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Journal of Molecular Catalysis B: Enzymatic 11 (2001) 961–965

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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A new experimental layout for non-aqueous enzymatic syntheses

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Abstract

The lipase-catalysed synthesis of geranyl caproate has been carried out in a stirred batch reactor.

A continuous flow of nitrogen is provided. The gas is pre-equilibrated by bubbling within different vessels upstream from the reactor, in order to insure controlled conditions in terms of water thermodynamic activity. In order to avoid a continuous stripping of the solvent, nitrogen is pre-saturated with the latter as well. Furthermore, nitrogen flow strips out the volatile reaction products, thus beneficially affecting reaction thermodynamics.

In order to further exploit the advantages of gas injection, a three-phase fluidised reactor configuration has been tested, where the gas-induced turbulence avoids mechanical stirring. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Lipase; Low-water system; Organic synthesis; Gas bubbling; Fluidised bed

1. Introduction

This study aims to analyse the effect of a novel experimental layout in non-aqueous enzymatic transformations with reference to three critical aspects.

1.1. Control of the thermodynamic activity of water (a_w)

Several methods for controlling a_w are described in literature [1]: based on the direct addition of activated molecular sieves or salt hydrates [2,3], the insertion of a cold trap at the exhaust line of the reactor [4], water evaporation through a membrane [5], water diffusion through silicone tubing [6], use of a vacuum reactor [4], rinsing of the immobilised enzyme by anhydrous acetone [2], and controlled leak of air into the reactor headspace [8]. Nevertheless, none of these techniques has been generally accepted as a practical solution for industrial-scale bioconversions. In this study, the control of a_w in a stirred batch reactor is performed by bubbling nitrogen, pre-equilibrated at a given a_w, within the non-aqueous mixture. Gas bubbling has been seldom used [2,7].

1.2. Product removal

Nitrogen bubbling can be used for the continuous removal of volatile products, in order to improve thermodynamically limited transformations. In this study, the lipase-catalysed transesterification of ethyl caproate and geraniol has been selected as a reference system, since it yields a product (ethanol) that is significantly more volatile than the other substrates and products. This is a fairly common situa-

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PII: S1381-1177(00)00174-0

tion, since short-chain alcohols are produced quite often in transesterification reactions of commercial interest. Whenever a solvent was used, the evaporation of the latter has been prevented by pre-saturating the nitrogen flow with the same solvent.

1.3. Mixing of the reaction mixture

In order to achieve an extremely simple reactor layout, gas bubbling has been exploited to fluidise the immobilised enzyme, as well. Thus, a mechanical stirrer is not required. It should be noted that, in non-aqueous enzymology, mixing is often critical, since proteins are not soluble in most organic solvents. In these three-phase fluidised reactors [9], use has been made of Novozyme 435 (Novo Nordisk), since its void fraction and density are such that the overall effective density is not far from that of the reacting mixture.

The simultaneous achievement of these objectives could obviously enhance the enzymatic synthesis in organic solvents.

2. Experimental

2.1. Chemicals

The transesterification of geraniol and ethyl caproate (usually 100 mM) has been carried out. Hexadecane (bp = 287°C) and octane (bp = 126°C) have been used as solvents. Lipase from *Candida antarctica*, immobilised on anion exchange resin beads (Novozyme 435), has been kindly supplied by Novo Nordisk.

2.2. Bioreactor

The experimental setup is described in Fig. 1. Upstream from the reactor, the carrier gas is dried by flowing through a column of molecular sieves. When higher levels of a_w are required, downstream from the molecular sieve column, water-free nitrogen bubbles within saturated water-solutions of $MgCl_2$ or NaCl, respectively. Gas pre-equilibration is completed by further bubbling within a vessel containing the same solvent used for the reaction mixture, to

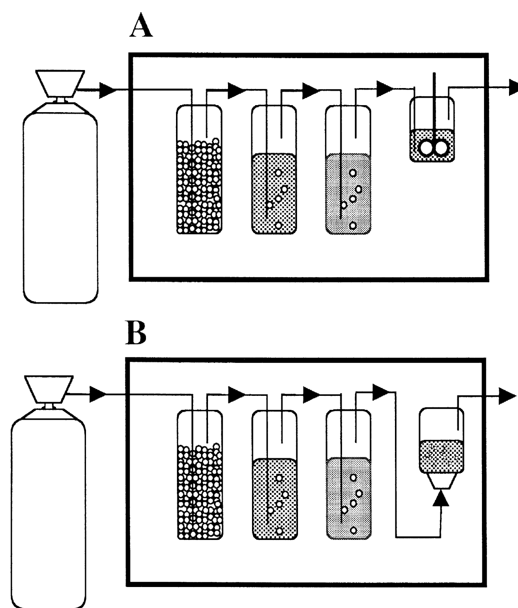

Fig. 1. Experimental layout. Stirred batch reactor (A) and fluidised-bed reactor (B).

keep a constant solvent volume in the reactor. In all tests described in the present work, substrate stripping due to the gas flow is negligible. All vessels used for equilibration are made of glass. All connecting lines are 3-mm ID Teflon tubing. Obviously, all the system is kept at the reaction temperature.

As regards the reactor, two different configurations have been tested. Initial runs have been performed by using mechanically stirred reactors with a submerged glass impeller (Fig. 1A). Nitrogen is injected via submerged tubing. Further runs have been carried out in a fluidised bed reactor (Fig. 1B). Obviously, no impellers were used, and the gas was distributed through a sintered glass septum at the bottom of the reactor. In either case, the bioreactors were cylindrical in shape (60 × 30 mm, $h \times$ ID) and made of glass.

Specific tests have been performed to confirm that, upon pre-saturation, equilibrium is reached in terms of water concentration within the reactor. This has been ascertained by direct measurements of water content (see Section 3). Solvent saturation is confirmed indirectly by the extremely limited solvent loss observed in the reactor, when a continuous nitrogen flow-rate of 36 ml/min is maintained for

24 h. Under these conditions, the amount of hexadecane stripped is less than 2% (less than 4% for octane).

2.3. Operation

Experimental tests are started by inserting the substrate solution (12 ml) into the reactor. Substrate is then equilibrated under gas bubbling (usually $Q =$ 36 ml/min) and stirring for 60 min, then the enzyme (500 mg) is added. The reaction is monitored by periodically drawing substrate samples.

2.4. Analyses

Samples collected from the reactor have been analysed by GLC equipped with an FID detector. Standard enzymatic activity measurements have been carried out in screw-capped tubes, at 37°C. The conversion is defined in terms of appearance of product. Water content of the reacting mixture has been measured by KF titration. Alcohol concentration determinations have been performed by means of a standard enzymatic kit (Boehringer 176290).

3. Results and discussion

The water content of the reacting mixture kept in the reactor at 50°C under pre-equilibrated nitrogen bubbling is reported in Fig. 2, as a function of time. Nitrogen has been pre-equilibrated in the presence of molecular sieves, or alternatively in the presence of saturated solutions of $MgCl_2$ ($a_w = 0.30$ at 50°C) and NaCl ($a_w = 0.74$ at 50°C). It can be seen that, in all tests, the water content is kept at a constant value for several days.

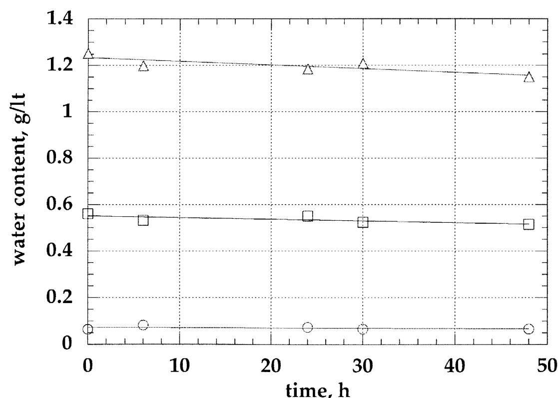

Fig. 2. Water content of the reacting mixture kept in the stirred batch reactor under pre-equilibrated nitrogen bubbling, as a function of time. $T = 50°C$, $Q_{nitrogen} = 36$ ml/h, solvent: octane. Nitrogen pre-equilibration over molecular sieves (○), $MgCl_2$ saturated solution (□) and NaCl saturated solution (Δ).

Table 1
Experimental values of the first order kinetic constant (K) of ethanol evaporation as a function of the nitrogen flow-rate

Q (ml/min)	K (/h)
18	0.77
36	0.88
48	1.02

In order to determine the rate of ethanol removal by gas stripping, experimental tests have been carried out by keeping under continuous nitrogen-bubbling a mixture containing a known amount of ethanol. The residual concentration of alcohol has been measured periodically. The experimental results indicate that the rate of ethanol evaporation follows first order kinetics with a kinetic constant K that depends on nitrogen flow rate. The results are summarised in Table 1.

Obviously, when the enzymatic reaction takes place, ethanol is continuously produced by the reaction and simultaneously stripped by nitrogen flow, according to these kinetics. A rough estimate of the maximum ethanol concentration achievable within the system can be performed by dividing the maximum observed experimental rate of alcohol production (0.02 moles/h/l) by the kinetic constant of removal corresponding to the standard nitrogen flow-rate of 36 ml/min. The calculated value is 23 mM.

The effect of gas bubbling on the conversion time profile is depicted in Fig. 3. The experimental data clearly show that an equilibrium conversion is reached, both in the presence and in the absence of gas bubbling. Ethanol stripping, however, improves both the final conversion and the initial reaction rate.

The effect of a_w is depicted in Fig. 4. At $a_w =$ 0.75 (pre-equilibration in the presence of a saturated NaCl solution at 50°C) the equilibrium conversion is significantly lower, probably because the presence of water promotes the hydrolysis of esters as a side-re-

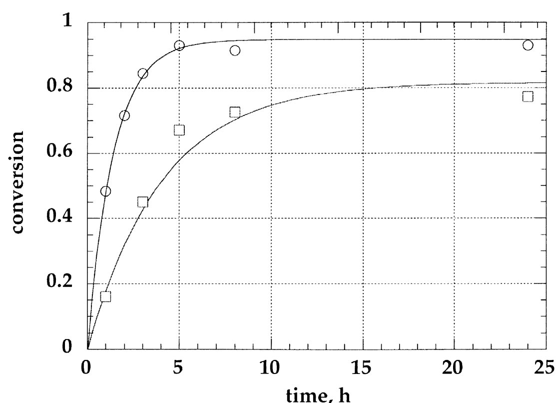

Fig. 3. Effect of nitrogen bubbling ($Q_{nitrogen} = 36$ ml/h, pre-equilibration over molecular sieves) on the conversion–time profiles. $T = 50°C$, solvent: octane. Reaction under nitrogen bubbling (○) and no bubbling (□).

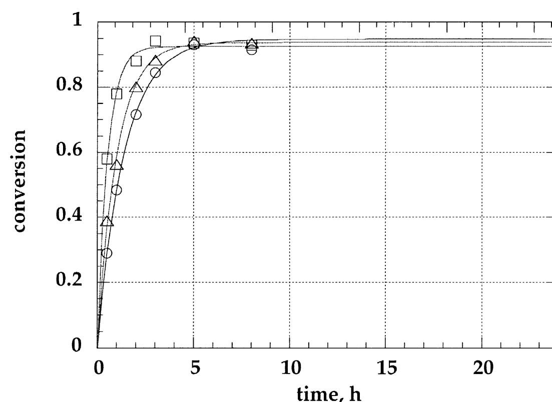

Fig. 5. Effect of the temperature and the solvent thermodynamic activity on the conversion–time profiles. $Q_{nitrogen} = 36$ ml/h, nitrogen pre-equilibration over molecular sieves. $T = 50°C$ (○) with octane, $T = 80°C$ with (□) and without (Δ) octane.

action. As an alternative, at higher water contents the enzyme might have undergone deactivation. Experimental tests are under way to identify the actual mechanism.

Further tests have been carried out under a variety of different experimental conditions, namely at different (higher) reaction temperatures (Fig. 5, curves a–b) and without solvent (Fig. 5, curve c). As expected, at 80°C the reaction rate is significantly higher, whereas the equilibrium conversion is still above 90%. In all instances, reactor operation was regular. The slower reaction rates observed in the absence of solvent are probably produced by substrate inhibition effects.

The thermal stability of Novozyme 435 both in the presence and in the absence of gas bubbling was monitored by collecting enzyme samples from the reactor periodically, and measuring their activity in a standard kinetic assay (see Section 2). Deactivation curves, depicted in Fig. 6, indicate that, under all the operating conditions tested, the enzyme is quite stable. Experimental data also indicate that enzyme stability is virtually unaffected by gas bubbling.

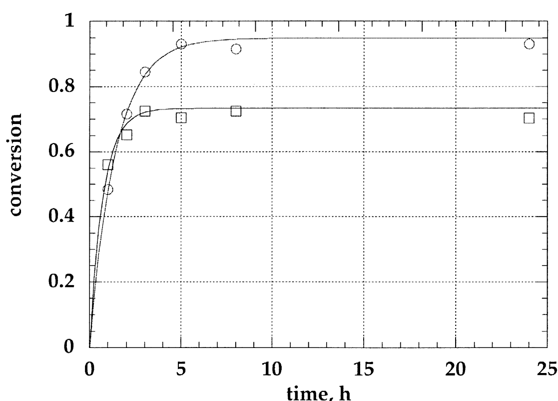

Fig. 4. Effect of the water thermodynamic activity on the conversion–time profiles. $T = 50°C$, $Q_{nitrogen} = 36$ ml/h, solvent: octane. Nitrogen pre-equilibration over molecular sieves (○) and NaCl saturated solution (□).

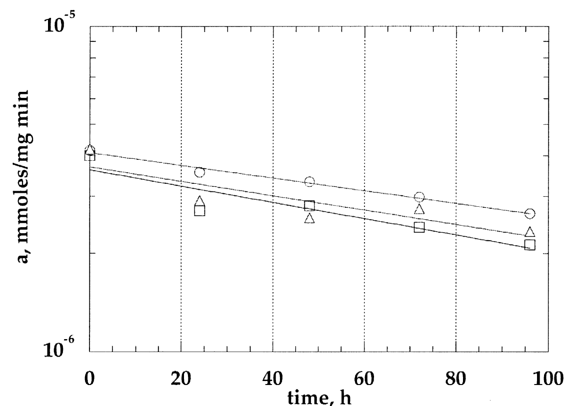

Fig. 6. Enzyme stability under different experimental conditions. $Q_{nitrogen} = 36$ ml/h, nitrogen pre-equilibration over molecular sieves, solvent: octane. $T = 50°C$ (○) with bubbling, $T = 80°C$ with (□) and without (Δ) bubbling.

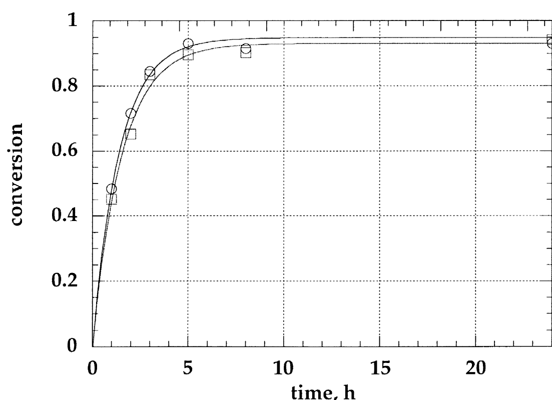

Fig. 7. Comparison between the three-phase fluidized bed reactor (□). and the batch reactor (○). Nitrogen flow-rates: 60 ml/h (fluidized bed) and 36 ml/h (batch). Nitrogen pre-equilibration over molecular sieves. $T = 50°C$, solvent: octane.

An experimental test has been carried out in a three-phase (solid-batch, liquid-batch) fluidised bed system, as described in Section 2. Preliminary tests at different flow-rates showed that the transition between fixed and fluidised bed conditions are quite gradual. As a consequence, a minimum fluidisation velocity can hardly be calculated. However, a flow rate of 60 ml/min, is clearly sufficient to achieve a fully fluidised state. Under these experimental conditions, the conversion–time curve, reported in Fig. 7, virtually coincides with that obtained in the impeller-stirred reactor. This result shows that, the gas-induced turbulence within the fluidised bed is sufficient to minimise the effect of external mass-transfer limitations.

4. Conclusion

This study shows that bubbling of pre-equilibrated nitrogen in a stirred reactor may become a feasible method to perform organic-phase enzymatic transformations keeping a constant value of the water content. Also, the gas bubbling allows an effective control of the volatile-products concentration, thereby improving thermodynamically limited transformations. Finally, nitrogen flow has been exploited to operate a three-phase (solid-batch, liquid-batch) fluidised bed system, thereby avoiding a mechanical stirrer.

Further improvements in the reactor layout can be designed to meet specific process requirements, e.g. when product removal is not required, the gas stream could be recycled, thus reducing the operation costs and making the control of water-content easier. Further interesting option might arise when dealing with gaseous substrates [10].

Obviously, the preliminary indications drawn by this study must be confirmed by further experiments with different enzymes, substrates, reactions, and operating conditions.

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