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Production of flavour ketones in aqueous-organic two-phase systems by using free and microencapsulated fungal spores as biocatalysts

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Abstract

The formation of 2-alkanones by free and microencapsulated *P. roquefortii* spores in an aqueous–organic two-phase system was investigated by using substrates supplied as a solution in decane. It was shown that the spores remained catalytically active after entrapment within permeable polyamide microcapsules and readily catalyzed the formation of 2-pentanone, 2-heptanone, and 2-undecanone from short-chain alkyl esters of hexanoic, octanoic, and lauric acid, respectively, with the rate of reaction being markedly dependent on the type and concentration of the ester substrate used. In general, the optimal concentration of the esters in decane was found to be much higher than that of the respective fatty acid substrates and, in the case of alkyl dodecanoates, the biotransformation could be carried out efficiently even in the absence of added solvent. Further analysis revealed a significant difference in the reaction rates observed with free and microencapsulated spores at 0.5 but not at 3.0 M methyl dodecanoate, suggesting that at high substrate concentrations the biotransformation was no longer limited by mass transfer.

Keywords: Aqueous-organic; Two-phase system; Biotransformation; Microencapsulation; Fungi; Spores; *P. roquefortii*; Solvent-free synthesis; Flavour; methyl ketone; 2-Alkanone

1. Introduction

The use of biocatalysts in nonaqueous organic solvents is now a well-established method for the preparation of products and intermediates for the pharmaceutical [1,2], fine chemical [3–7], and food industries [8–10]. One of the advantages of this approach is that it enables bioprocess operation at much higher concentrations of poorly water soluble substrates, thus making both the synthesis and product recovery more attractive from a practical standpoint [11,12]. However, many enzymes, and certainly microorganisms, still require a substantial amount of water present to maintain their catalytic activity at synthetically useful levels. In these cases, conventional aqueous-organic two-phase systems are still widely used, although the rates of mass transfer and inactivation of biocatalysts at the interface can be a serious drawback [13–16].

Recently, we suggested an alternative approach to biotransformations in aqueous-organic two-phase systems, where the aqueous phase is contained within permeable polyamide microcapsules suspended in organic solvent. The encapsulation of Baker's yeast [17] and β-glucosidase [18] was performed by using mild methods of interfacial polymerization, and the resulting biocatalysts were used successfully for the reduction of 1-phenyl-1,2-propanedione to 2-hydroxypropiophenone and the synthesis of anomerically pure glycosides respectively. In both cases, a noticeable improvement over conventional aqueous-organic two-phase systems was observed. Here we demonstrate the use of yet another class of microencapsulated biocatalyst, fungal spores, for the preparation of 2-alkanones. These compounds are found in many traditional food aromas, e.g. blue cheeses [19] and are of interest as natural flavourants [13]. The formation of several 2-alkanones by *P. roquefortii* spores by using short-chain alkyl esters of fatty acids as starting materials is reported here.

2. Materials and methods

Methyl dodecanoate, methyl hexanoate, methyl octanoate, ethyl hexanoate, ethyl octanoate, propyl hexanoate,

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dodecanoic acid, octanoyl chloride, *n*-propanol, 2-pentanone, 2-heptanone, 2-undecanone, 3-decanone, triethylamine, polyoxyethylene sorbitan monooleate (Tween 80), high-molecular-weight poly(allylamine hydrochloride) (PAA), 1,6-hexanediamine, and dodecanedioyl chloride were obtained from Aldrich Chemical, (Dorset, UK). Sorbitan trioleate (Span 85) and 3-[*N*-morpholino]-propanesulfonic acid (MOPS) were supplied by Sigma (Dorset, UK). Ethyl dodecanoate and *n*-decane were purchased from Fluka (Dorset, UK). Potato dextrose agar was obtained from Oxoid (Basingstoke, UK). All organic solvents used in this study were of the highest purity available and stored over 4-Å molecular sieves (Aldrich).

2.1. Spore growth and culture medium

A suspension of *Penicillium roquefortii* spores (ATCC 64383) (200 μl) containing 1×10^9 spores/ml was used to inoculate potatoe dextrose agar plates and mycelial growth and sporulation was initiated at 25°C over 7 days. The spores were harvested by the addition of 0.05% Tween 80 (10 ml) to the plate which was agitated to remove spores. The resulting solution (containing 1.73×10^{10} spores/ml) was collected and stored at −20°C. Calculation of the spore concentrations in suspension was by serial dilution of this stock suspension followed by cell counting by using a hemocytometer (Gelman Hawksley, Lancing, UK).

2.2. Preparation of polymeric microcapsules containing P. roquefortii spores

The stock spore suspension (2.8 ml) was centrifuged, the supernatant removed and the spores resuspended in 0.3 M MOPS buffer, pH 7.2, (2.0 ml) containing 0.1 M PAA and 0.1 M 1,6-hexanediamine (note that the PAA was dialyzed against 1 mM hydrochloric acid and lyophilized prior to use). This mixture was added dropwise to decane (20 ml) containing Span 85 (300 μl) and emulsified at room temperature by using a conventional magnetic stirrer operating at 400 rpm (IKA RETbasic). To the resulting dispersion (30–50-μm diameter microspheres), dodecanedioyl chloride (22.5 μl) in dry decane (15 ml) was added drop-wise over 10 min and the mixture was stirred for a further 20 min. The solvent was decanted from the microcapsules (30–50-μm diameter), which were then rinsed with water-saturated decane (2×20 ml) and used for the biotransformations.

2.3. Synthesis of 2-alkanones in decane and a solvent-free system

In a typical experiment, encapsulated spores (200 μl) or buffer (200 μl of 300 mM MOPS, pH 7.2) containing 4.84×10^{10} cells were added to 1.8 ml of *n*-decane containing 2.5 mM to 4.6 M carboxylic acid or ester substrate in sealed 10-ml Wheaton vials. Solvent free reactions were performed by replacement of decane with neat liquid substrate (1.8 ml). The reactions were performed at 25°C in a Luckham R300 shaker-incubator at 150 rev./min and the reactions were monitored by removal of 50-μl aliquots of the organic layer at regular intervals. These samples were diluted 10-fold with dichloromethane containing 3-decanone as an internal standard and analyzed by GC. All experiments were performed at least in duplicate.

2.4. Chemical synthesis of propyl octanoate

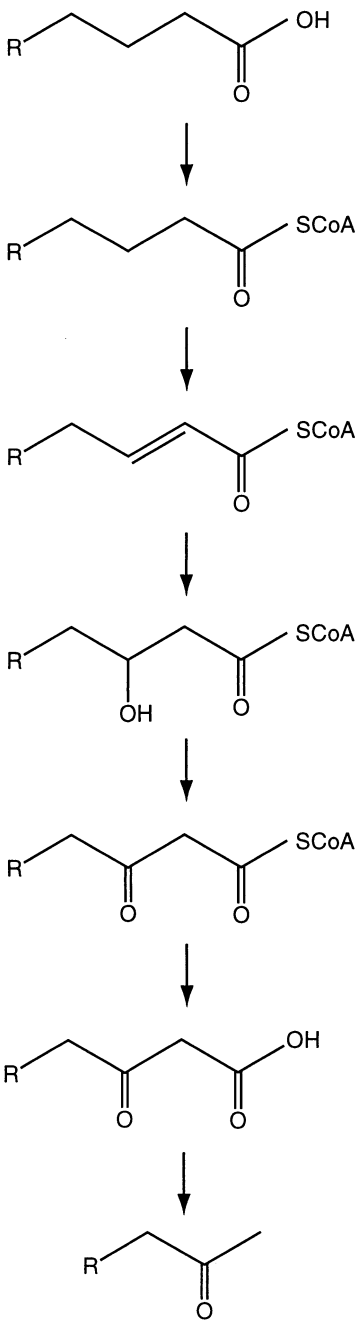
Octanoyl chloride (9.53 g, 59 mmol) was dissolved in dry dichloromethane (60 ml) at 25°C and dry triethylamine (5.81 g, 59 mmol) was added with stirring under dry nitrogen followed by the addition of *n*-propanol (3.55 g, 59 mmol). The reaction was stirred overnight, filtered and the organic phase washed with 5% aqueous sodium hydrogen carbonate (2×100 ml) and 1 mM hydrochloric acid (2×100 ml). The organic layer was then dried over anydrous sodium sulfate, filtered, and the solvent removed in vacuo to obtain a pale yellow liquid (10.57 g, 96.2%). The purity of the ester was shown to be 95.2% by GC analysis (retention time 9.14 min).

2.5. Gas chromatographic analysis

This was performed by using an HP 5890 GC (Split) System with a capillary column, methyl silicone gum, HP-1 ($25 \text{ m} \times 0.32$ mm; film thickness = 1.05 μm) connected to a FID detector, and by using hydrogen as a carrier gas at a flow rate of 0.7 ml/min. The injection temperature was set to 250°C and the detector was at 280°C. The oven temperature was programmed at 80°C for 2 min, increased to 200°C at 10°C/min and maintained at 200°C for 2 min. Samples were injected by using a split/splitless injector, split ratio 1/100. The retention times of 2-pentanone, 2-heptanone, and 2-undecanone were 1.2, 2.8, and 9.1 min, respectively.

3. Results and discussion

In order to assess the potential of microencapsulated fungal spores as a catalyst for the transformation of poorly water soluble substrates, *P. roquefortii* spore-mediated conversion of fatty acids to 2-alkanones (Scheme 1) was selected as a model reaction. This microbial biotransformation has been known for many years [20] and it was shown that both spores [21] and mycelia [22] of *P. roquefortii* can be used to form the 2-alkanone flavor ketones. Subsequently, there has been significant interest in this biotransformation, including the selection of alternative fungal strains [23,24] and optimization of the reaction conditions [25,26] for 2-alkanone production (note that under current legislations enzymatically prepared ketones can be sold as natural flavors). Therefore, this reaction was a good model system to assess the performance of microencapsulated spores.



Scheme 1. Bioconversion of carboxylic acids to alkyl methylketones by using *Penicillium roquefortii* spores (*R* = ethyl, *n*-butyl, and *n*-octyl).

The encapsulation of *P. roquefortii* spores was carried out as described in Section 2. Decane was selected as a suitable nontoxic organic solvent for both microencapsulation and as the reaction media because it was shown in our previous work and by other workers to have minimum deleterious effects on the viability of microorganisms [27–31]. Initially the reaction was carried out at 2.5, 10, and 50 mM octanoic acid by using free spores and, in accordance with other workers, we observed severe inhibition at higher substrate concentrations. The data presented in Fig. 1a shows that, although more product was formed with 10 mM as compared to 2.5 mM of octanoic acid, the reaction rate was slower and virtually no 2-heptanone was detected in the reaction mixture containing 25 mM (not shown) or 50 mM substrate. In an attempt to minimize the toxicity/inhibitory effect of octanoic acid, we also tested methyl octanoate as a possible substrate (we have found no examples in the literature of simple alkyl esters being used for this biotransformation). It appeared that methyl octanoate was readily converted to 2-heptanone by *P. roquefortii* spores, albeit at much lower rate under the same experimental conditions (compare ● and ○, Fig. 1a). At 50 mM concentration of methyl octanoate microencapulated spores catalyzed the reaction noticeably faster than free spores and the rate and final yield could be further improved by increasing the substrate concentration to 500 mM (compare ○ and △, Fig. 1a). The reactions described in Fig. 1a were stopped after 4 days of incubation but as shown in Fig. 1b, the spores were active for a considerably longer period of time.

The tolerance of spores to high concentrations of methyl octanoate implied that the lipase activity associated with spores was low and indeed no accumulation of octanoic acid in the media was observed by GC under the conditions used for the biotransformation. This result suggested that methyl octanoate was hydrolyzed intracellularly and then converted to the final product. If so, one would expect other esters also to be suitable substrates for *P. roquefortii*, with the rate of reaction being dependent on the concentration of the ester in the aqueous phase. One would also expect to observe a shift in the optimal concentration of homologous fatty acid esters in accordance with their partition coefficients between organic and aqueous phase. In other words, with no additional factors at play, the optimal concentration of substrate in the organic phase must be higher for less water soluble esters. This was indeed shown to be the case and the optimal concentration of methyl hexanoate, methyl octanoate, and methyl dodecanoate in decane was found to be 0.25, 1.25, and 3.0 M, respectively (Fig. 2a). A similar “shift” from 0.25 M to 0.5 M and 1.0 M was observed when methyl hexanoate was replaced by ethyl- and propyl hexanoate, respectively (Fig. 2b), and essentially the same dependence was obtained with short-chain alkyl esters of octanoic (Fig. 2c) and dodecanoic acids (not shown). It is interesting to note that in some cases the spores were able to catalyze the formation of the product even in the absence of decane, i.e. when neat methyl or ethyl dodecanoate or propyl octanoate were used as substrates and reaction solvent (Figs. 2c and 3). To the best of our knowledge, this is the first example of a solvent free biotransformation of substrate involving a fungal biocatalyst.

This observation was also of interest from a practical standpoint given the restrictions on the use of solvents in the food industry. Hence, the possibility of solvent free biotransformations of methyl decanoate, ethyl decanoate, and dodecanoic acid was investigated further. Fig. 3 depicts the dependence of 2-undecanone formation at different concen-

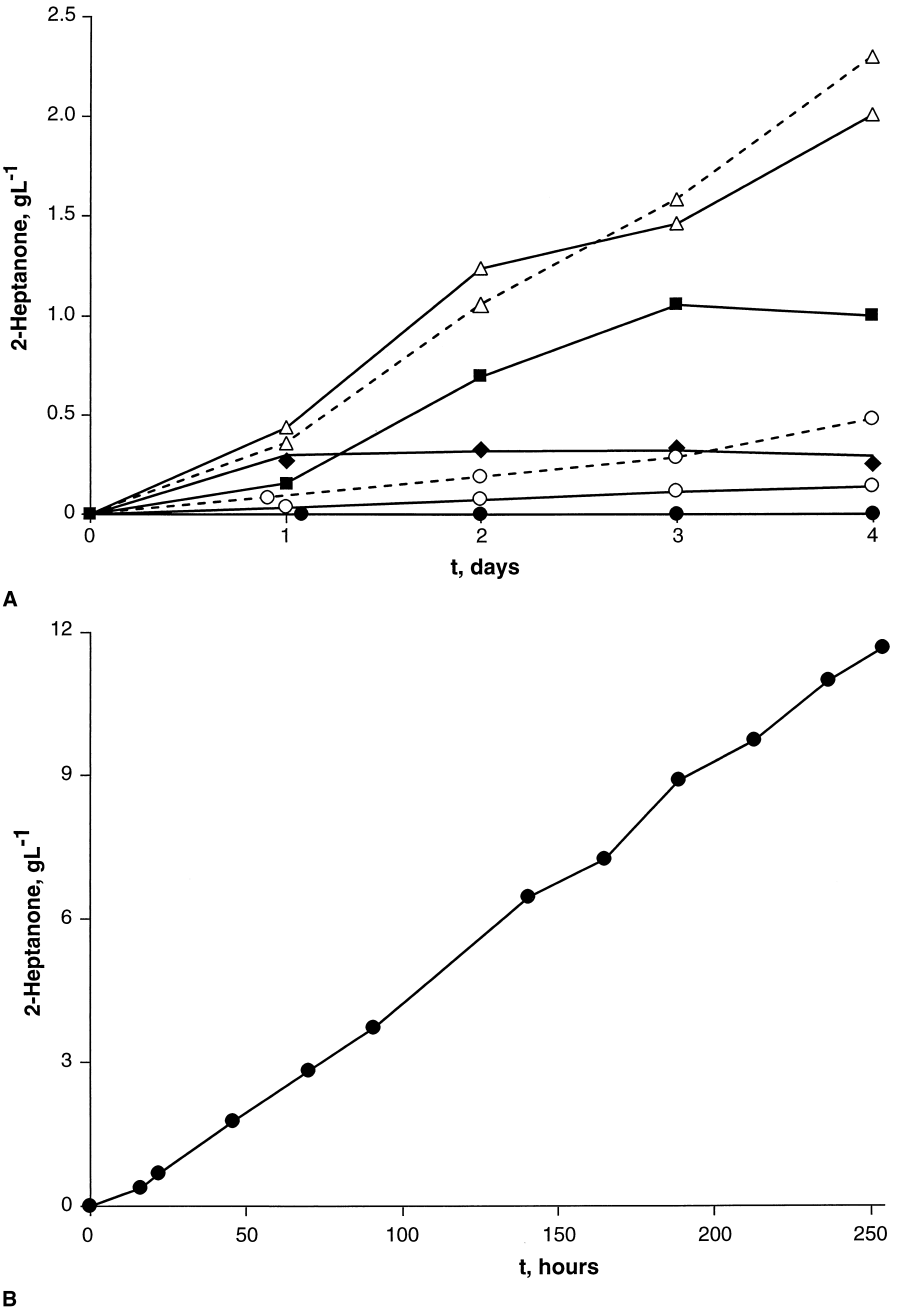


Fig. 1. (a) Bioconversion of 2.5 (♦), 10 (■), and 50 (●) mM octanoic acid and 50 (○) and 500 mM (△) of methyl octanoate to 2-heptanone by using free (—) and microencapsulated (----) *P. roquefortii* spores (see Section 2 for further details) and (b) kinetics of 2-heptanone formation catalyzed by encapsulated spores at 1 M methyl octanoate (●) in decane over a period of 14 days.

trations of all three substrates (dodecanoic acid was not soluble in decane at concentrations much higher than 2 M). It is evident from the data that rather similar yields were obtained with the ester substrates in a range of concentrations from 1 to $>$ 4 M (no added solvent) and that, contrary to the results presented in Fig. 1a for octanoic acid/methyl octanoate, dodecanoic acid was a better substrate for the spores than its esters even at very high concentration. Presumably this is because the solubility of dodecanoic acid in water is much less than that of octanoic acid [25] and the former is not toxic to the cell even at the saturating concentration.

Another interesting conclusion can be drawn from this set of data, i.e. it is probable that oxidative cleavage of dodecanoic acid, either imported into the cell or formed intracellularly from the esters, is the rate limiting step in this biotransformation. Two arguments can be advanced to support this view. Firstly, the initial rates of transformation of dodecanoic acid and methyl dodecanoate were not too dissimilar but there was a significant difference between the

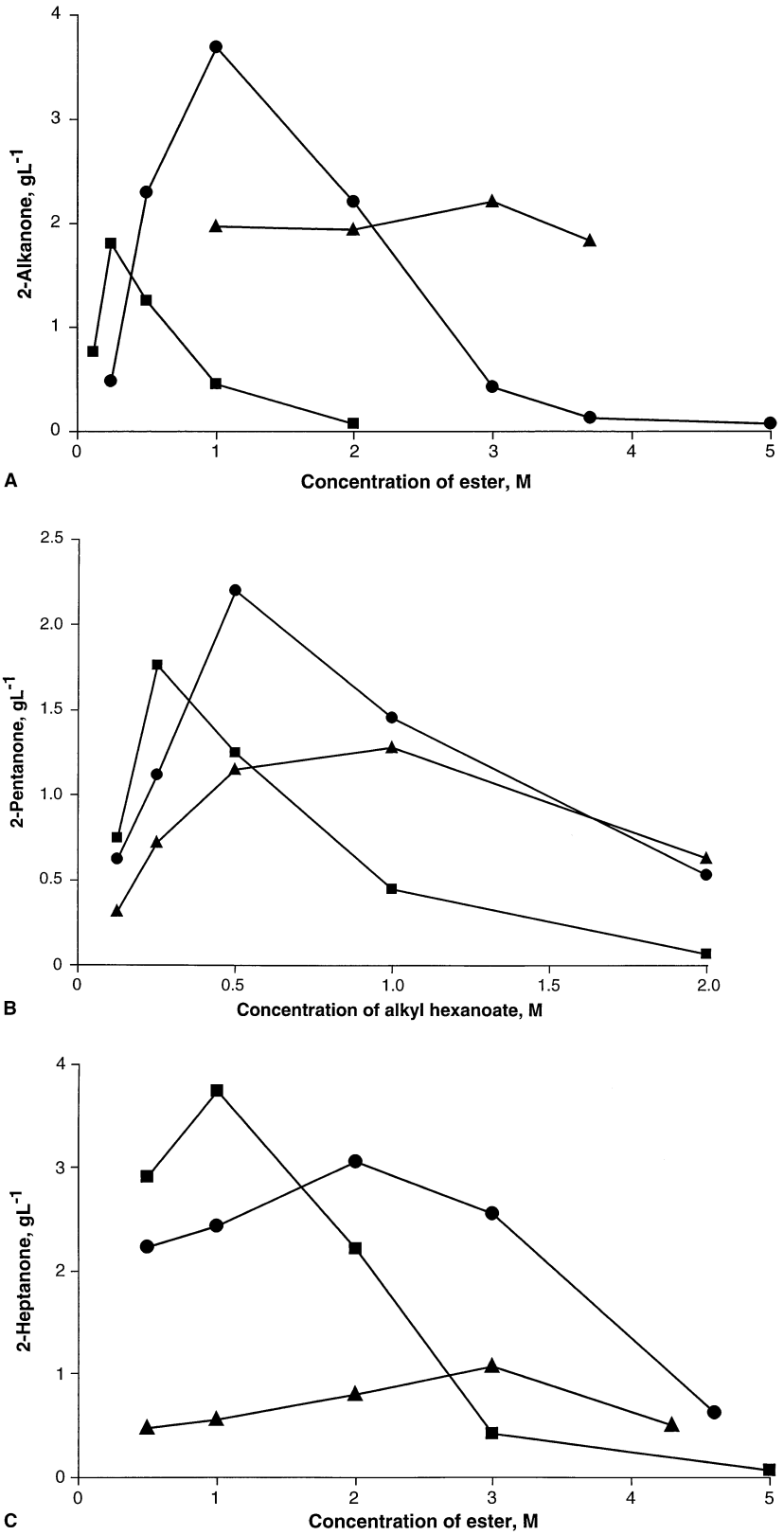


Fig. 2. Dependence of ketone formation catalyzed by microencapsulated spores on the concentration of ester substrates: (a) formation of 2-pentanone (■), 2-heptanone (●), and 2-undecanone (▲) from methyl hexanoate, methyl octanoate, and methyl dodecanoate, respectively; (b) formation of 2-pentanone from methyl (■), ethyl (●), and propyl (▲) esters of hexanoic acid; (c) formation of 2-heptanone from methyl (■), ethyl (●), and propyl (▲) esters of octanoic acid. All reactions were performed over a period of 4 days as described in Section 2.

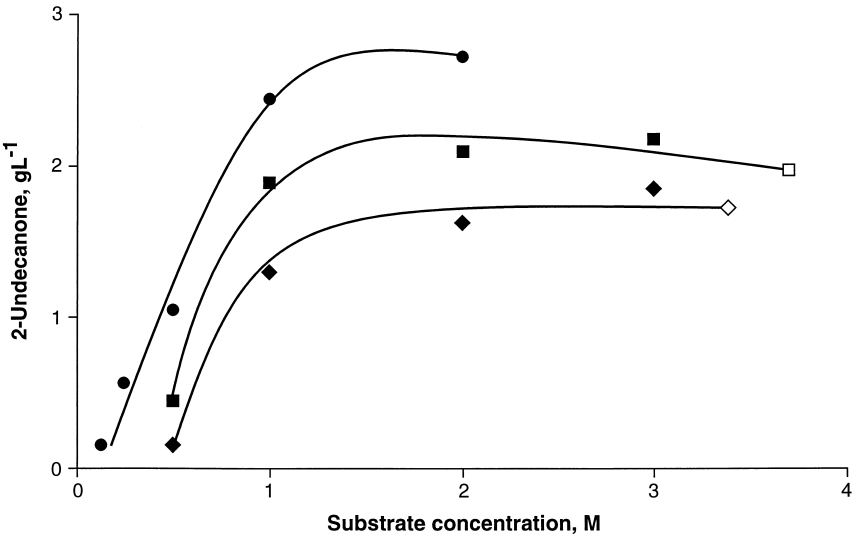


Fig. 3. The formation of 2-undecanone by encapsulated *P. roquefortii* spores at varying concentrations of dodecanoic acid (●), methyl dodecanoate (■), and ethyl dodecanoate (◆) as a solution in decane and in a solvent-free reaction (□, ◇).

rate of formation 2-undecanone as compared to that of 2-pentanone and especially 2-heptanone when all the respective substrates were used at optimal concentrations (Fig. 2). This implies that the overall rate of the biotransformation is probably defined by the specificity of the dehydrogenase, which has a preference for a C-8 chain-length substrate relative to C-6 or C-12. Secondly, all the esters were used at optimal concentrations and therefore mass transfer between the organic and aqueous phase and the transportation into the cell should not be limiting. Furthermore, a rapid decline in the reaction rates at higher concentration of alkyl hexanoate and octanoate, suggests that under these conditions the intracellular hydrolysis becomes too fast and the free acid released starts to inhibit the enzyme system.

In order to verify this analysis, we performed another set of experiments and compared the rate of transformation of methyl decanoate at optimal and suboptimal substrate concentrations by using both microencapsulated and free spores. We reasoned that if the mass transfer was not limiting, the two preparations should give us very similar results. However, if it was, there should be a significant difference in favor of the microcapsules due to their smaller droplet size. Hence, the prediction was that there would be no or very little difference in the reaction rate between the microcapsules and free spores at 3 M methyl dodecanoate, but at the lower concentration of 0.5 M the microcapsules should perform noticably better than free spores. The result of this experiment is shown in Fig. 4 and it is evident from the data that the outcome was exactly as predicted. Whereas microcapsules and free spores catalyzed the formation of 2-undecanone at more or less the same rate by using 3 M methyl dodecanoate, more than two times higher rate of conversion was observed with microcapsules at 0.5 M concentration of substrate.

The above clearly shows that the use of a microencapsulated biocatalyst cannot be an efficient strategy for every biotransformation involving poorly water soluble substrates and some preliminary knowledge of the limiting step(s) (or evidence for mass transfer limitations) is necessary. On the other hand, the approach of "dispersing" and "freezing" the aqueous phase in the form of small droplets containing a biocatalyst, i.e. microencapsulation, is a simple test that one can apply to a two-phase system to determine whether mass transfer limitations exist in a particular biotransformation. Furthermore we found in the course of our work with *P. roquefortii* that the analysis and optimization of the biotransformation was much easier to perform with microencapsulated spores than a conventional two-phase system. It was possible to carry out the reaction without potential complications caused by the adequacy of mixing, and excellent reproducibility was observed from one experiment to another.

In conclusion, we have demonstrated that fungal spores can be successfully encapsulated within polyamide microcapsules with no loss of biocatalyst activity. *P. roquefortii* spores, used as a model, were shown to catalyze the synthesis of methyl ketones from respective free fatty acids and their alkyl esters. Unexpectedly, it was found that short-chain alkyl esters are in fact as good, if not better, substrates than free acids provided they are used at optimal concentrations. As a large excess of esters is used to obtain high yields of products, there is no need for continuous feed of substrates and monitoring of the concentration of free acids during the reaction. The preparation of 2-undecanone can be carried out in a solvent-free process with methyl or ethyl dodecanoate. It was also shown that in the presence of a high concentration of ester substrates the biotransformation is no longer limited by mass transfer and, consequently, there is no difference in the overall bioconversion rates

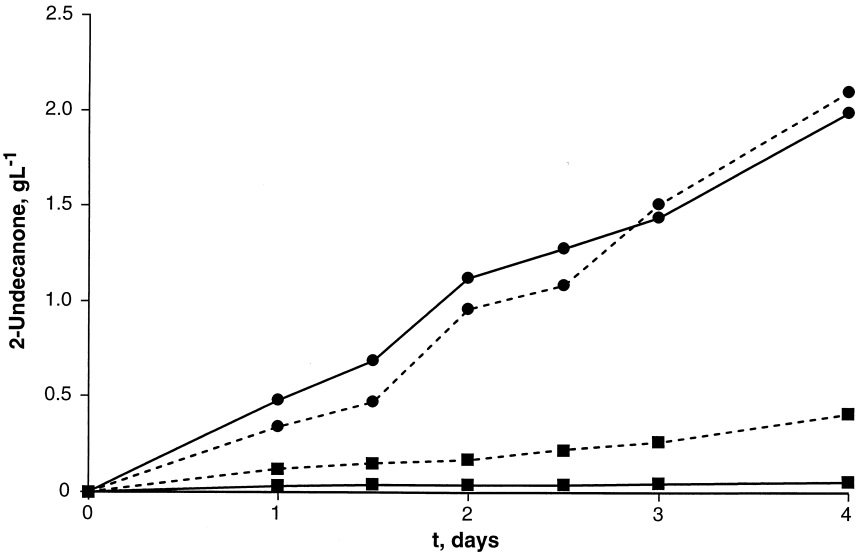


Fig. 4. Kinetics of 2-undecanone formation by using free (——) and encapsulated (----) spores at 0.5 (■) and 3.0 M (●) concentration of methyl dodecanoate in decane over a period of 4 days.

observed with microencapsulated and free spores. However, if the reaction is carried out under conditions of diffusional limitations, microcapsules performed significantly better.

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