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Enzymatic catalysis in gel-stabilized two-phase systems: improvement of the solvent phase

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Abstract

Gel-stabilized aqueous phases entrapping enzymes and surrounded by organic solvents have become promising tools for the biocatalytic conversion of hydrophobic compounds. In this work, we provide methods for an improvement of the solvent phase with special regard to the avoidance of gel agglomeration in batch as well as fluidized-bed reactors, and resulting effects on the catalyzed reaction. With alginate beads entrapping a lipase from *Candida rugosa* as investigation system, it was demonstrated that increasing the solvent polarity was only a limited measure to separate agglomerated beads, as water-unsaturated polar solvents extracted large amounts of water from the hydrogel. Water-saturated alcohols, however, were incorporated into side product esters by the entrapped enzyme. With non-polar solvents, like hexane, bead separation in batch reactors was achieved by the addition of certain surfactants to the organic phase. Best results were obtained with the cationic surfactant cetyl trimethyl ammonium chloride (CTAC), which in contrast to other surfactants only slightly affected the entrapped lipase and revealed no effects on the hydrogel structure. For the suspension of alginate beads in a fluidized-bed reactor, not only CTAC, but an additional increase in the solvent density was necessary, which affected the system's productivity.

Keywords: Immobilization; Entrapment; Hydrogel; Agglomeration; Two-phase system

1. Introduction

Organic solvents are versatile media to expand the application range and efficiency of biocatalytic conversions as they (I) increase the solubility of many industrially interesting compounds compared to aqueous solutions, (II) allow the catalysis of reactions that are unfavorable in water, e.g. the reversal of hydrolysis reactions in favor of synthesis, (III) ease the product recovery, (IV) ease the recovery of the biocatalyst and (V) increase the biocatalyst thermostability [1].

Many different types of organic solvent systems have already been used. However, with regard to the classification of Davison et al. [2], monophasic systems, meaning organic solvents with only few water molecules and two-phase systems of immiscible liquids are most frequently investigated. In monophasic systems, aggregates of solid enzymes perform the catalysis, while in liquid two-phase systems the biocatalysts are usually dissolved in an aqueous phase. Unfortunately, only few enzymes, mainly lipases, are

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really stable in any of the two systems [3]. Profound losses in activity often occur due to the direct exposition of the biocatalysts to the organic phase or to organic–aqueous interfaces [4,5]. This can also be observed, when two-phase systems are stabilized by the formation of reverse micelles [6].

A common tool to improve the operational stability of biocatalysts in organic synthesis is their appropriate immobilization [7]. In reactions with low water content, lipases have most often been immobilized by adsorption on hydrophobic solid surfaces [8], while, in spite of the advantageous effects described later, entrapment into natural or synthetic hydrogels has only occasionally been performed [9–11]. By forming a solidified water phase in an aqueous-organic two-phase system, hydrogels protect the entrapped biocatalyst against detrimental effects of the environment. The gel composition can be adapted to the specific demands of different biocatalysts with regard to ionic strength, pH, etc., while the leakage of enzymes from the hydrogel into the surrounding phase is prevented by their insolubility in hydrophobic organic solvents. Like in all multi-phase systems, molecules partition between the stabilized aqueous and the organic phase. At high substrate concentrations inside the hydrogel and a partition of the reaction products into the organic solvent, the chemical equilibrium of the biocatalytic reaction is shifted to full-conversion [12] and a high productivity is achieved without a concomitant product inhibition of the biocatalyst.

However, in non-polar organic solvents, like hexane or heptane, which are preferably used in non-aqueous biocatalysis [13], hydrogel particles spontaneously agglomerate and often cannot be separated by stirring or shaking without considerable abrasion from the gel matrix. Thus, biocatalytic processes with gel-stabilized two-phase systems are restricted to and have since today been performed in fixed-bed reactors [9,14]. If the overall reaction rates are limited by the amount of available enzyme, fixed-bed reactors are appropriate to achieve a high volumetric productivity due to the dense loading with biocatalysts [7]. However, when entrapped enzymes are used, the overall reaction rates are often affected by mass transfer limitations within the entrapment matrix [9] and restrictions of the surface available for the transfer of substrates and products [15]. Subsequently, for the improvement of the volumetric productivity in such reaction systems, reactor concepts are necessary that are able to handle separated small gel particles with favorable surface-to-volume-ratio, like batch or fluidized-bed reactors.

The aim of this work was the improvement of future applications of gel-stabilized two-phase systems in the conversion of hydrophobic compounds by developing and investigating methods to separate or fluidize agglomerated hydrogel beads in organic solvents in batch and fluidized-bed reactors, respectively, without changes in the hydrogel composition and effects on the system's productivity. As a model reaction, the synthesis of the short-chain flavor ester butyl propionate by a lipase from *Candida rugosa* entrapped in an alginate matrix was investigated.

2. Experimental

2.1. Chemicals

Manugel® DJX sodium alginate was from Monsanto (Waterfield, UK). The lipase from *C. rugosa* (E.C.3.1.1.3, type VII, L-1754) and the Karl–Fischer calibration standard, Hydranal® water standard, were obtained from Sigma–Aldrich (Seelze, Germany). The Karl–Fischer reagents 1,2-dichloroethane and dichloromethane were from Merck Eurolabs (Darmstadt, Germany). All other chemicals were purchased from Fluka (Buchs, Switzerland).

2.2. Immobilization procedure

A 2% (w/v) sodium alginate solution buffered with 0.1 M Tris–HCl, pH 7.5 and containing 5 mg/ml (3730 U/ml) crude lipase was dropped into an equally buffered continuously stirred 0.18 M $CaCl_2$ solution. After curing in the hardening bath for 1 h, the beads were filtered, slightly dried on paper and stored in hexane at 4 °C. Alginate beads without enzyme were produced accordingly.

2.3. Determination of the bead size

Bead diameters were measured with the automatic particle sizer "Accusizer 780" (Particle Sizing Systems, St. Barbara, USA), equipped with the sensor

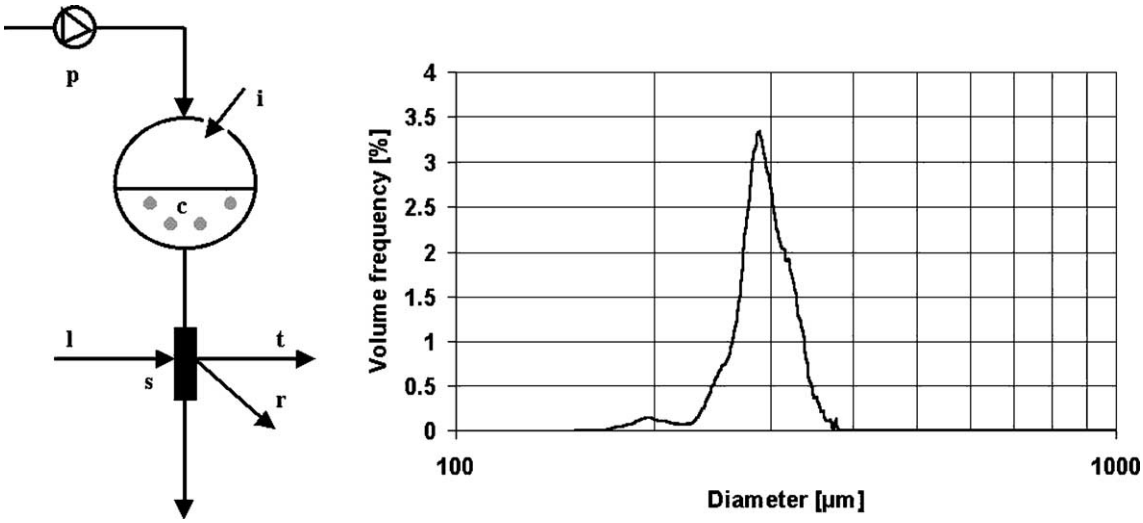

Fig. 1. Principle of the particle size analysis with an "Accusizer 780" (Particle Sizing Systems, St. Barbara, USA) (left). Particles diluted in water are injected (i) into the sample cell (c) and pumped (p) through a laser beam (l). Reflected light (r) and transmitted light (t) are detected by a sensor (s) and converted into a bead size by an automatic plotting unit. Typical size distribution of the investigated alginate beads (right).

LE2500-3. Every measurement included at least 2000 particles and was finished within 30 s after introducing the sample into water. The principle of the particle size-analysis and a typical bead size distribution is shown in Fig. 1. The average diameters of the investigated beads were 200–400 μm.

2.4. Determination of bead separation

Bead separation was defined as the ability of single beads to move in mixed organic phases without permanent contact to other hydrogel beads. In pre-experiments using a 10-fold magnification to observe the beads it was obvious that, as a consequence of changes in the surrounding phase, large gel agglomerates either remained unchanged or dissolved completely, but never formed smaller aggregates. For further investigations, the separation point was therefore determined by visually checking for the loosening of alginate agglomerates.

2.5. Determination of the ester productivity

250 mg of alginate beads were added to 10 ml solvent containing 50 mM *n*-butanol, 50 mM propionic acid and 20 mM decane as internal standard. After 6 h of incubation at 30 °C in an overhead shaker, stirred vessel or fluidized-bed reactor, the butylpropionate concentration was determined. The ester productivity was expressed as butylpropionate produced per hour (mM/h).

2.6. Analysis of substrate, product and water concentrations

Substrate and product concentrations were measured by gas chromatography using an HP 5890 series II gas chromatograph (Hewlett-Packard, Walbronn, Germany), equipped with an autosampler and a flame ionization detector (FID), on a FS–FFAP fused silica capillary column (25 m × 0.5 mm i.d.; film thickness 0.25 μm; CS GmbH, Langerwehe, Germany) with nitrogen as carrier gas. Mass spectra of side products were obtained using an equal gas chromatograph, equipped with a MS 5971A, electron impact (70 eV) mass spectrometer (Helwett Packard, Walbronn, Germany), and a SE-54-CB capillary column (25 m × 0.5 mm i.d; film thickness 0.25 μm; CS GmbH, Langerwehe, Germany) with hydrogen as carrier.

The water content of the organic phase was determined by triplicate Karl–Fischer titrations with a Titroline alpha autotitrator (Schott, Hofheim a. T., Germany).

2.7. Overhead shakers and stirred tanks

An overhead shaker REAX 2 (Heidolph, Schwalbach, Germany) was used to move alginate beads in solvent filled glass tubes (16 cm × 1.25 cm i.d.) by turning the tubes upside down at a rotation velocity of 90 turns/m. The stirred tank reactors were built from 50 ml screw flasks (4.5 cm × 4 cm i.d.) containing 1.25 g of gel beads and 50 ml of organic solvents, placed on a magnetic stirrer.

2.8. Fluidized-bed reactor

A tubular glass reactor (20 cm × 0.9 cm i.d.), filled with 500 mg wet alginate beads and 20 ml of the respective solvent mixture, was linked to a peristaltic pump (Braun, Melsungen, Germany) performing a flow up to 300 ml/min. Flow distribution at the reactor entry was achieved by passing through a porous polymer frit (28 μm pore size). Experimental data considering the separation of beads in solvent mixtures were drawn from systems with circulating organic phase.

3. Results and discussion

3.1. Bead separation in stirred tank reactors and overhead shakers

3.1.1. Changing the solvent polarity

Supposing that bead agglomeration is a result of the difference in polarity between the hydrogel and the organic solvent, a reduction of this difference by the use of increasingly polar organic solvents should result in bead separation. As polarity itself has no distinct definition, but results from a combination of physical and chemical parameters such as dipole moment, hydrogen bonding, entropy, and enthalpy, the solvent log P was chosen to compare and systematically investigate polarity properties necessary for bead separation. Specifically, the log P describes the hydrophilicity of organic compounds [16], but in common literature, hydrophilicity and polarity are often used synonymously since hydrophilic molecules usually bear polar features [17]. The log P is a dimensionless value that is arbitrarily measured in an octanol–water two-phase system and defined as the logarithm of the concentration of a partitioning compound in octanol divided by the concentration of the same compound in water ($\log P = \log[\text{concentration}_{\text{octanol}}/\text{concentration}_{\text{water}}]$) [18]. It decreases with increasing hydrophilicity.

However, when twelve organic solvents with a log P ranging from 3.8 to −0.7 were tested for their ability to separate alginate beads, no distinct log P, above or below which separation or agglomeration of added hydrogel beads occurred, was found. Table 1 demonstrates that the five organic solvents that succeeded in bead separation had rather different log P values, while solvents with a log P similar to a successful solvent did not provide bead separation.

A closer look to the chemical properties of the solvents successfully separating alginate beads revealed, that they all included hydroxyl functions, which indicated an important role of such groups in the separation/agglomeration behavior of the investigated system. As hydroxyl functions are able to form strong hydrogen bonds and thus increase the water uptake of a solvent (Table 1) and its miscibility with aqueous phases, they might decrease the intermolecular repulsion between the hydrogel and the organic solvent in the investigated two-phase system. However, not unexpectedly, this behavior revealed additional effects on the gel-stabilized aqueous phase. In pure octanol, hexanol or butanol the alginate bead sizes reduced to 10, 14 and 13%, respectively, of their initial diameters. The distinct conditions under which bead separation and this side effect occurred were investigated by slowly increasing the octanol concentration in an inert hexane phase. Fig. 2 demonstrates the bead separation, loss of water and size reduction for mixtures of hexane with increasing concentrations of octanol. While bead separation required octanol concentrations above 40%, the size of the alginate beads was already reduced at very small amounts of octanol. As the changes in the particle size directly correlated with the water loss from the beads, it can be assumed, that water is drawn from the alginate beads without a concomitant uptake of the octanol into the hydrogel matrix. Similar results were obtained for hexanol and butanol. In contrast, the use of neat ethanol or methanol

Table 1
Physical properties of organic solvents and classification according to the EU Directive 88/344/EEC for food stuff [26]

Solvent	Bead Separation	log *P*	Water uptake ($mg_{water}/mg_{solvent}$)[a]	Density (g/ml)	Classification[b]
Hexane	–	3.90	0.0684	0.660	A/B
n-Bromohexane	–	3.80	4.0120	1.175	C
Cyclohexane	–	3.44	0.0828	0.770	B
Chlorocyclohexane	–	3.36	5.4000	1.000	C
Bromocyclohexane	–	3.20	3.4000	1.355	C
Octanol	+	3.07	27.500	0.820	C
Hexanol	+	2.03	41.680	0.820	C
Chloroform	–	1.97	1.2200	1.480	C
Dichloromethane	–	1.25	2.1800	1.325	A/B
1,2-Dichloroethane	–	1.25	2.5300	1.253	C
Butanol	+	0.84	200.70	0.810	B
Ethanol	+	−0.30	∞	0.810	A
Methanol	+	−0.74	∞	0.791	B
Water	+	−1.38	∞	1.000	A

[a] Determined by Karl–Fischer titration.
[b] A: allowed for all extraction procedures; B/C: allowed under specific conditions.

increased the water content in the organic phase with only small changes in the bead size (Fig. 3), indicating that the water inside the hydrogel matrix had partly been exchanged for the co-solvents. A direct effect on the entrapped enzyme might be the result [19].

When alcohols with a limited water miscibility, like octanol, hexanol or butanol, were saturated with water, bead separation was achieved without apparent loss of water from the hydrogel. However, due to the broad range of alcohols representing a substrate for the entrapped lipase from *C. rugosa* [20], side products of the enzymatic conversion, like propionic octyl esters, occurred when such reaction media were used in combination with the investigated system. Nevertheless, if

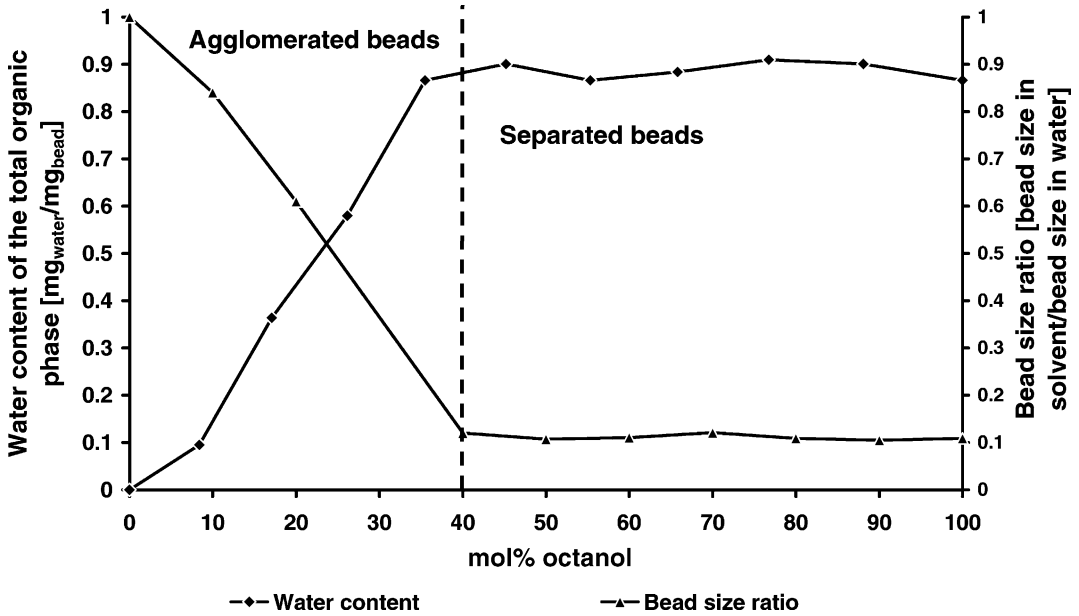

Fig. 2. Water content of the total organic phase (10 ml) and bead size ratios in hexane:octanol mixtures after equilibration.

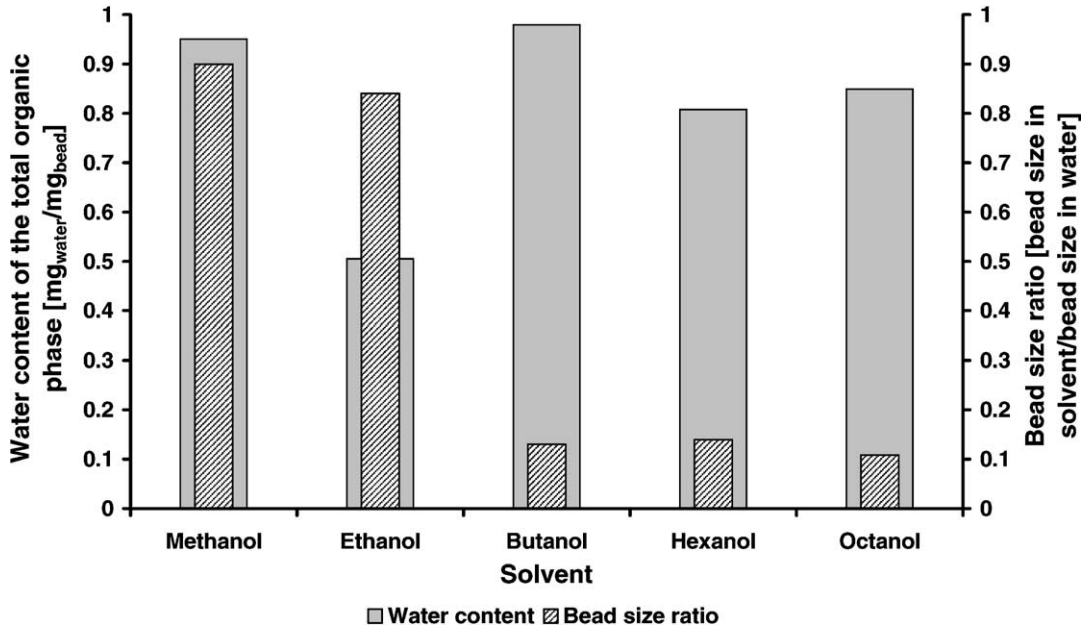

Fig. 3. Water content of the total organic phase (10 ml) and bead size ratios in pure organic solvents after equilibration.

an esterification can be found that regularly utilizes the alcoholic solvent as a substrate, or if a biocatalyst with a substrate range not including alcohols is entrapped, the use of the water saturated alcohols with gel-stabilized aqueous phases might be an appropriate measure to prevent the agglomeration of hydrogel compartments.

3.1.2. Addition of surfactants

Due to their amphiphilic structure, consisting of hydrophilic head and hydrophobic tail or vice versa, surfactants are located at the interface between polar and non-polar phases. When added to a stabilized two-phase system, the hydrophilic parts bind to the hydrogel while the hydrophobic groups are exposed to the organic solvent. Thus, they may counteract the affinity of the hydrophilic surfaces. Effects of small concentrations (0.1%, w/v or v/v) on the stabilized two-phase system were tested for a variety of surfactants with different neutral, positively or negatively charged head groups.

When the neutral surfactants Brij®30V, Brij®35V, Brij®56V, Brij®76V, Brij®96V (all polyoxyethylene ethers), Span®85 (sorbitan trioleate), TWEEN®80 (polyoxyethylenesorbitan monoleate) and Triton® X-100 (*t*-octylphenoxypolyethoxyethanol) were added to alginate beads suspended in hexane, Span®85 and Brij®96V led to bead separation. Almost no influence on the ester productivity could be observed, however, in the presence of Brij®96V the gel structure was destructed within 24 h (Table 2). Consequently, only Span®85 was further investigated.

With 0.1 vol.% Span®85 in the hexane phase, a number of side products of the lipase catalyzed

Table 2
Ester productivity in the presence of surfactants and evaluation of their effects

Surfactant	Ester productivity (%)	Drawbacks
None	100	No separation
Neutral		
TWEEN®80	n.d.	No separation
Triton®X-100	n.d.	No separation
Span®85	100	Side products
Brij®96V	105	Bead destruction
Ionic		
CTAB	40	Productivity loss
CTAC	69	Productivity loss
AOT	0	No residual productivity

reaction were observed. For example, GC–MS measurements revealed a 281 and a 338 Da molecule (data not shown), correlating in retention time and mass spectra to oleic acid and oleic acid butyl ester, respectively. Obviously, Span®85 was hydrolyzed to oleic acid and sorbitan mono- and/or diesters, as well as transesterified with butanol to oleic acid butyl ester. Due to the heterogeneity of fatty acids in commercially available Span®85 (oleic acid 74%, linoleic acid 7%, palmitoleic acid 7%, palmitic acid 7%, linolenic acid 2%) the formation of further side products can be expected.

From the class of ionic surfactants, the anionic AOT (sodium dioctylsulfosuccinate) and sodium dodecylsulfate (SDS) and the cationic cetyl trimethyl ammonium bromide (CTAB) and cetyl trimethyl ammonium chloride (CTAC) were tested (Table 2). Bead separation was achieved with AOT, CTAB and CTAC. For CTAB and CTAC this could be explained by the interactions between their positive charges and the carboxylic functions of the alginate as described by Babak et al. [21]. In the presence of AOT, no residual enzyme activity was detectable, while with CTAB and CTAC the overall productivity decreased 60 and 30%, respectively. As an explanation, a direct interaction between the lipase and the surfactants [21], the occupation of the interface by the surfactants preventing the lipase to adsorb and/or changes in the substrate concentrations inside the alginate beads [22,23] are likely.

Considering the effects on the alginate beads and the ester productivity, as well as the toxicity to environment and health, CTAC was chosen for all further experiments.

3.2. Fluidization of beads in a fluidized-bed reactor

The efficient mass transfer from the liquid phase to the catalyst particles and the low mechanical shear forces, make fluidized-bed reactors favorable for a biotechnological process using hydrogel matrices. The velocity of the upward stream of the solvent phase necessary for the fluidization of an initially stationary bed of non-agglomerated alginate beads and their maintenance of fluidization, depends on the density difference between the alginate bead and the liquid, the bead size and the drag coefficient. The minimum fluid velocities decrease with increasing density of the solvents. For the maintenance of fluidization, they were calculated from the five equations (given later), the experimental correlation for the drag coefficient ξ from [24], and solvent densities and viscosities from the commercial database DETHERM [25].

$$F_G = F_A + F_W \quad (1)$$

$$F_G = \tfrac{4}{3}(\pi) d_{bead}^3 \rho_{bead} g \quad (2)$$

$$F_A = \tfrac{4}{3}(\pi) d_{bead}^3 \rho_{solvent} g \quad (3)$$

$$F_W = \xi \rho_{solvent} \tfrac{1}{4}(\pi) d_{bead}^2 \tfrac{1}{2} u^2 \quad (4)$$

$$\xi = f\left(Re = \frac{u d_{bead}}{\nu_{solvent}}\right) \quad (5)$$

where F_G is the gravity force, F_A the upward hydrostatic force, F_W the drag force, d_{bead} the diameter of the bead, ρ_{bead} the bead density, $\rho_{solvent}$ the solvent density, g the acceleration of gravity, u the superficial velocity, i.e. relative velocity between the particle and the fluid, Re the particle Reynolds number, ξ the drag coefficient of a single particle and $\nu_{solvent}$ the solvent viscosity.

Results for alginate beads with a diameter of 200 μm (density 1.022 g/l) in selected solvents and solvent mixtures are given in Table 3. It is obvious that a fluidization of the beads at reasonable fluid velocities cannot be obtained in neat hexane, but requires a solvent density above 900 g/l. This was achieved by mixing hexane or cyclohexane with

Table 3
Superficial fluid velocity required for the maintenance of fluidization of alginate beads (Ø: 200 μm, ρ_{bead}: 1.022 kg/m^3) in some organic solvents or solvent mixtures

Composition solvent (v/v)	Density ($\rho_{solvent}$, kg/m^3)	Viscosity (ν, kg/m/s)	Superficial velocity (u, m/s)
Hexane	660	3.1×10^{-4}	**0.02**
Cyclohexane	775	8.9×10^{-4}	**0.01**
Chloroform:hexane (1:3)	840	3.4×10^{-4}	**0.008**
Chloroform:cyclohexane (1:3)	965	6.8×10^{-4}	**0.002**

Table 4
Influence of solvent mixtures ($d = 0.9\,g/cm^3$) on the ester productivity

Solvent mixture[a]	Volumetric ratio	Ester productivity (%)[b]
Hexane:bromocyclohexane	1.4:1	78.2
Cyclohexane: bromocyclohexane	2.3:1	86.6
Chlorocyclohexane:hexane	5:1	8.8
Chlorocyclohexane: cyclohexane	3.8:1	6.1
Bromohexane:hexane	1.6:1	46.4
Bromohexane:cyclohexane	1:1	109.8
Hexane:chloroform	3:1	14.4
Cyclohexane:chloroform	4:1	16.2
Hexane:dichloromethane	2.6.1	7.8
Cyclohexane: dichloromethane	3.7:1	10.7
Hexane:1,2-dichloroethane	7:1	4.6
Cyclohexane: 1,2-dichloroethane	2.4:1	12.3

[a] All mixtures contained CTAC to enable bead separation.
[b] Reference productivity: productivity in pure hexane containing CTAC, 1.03 mM/h, with an experimental setting described in Section 2.

appropriate high-density solvents, like chloroform, bromohexane or dichloromethane (various densities are given in Table 1). However, compared to the initial ester productivity in neat hexane with small amounts of CTAC (0.1%) the productivity in such mixtures decreased unpredictably (Table 4). While in cyclohexane:bromocyclohexane a reduction of about 14% was detectable, a residual ester productivity of only 4.6% remained in hexane:1,2-dichloroethane. An increase in productivity was obtained with a mixture of 1-bromohexane and cyclohexane. Nevertheless, as the application of such a solvent mixture is restricted by EU legislation [26], e.g. in the production of food stuff, further investigations of alternative solvents with appropriate density and their resulting effects on a gel-stabilized biocatalytic system are necessary.

4. Conclusions

The separation or fluidization of alginate beads in organic solvents in batch and fluidized-bed reactors, respectively, is a challenging task due to the multiple effects of changes of the organic phase on the aqueous phase and the productivity of a gel-stabilized two-phase system. Obviously, the success of preventing gel agglomeration as well as many side effects depend on special properties of the investigated system, like the entrapped enzyme and its catalytic specificities or the nature of the gel matrix. Thus, the prediction of conditions under which a certain gel-stabilized two-phase system can successfully be applied in batch or fluidized-bed reactors remains difficult and requires individual adaptation. As a rule for the mere separation of hydrogel particles in both reactor concepts, the need for water saturation of very polar organic solvents, like alcohols, can be stated. Promising results can generally be expected for the addition of surfactants to a non-polar organic solvent. Regarding the fluidization of gel particles in a fluidized-bed reactor, however, the need for halogenated solvents to fluidize even small beads is a considerable drawback, as in spite of the permission to use such solvents in pharmaceutical and chemical production processes most companies will probably be reluctant to apply them.

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