungal walls contain some lipids that make them hydrophobic [2] and so Equation 4 could apply to the complex wall-membrane. Calculation of octanoic acid concentration in the cytoplasmic solute needed an estimation of the weight fraction, α, of this complex in spores of *P. roquefortii*. This estimate was made from Equation 5:

$$\alpha = \frac{\rho_c V_c - \rho_i V_i}{\rho_c V_c} \qquad (5)$$

Table 1 Summary of initial intrasporal octanoic acid concentrations achieved under various experimental conditions

External pH	Bioconversion medium	Spore concentration (10^8 spores ml^{-1})	Total octanoic acid concentration (mmol L^{-1})		Internal/external ratio
			external	internal	
5.5	Potassium phosphate buffer	3	4.42	32.0	7.24
		3.3	9.10	66.9	7.35
	Sodium phosphate buffer	6.1	4.63	29.1	6.29
	Water, pH adjusted with NaOH	3	5.05	31.8	6.30
6.5	Potassium phosphate buffer	5.5	4.63	9.8	2.12
	Water, pH adjusted with NaOH	2	4.87	8.6	1.77

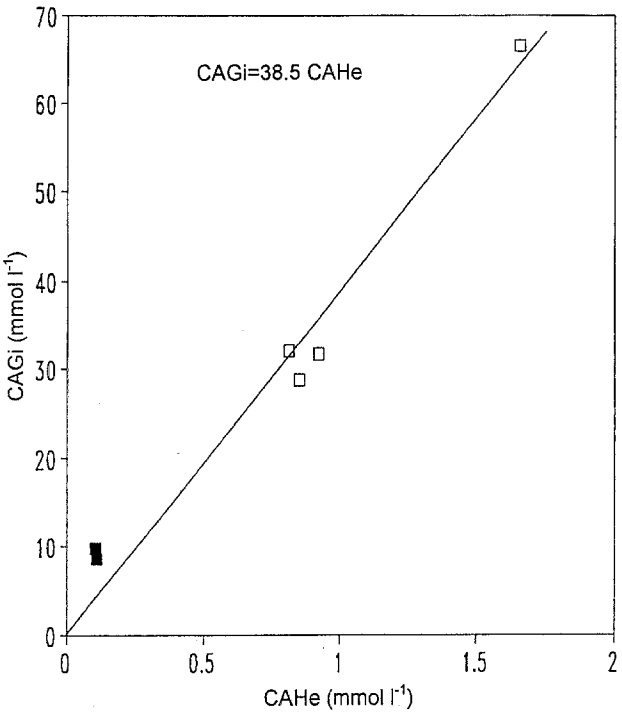

Figure 3 Total internal concentration of octanoic acid C_{AGi} plotted against the extrasporal concentration of undissociated substrate C_{AHe}. C_{AHe} is calculated as $\frac{C_{AGe}}{1 + 10^{pH_e\ pK}}$ where C_{AGe} is the total extracellular octanoic acid concentration, pH_e the external pH and pK is taken as 4.85. Data are from Table 1; (■--■), $pH_e = 6.5$; (□--□), $pH_e = 5.5$

where V_c was the spore volume obtained from experiments involving image analysis, V_i the internal spore volume, ρ_i the internal solute density and ρ_c the spore density, calculated from settling experiments. Data in Table 2 showed that α could be estimated at near 0.43.

Material balance on total internal octanoic acid could be written as:

$$C_{AGc}\ T_i + C_{AGm}\ \alpha = C_{AGi}\ T_i \qquad (6)$$

where C_{AGc} was the cytoplasmic octanoic acid concentration and T_i the cell water content with respect to dry matter (666 μl g DW^{-1}, see above). Combining Equations 4 and 6 afforded Equation 7:

Table 2 Calculation of the weight fraction of wall-membrane complex α from spore characterizations

Parameter	Value	Method
Perimeter (μm)	12.34	Image analysis
Diameter D_c (μm)	3.93	Image analysis (Eqn 3)
Total volume V_c (μm^3)	32	Image analysis (Eqn 3)
Cell density ρ_c (g ml^{-1})	1.32	Settling experiments[a]
Internal volume V_i (μm^3)	13.8	From ref 18[b]
α	0.43	Equation 5[c]

[a]ρ_c is deduced from the following equation, valid for the settling of spherical particles in the laminar region [30]: $\rho_c = \rho_w + \frac{3\pi\mu\nu D_c}{g\ V_c}$, where ρ_w is the water density, μ its viscosity (10^{-3} kg m^{-1} s^{-1}), ν the free settling velocity (9.98 mm h^{-1}) measured as the slope of the straight line obtained by plotting the height of settling as a function of time. The high value calculated for ρ_c is due to the low water content of spores. For example, the density of fresh *Saccharomyces cerevisiae* cells has been reported [13] to be close to 1.12 g ml^{-1} while the value for dry cells was 1.48 g ml^{-1}. These cells contained 1940 μl water g DW^{-1}, to be compared to 666 μl water g DW^{-1} for spores of *P. roquefortii*

[b]Calculated from the internal water content of spores

[c]The spores of *P. roquefortii* contain minor amounts of lipids [9], and ρ_i was taken as 1 g ml^{-1}

$$C_{AGe} = C_{AGi} - \frac{0.19\ \alpha\ P^{0.84}}{T_i}\ C_{AHe} \quad (7)$$

where the term $0.19\alpha P^{0.84}/T_i$ was close to 35.5. Equation 7 may be written, from data in Figure 3, as:

$$C_{AGe} = (38.5 - 35.5)C_{AHe} = 3\ C_{AHe} \quad (8)$$

showing that about 92% of total initial internal octanoic acid was located in the wall-membrane complex. Other possible phenomena, such as binding to cytosolic proteins or droplet formation are thus considered as negligible with spores of *P. roquefortii*.

Evidence for an active transport: The time-course of base addition needed to adjust the initial pH of non buffered media showed that this allowed full substrate solubilization (Figure 4). However, this addition continued up to 200 min, and the final amount exceeded that theoretically needed to hold the pH at the desired value. In the typical example given in Figure 4, the quantity added was close to 5.75 mmol L^{-1} sodium hydroxide while the requirement was only 4.89 mmol L^{-1}. This excess could not be attributed to some CO_2 dissolution [33] and it was concluded that this behavior reflected a proton efflux from the spores, linked to octanoic acid entry. Also, the initial pH equilibration took more time than needed to achieve maximal internal concentration.

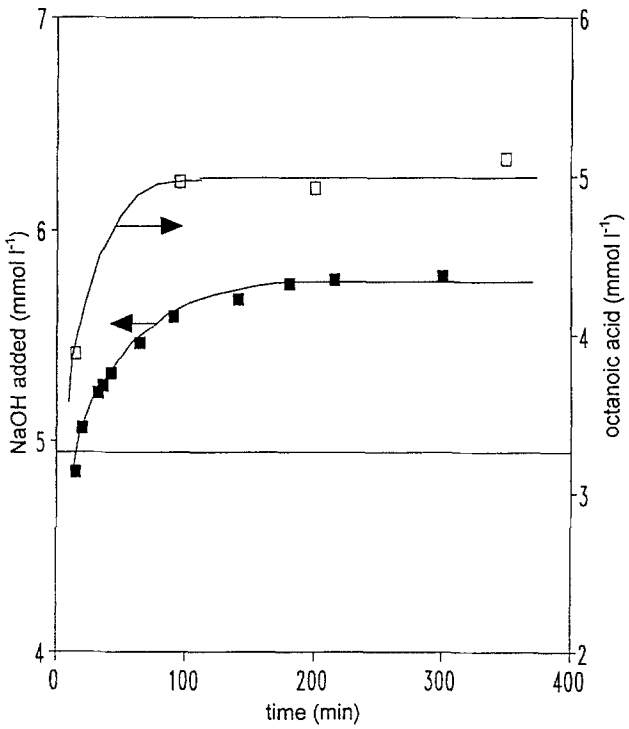

Figure 4 Sodium hydroxide addition to a non-buffered medium to adjust the external pH at 6.5 (■--■) and soluble octanoic acid concentration (□--□) plotted against the time. The horizontal line denotes the theoretical NaOH requirement, calculated as $\frac{K_w}{10^{pH_e}} + \frac{C_{AGe}\ 10^{-pK}}{10^{pH_e} + 10^{pK}} - 10^{-pH_e}$. K_w is the ionic product of water and the other terms are defined in the legend to Figure 3. Experiment carried out in the reactor, volume 750 ml, stirring speed 300 rev min^{-1}, T = 27°C, aeration rate (2.7 L h^{-1}) started 25 min after substrate addition, biocatalyst content 2.35×10^8 spores ml^{-1}

These results were consistent with data obtained during fed-batch reactions. Hence, these experiments were carried out by controlling the pH by direct substrate addition. Although this design was expected to give constant octanoic acid in the medium, it was not the case, and a decrease was observed after the lag phase [19].

This proton efflux had already been reported during butyric acid penetration in the fungus *Neurospora crassa* [31] and pointed to an active transport of this compound into the cells, in contrast with results reported for yeasts [15].

Time-course of internal concentrations during reaction

The internal octanoic acid concentration decreased linearly from the beginning of the reaction, and came close to zero before the end of the process (Figure 5). This decrease took place before the establishment of the constant rate of decrease in the external concentrations. As a result, plotting internal against external concentrations gave a straight line showing a deviation for the first points (Figure 6).

These results demonstrate that the membrane accumulation evidenced above was reversible, as reported for Ehrlich ascites cells [34]. The deviation from the line in Figure 6 could be interpreted as an exhaustion of the cytoplasmic content at the start of the reaction, and so the biotransformation mainly took place with a cytoplasmic octanoic acid concentration close to zero.

It could thus be concluded that internal concentrations *per se* did not govern the process.

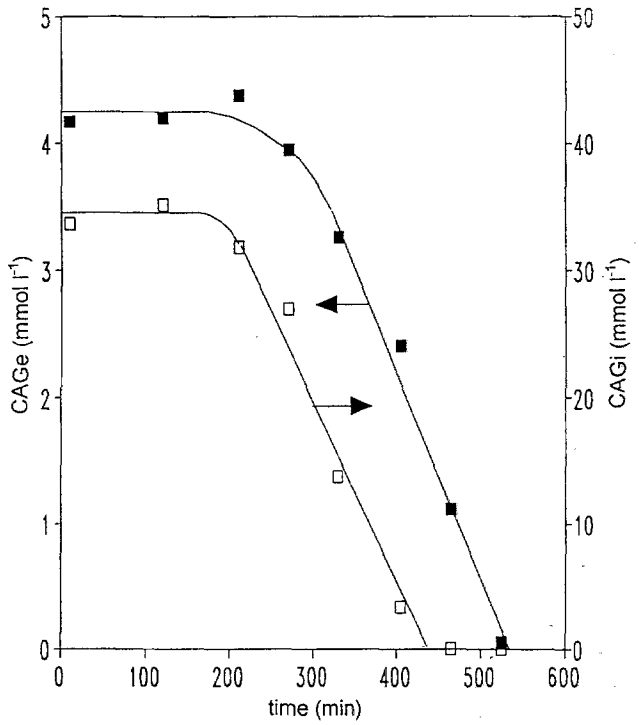

Figure 5 Time-course of external (■--■) and internal (□--□) concentrations of octanoic acid during a reaction. Experiment carried out in an Erlenmeyer flask containing sodium phosphate buffer and 6.1×10^8 spores ml^{-1}; other conditions as in the legend to Figure 1

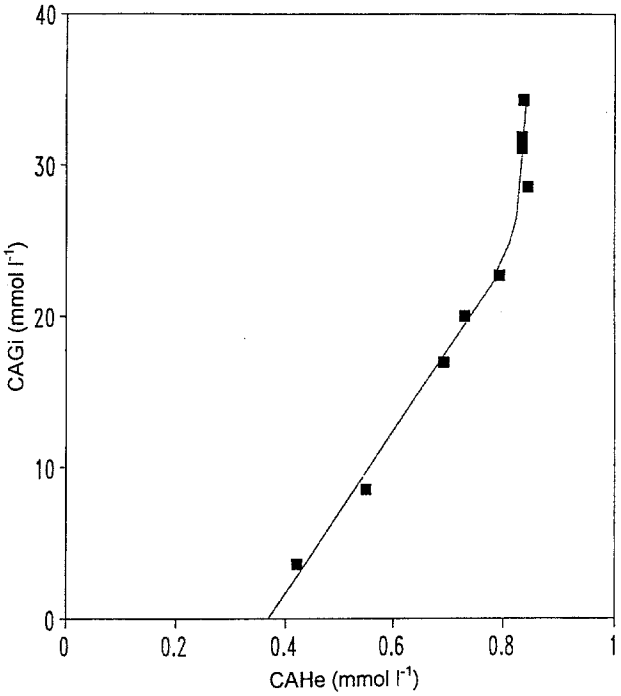

Figure 6 Internal octanoic acid concentration plotted against external undissociated substrate concentration, from data in Figure 5

Discussion

Results obtained in this work show that octanoic acid enters the spores in its protonated form, and that it displays a marked heterogeneity in its internal distribution. It strongly accumulates in the wall-membrane complex and the initial fast and intensive substrate penetration may thus be considered as a reversible solubilization or adsorption on the spore wall and membrane; this has already been postulated elsewhere [23].

The 'true' substrate penetration, which entails an enrichment of the cytoplasm, is a more lengthy phenomenon. It is associated with a proton efflux, which indicates an active mechanism. This active transport is also evidenced by oxygen consumption that takes place on adding the substrate to the medium, before the start of the reaction [19]. It is also consistent with the endogenous oxygen consumption and carbon dioxide evolution that occurs during the course of a process [17,23]. Penetration of organic acids in cells generally involves two phenomena, a carrier-mediated transport and simple diffusion [15]. The first mechanism may be an active transport process or an energy-independent facilitated transport and operates at low acid concentrations. The second one is believed to be the main way of passage of fatty acids through the membrane at high substrate concentrations. In the case of spores of *P. roquefortii* the main phenomenon is an active transport, as already described for the penetration of butyric acid into *Neurospora crassa* [31]. This reported system involves an electrogenic proton pump that generates a proton efflux from the cells connected to an ATP hydrolysis.

The substrate concentration in the cytoplasmic solvent thus increases during the overall lag phase to reach its final value at the time corresponding to the beginning of the reaction, ie near 200 min (see Figures 1 and 4). This lag phase may therefore correspond to the time required to achieve a critical cytoplasmic concentration necessary to start the enzymatic chain responsible for the methyl ketone synthesis. This critical concentration, also encountered during butanol synthesis from butyric acid by *Clostridium acetobutylicum* [29], is necessarily low. It can be reached even with the smallest external substrate concentrations used in this work, which explains why the lag phase duration does not depend on the external content (Figure 1). Preliminary attempts to explain this period, which involved permease activation [23] or substrate inhibition [14] are inadequate.

The kinetic behavior of the system is quite unusual, since it is governed by the initial external concentration of the substrate. This phenomenon might be expected if the internal fatty acid contents were constant, but this is not the case. The cytoplasmic solute becomes rapidly exhausted, and the wall-membrane accumulation also decreases and is nullified before the end of the experiment. The reaction is therefore limited by the transport of the substrate into the cells, and it is this rate of penetration that remains constant and is limited by the initial external concentration. The parameters r_{AGmax} and K_m given in Figure 2 are thus related to the transport system and not to the enzymatic chain that carries out the reaction.

The constant rate of operation of the carrier system indicates that the substrate's concentration in the bulk medium is not its main limiting factor. These considerations suggest that the system is governed by the driving force that allows the fatty acid penetration into the spores, ie the electrochemical gradients of solutes, H^+ and undissociated octanoic acid [8]. This driving force is an electrical potential, $\Delta\psi$, if the transport involves the exchange of a net charge and mainly a ΔpH if the process leads to proton transfer. Since such a transfer is evidenced, we may postulate that octanoic acid uptake is ΔpH-driven, as it is for lactate in *Escherichia coli* at pH 5.5 [8]. The proton efflux would then result from the action of a H^+-ATPase located in the cell membrane, just as in the energy-recycling model described by Michels *et al* [25]. This implies that the internal pH, pH_i, remains constant throughout an experiment, since the external pH is controlled.

Fatty acids are known to have an uncoupling effect [35] that may modify the stoichiometry of the H^+/octanoic acid exchange. This stoichiometry controls the pH_i and would therefore be set by the initial substrate concentration.

References

1 Allen KG, J McGee, ME Fellows, PA Tornheim and KR Wagner. 1984. A new procedure to analyze free fatty acids. Application to 20-mg brain tissue samples. J Chromatog 309: 33–42.
2 Bartnicki-Garcia S. Cell wall chemistry, morphogenesis, and taxonomy of fungi. 1968. Ann Rev Microbiol 22: 87–108.
3 Borst-Pauwels GWFH. 1981. Ion transport in yeasts. Biochim Biophys Acta 650: 88–127.
4 Cheetham PSJ. 1993. The use of biotransformations for the production of flavours and fragrances. Trends Biotechnol 11: 478–488.
5 Chu IM, TA Keuer and ET Papoutsakis. 1987. Formate transport, growth inhibition and the membrane protonmotive force in two methylotrophs (T15 and L3). Appl Microbiol Biotechnol 26: 70–77.
6 Creuly C, JB Gros and C Larroche. 1990. Procédé microbiologique d'obtention de methylcétones. Eur Patent 0 390 624.
7 Creuly C, C Larroche and JB Gros. 1990. A fed-batch technique for

2-heptanone production by spores of *Penicillium roquefortii*. Appl Microbiol Biotechnol 34: 20–25.

8 Driessen AJM and WN Konings. 1990. Energetic problems of bacterial fermentations: extrusion of metabolic end products. In: The Bacteria, vol 12 (Gunsalus IC, JR Sokatch and LN Ornston, eds), pp 449–478, Academic Press, New York.

9 Fan TY and JE Kinsella. 1976. Changes in biochemical components during the germination of spores of *Penicillium roquefortii*. J Sci Food Agric 27: 745–752.

10 Gehrig RF and SG Knight. 1958. Formation of ketones from fatty acids by spores of *Penicillium roquefortii*. Nature 182: 1237.

11 Jolly R and FV Kosikowski. 1975. A new blue cheese food material from ultra-filtrated skim milk and microbial enzyme-mold reacted fat. J Dairy Sci 58: 1272–1275.

12 Jolly R and FV Kosikowski. 1975. Blue cheese flavor by microbial lipases and mold spores utilizing whey powder, butter and coconut fats. J Food Sci 40: 285–287.

13 Ju LK and CS Ho. 1988. Correlation of cell volume fractions with cell concentrations in fermentation media. Biotechnol Bioeng 32: 95–99.

14 Kinsella JE and DH Hwang. 1976. Enzymes of *Penicillium roquefortii* involved in the biosynthesis of cheese flavor. CRC Crit Rev Food Sci Nutr 8: 191–228.

15 Kohlwein SP and F Paltauf. 1983. Uptake of fatty acids by the yeasts, *Saccharomyces uvarum* and *Saccharomycopsis lipolytica*. Biochim Biophys Acta 792: 310–317.

16 Laane C, S Boeren, R Hilhorst and C Veeger. 1987. Optimisation of biocatalysis in organic media. Stud Org Chem 29: 65–89.

17 Larroche C, M Arpah and JB Gros. 1989. Methyl-ketone production by Ca-alginate/Eudragit RL entrapped spores of *Penicillium roquefortii*. Enzyme Microb Technol 11: 106–112.

18 Larroche C, I Besson, CG Dussap, F Bourrust and JB Gros. 1993. Characterization of water distribution in cell pellets using nonlabeled sodium thiosulfate as interstitial space marker. Biotechnol Prog 9: 214–217.

19 Larroche C, I Besson and JB Gros. 1994. Behavior of spores of *Penicillium roquefortii* during fed-batch bioconversion of octanoic acid into 2-heptanone. Biotechnol Bioeng 44: 699–709.

20 Larroche C and JB Gros. 1989. Batch and continuous 2-heptanone production by Ca-alginate/Eudragit RL entrapped spores of *Penicillium roquefortii*. Biotechnol Bioeng 34: 30–38.

21 Larroche C and JB Gros. 1992. Characterization of the growth and sporulation behavior of *Penicillium roquefortii* in solid substrate fermentation by material and bioenergetic balances. Biotechnol Bioeng 39: 815–827.

22 Larroche C, B Tallu and JB Gros. 1988. Aroma production by spores of *Penicillium roquefortii* on a synthetic medium. J Ind Microbiol 3: 1–8.

23 Lawrence RC. 1966. The oxidation of fatty acids by spores of *Penicillium roquefortii*. J Gen Microbiol 44: 393–405.

24 Metz B, EW de Bruijn and JC van Suijdam. 1981. Method for the quantitative representation of the morphology of molds. Biotechnol Bioeng 23: 149–162.

25 Michels PAM, JPJ Michels, J Boonstra and WN Konings. 1979. Generation of an electrochemical proton gradient in bacteria by the excretion of metabolic end products. FEMS Microbiol Lett 5: 357–364.

26 Moo-Young M, AR Moreira and RP Tengerdy. 1983. Principles of solid-substrate fermentation. In: The Filamentous Fungi, vol 4 (Smith JE, DR Berry and B Kristiansen, eds), pp 117–144, Edward Arnold, London.

27 Nelson JH. 1970. Production of blue cheese flavor via submerged fermentation by *Penicillium roquefortii*. J Agric Food Chem 18: 567–569.

28 Osborne SJ, J Leaver, MK Turner and P Dunnill. 1990. Correlation of biocatalytic activity in an organic-aqueous two-liquid phase system with solvent concentration in the cell membrane. Enzyme Microb Technol 12: 281–291.

29 Papoutsakis ET, CM Bussineau, IM Chu, AR Diwan and M Hesmemann. 1987. Transport of substrates and metabolites and their effect on cell metabolism (in butyric-acid and methylotrophic fermentations). Ann NY Acad Sci 506: 24–50.

30 Sakiadis BC. 1984. Fluid and particle mechanics. In: Perry's Chemical Engineers Handbook, 6th edn (Green DW and JO Maloney, eds), Section 5, pp 1–68, McGraw Hill Book Co, New York.

31 Sanders D, UP Hansen and CL Slayman. 1981. Role of the plasma membrane proton pump in pH regulation in non-animal cells. Proc Natl Acad Sci USA 78: 5903–5907.

32 Sanders D and CL Slayman. 1982. Control of intracellular pH-predominant role of oxidative metabolism, not proton transport, in the eukariotic microorganism *Neurospora*. J Gen Physiol 80: 377–402.

33 Schumpe A, G Quicker and WD Deckwer. 1982. Gas solubilities in microbial culture media. Adv Biochem Eng 24: 1–38.

34 Spector AA, D Steinberg and A Tanaka. 1965. Uptake of free fatty acids by Ehrlich ascites tumor cells. *J Biol Chem* 204: 1032–1041.

35 Stevens S and JH Servaas Hofmeyr. 1993. Effects of ethanol, octanoic and decanoic acids on fermentation and the passive influx of protons through the plasma membrane of *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 38: 656–663.

36 Vezina C. 1987. Biotransformations. In: Basic Biotechnology (Bu'Lock J and B Kristiansen, eds), pp 463–482, Academic Press, New York.

37 Welsh FW, WD Murray and RE Williams. 1989. Microbiological and enzymatic production of flavor and fragrance chemicals. CRC Crit Rev Biotechnol 9: 105–169.