Catalyst Recycling

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Biocatalytic Reaction And Recycling by Using CO_2-Induced Organic–Aqueous Tunable Solvents**

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Enzymes display high substrate specificity and can catalyze reactions that are not possible in a single step through traditional synthesis.[1] In addition, biocatalysts usually function at relatively mild aqueous conditions with moderate temperature, pressure, and pH, and thus can allow for process routes that can potentially replace less environmentally friendly steps in chemical synthesis. The ability, or in many cases, the need to function in relatively mild reaction media can also limit the utility of biocatalysts. Many interesting, often prochiral, compounds are water insoluble and thus unavailable to biocatalytic conversion. Numerous schemes have been developed to use biocatalysts to transform water-insoluble substrates.[2] These schemes employ soluble and immobilized enzymes in simple one- and two-phase organic–aqueous mixtures or more-complex mixtures by using reversed micelles,[3] supercritical fluids, and ionic liquids.[4] Two-phase approaches, either liquid–liquid or solid–liquid (as in the case of immobilized enzymes), can suffer from reduced reaction rates owing to interphase mass-transfer limitations. Furthermore, immobilized enzymes are susceptible to activity loss owing to the immobilization process and leaching of the enzyme from the solid support. Monophasic systems can avoid these limitations; however, recovery and reuse of the biocatalyst, which is imperative for large-scale processes or the isolation of pharmaceutical products, is more challenging.

Herein, we demonstrate an approach to take advantage of the higher reaction rates of homogeneous biocatalysis while providing a simple method for biocatalyst recycling by using organic–aqueous tunable solvent (OATS) systems. OATS mixtures are engineered to couple a reaction and separation as shown in Figure 1. As in other latent biphasic systems,[5]

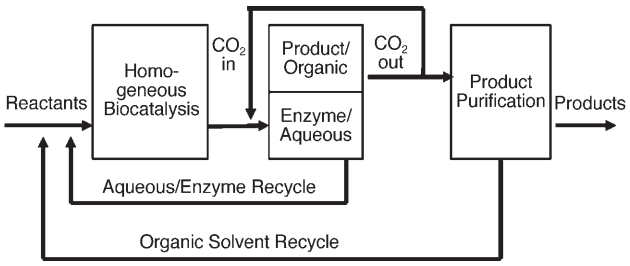

Figure 1. A Proposed OATS process for biocatalyst recycling.

OATS mixtures allow homogeneous reactions between hydrophobic and hydrophilic components, therefore eliminating mass-transfer limitations. CO_2 can be added to split the reaction mixture into a gas-expanded liquid organic phase containing hydrophobic components and an aqueous phase containing the hydrophilic catalyst[6,7] The CO_2-induced separation allows for a one-pot reaction and separation scheme.

The successful application of CO_2 as a reversible switch to modulate miscibility of aqueous and organic phases and the phase-separation behavior for a number of OATS systems has previously been studied with solvents such as acetonitrile, THF, and dioxane.[6,7] Recent investigation of this miscibility switch as a vehicle for catalyst recovery was tested on the hydrophobic substrate 1-octene with a water-soluble Rh–triphenylphosphine tris-sulfonated salt (TPPTS) complex as the catalyst. The use of an OATS system increased the

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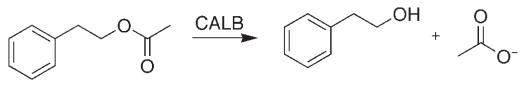
Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

catalytic efficiency by a factor of 65 as compared to the industrially used aqueous biphasic system, which is limited by the substrate solubility.[8]

We tested the feasibility of OATS mixtures for biocatalytic reactions and separations with soluble *Candida antarctica* lipase B (CALB) (E.C. 3.1.1.3). This versatile enzyme has been used to catalyze esterifications of hydrophobic acids and alcohols as well as ester hydrolyses in a range of solvents[9] and supercritical CO_2.[10] This therefore renders it an attractive choice for use in an OATS process. The hydrolysis of 2-phenethyl acetate (PEA) to 2-phenylethanol (2PE) and acetate (Scheme 1) was selected as a model reaction as both PEA and 2PE both have extremely low water solubility and can be easily detected with gas chromatography to assay the reaction progress.



Scheme 1. CALB test reaction.

An OATS process will be most effective when three requirements are met: 1) The use of an OATS mixture must provide acceptable enzyme reactivity in the reaction medium; 2) The biocatalyst must survive the CO_2-pressurized separation process; 3) The biocatalyst should be retained in the aqueous phase and reaction products should partition favorably into the organic phase. Thus, the reactivity, pH stability, recycleability of the biocatalyst, and partitioning behavior of the substrate and product in a water–dioxane OATS mixture were tested. By satisfying these requirements, we have developed a system that allows 80% recovery of reaction products in the organic phase, and displays less than 10% apparent biocatalyst activity loss after recycling six times.

Enzymatic reaction rates in aqueous buffer–dioxane mixtures were evaluated to choose a suitable solvent composition for future recycling experiments (Figure 2). Since PEA is nearly insoluble in water, monophasic reaction rates in buffer alone are negligible. By taking advantage of the higher substrate solubility afforded by the addition of water-miscible organic solvents, higher specific reaction rates can be obtained than in purely aqueous solvent. Although the rates seen in 8 mM PEA in water–dioxane mixtures decreased as the dioxane content increased, the specific rate in water–dioxane mixtures that are nearly saturated with PEA substrate improved over the range of dioxane content tested owing to the higher substrate solubility (up to 56 mM PEA in 40% dioxane).

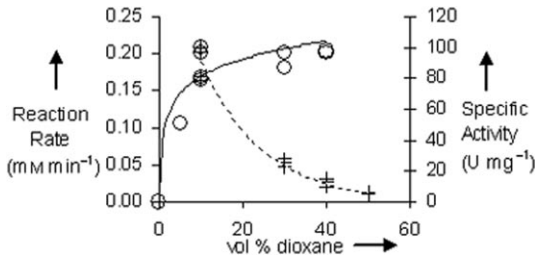


Figure 2. Monophasic reaction rates in dioxane OATS mixtures containing 8 mM (+) and near-saturating concentrations (○) of PEA (4, 8, 32, 56 mM for 5, 10, 30, 40% dioxane, respectively). Assumed trend lines for 8 mM (-----) and near-saturated PEA mixtures (——) have been added. Note that 8 mM PEA is also the near-saturation concentration in 10% dioxane mixtures. The specific reaction rates ($U\,mg^{-1} = \mu mol\,min^{-1}\,mg\ enzyme^{-1}$) are shown on the secondary axis.

A 40% dioxane mixture was chosen for use in further experiments because this mixture provides increased substrate solubility and achieves the highest observable reaction rate. The 40% dioxane mixture is also favorable for the phase separation—upon CO_2 addition it provides a larger organic-phase volume to aid extraction of reaction products than lower dioxane levels, thus improving separation. At greater than 40% dioxane concentration, buffer precipitated out of solution and further study is needed to see if this lower buffer concentration can adequately maintain sufficiently high pH levels. The importance of adequately buffering the reaction mixture is discussed below.

To survive the CO_2-pressurized separation process, the enzyme must withstand the pressure used for separation as well as the associated pH fluctuations. The pressure required to unfold enzymes (>2000 bar)[11] is one to two orders of magnitude higher than that required to separate OATS mixtures (10–50 bar), so it is unlikely that hydrostatic pressure alone will damage the enzyme. However, it is well documented that CO_2 addition to aqueous mixtures leads to a dramatic drop in solution pH owing to the formation of carbonic acid.[12] Such low pH values can have detrimental effects on enzyme activity and stability. We measured pH values of the aqueous phase of CO_2-separated OATS mixtures as a function of CO_2 pressure by using a method similar to Holmes et al,[13] as shown in Figure 3. In unbuffered

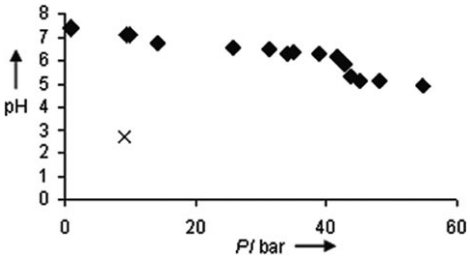


Figure 3. The pH of unbuffered (×) and buffered (150 mM phosphate buffer solution (NaH_2PO_4) (◆) dioxane/water (30:70%) solutions

mixtures, the addition of 9.5 bar of CO_2 lowers the pH to below 3. With 150 mM sodium phosphate buffer solution in the aqueous component, the pH decreases only to ≈5 under 50 bar CO_2. A similar result was also found in previous work.[13] As the typical time required to pressurize, mix, and separate OATS mixtures with CO_2 is approximately 30 min, the aqueous phase containing enzyme is exposed to pH 5 for a similar amount of time. Enzyme tolerance to low-pH exposure was tested by incubating CALB samples in buffered low-pH solutions and assaying for activity at pH 7 (see the Supporting Information). Even after 2 h of exposure to a solution of pH 4, CALB samples showed no loss in activity.

Clearly, use of 150 mM phosphate buffer solution is sufficient to prevent the pH of the pressurized aqueous phase from decreasing to low levels, and CALB is not detrimentally affected by short exposures to pH 5 that are encountered during phase separation.

As the addition of CO_2 and accumulation of acetate ions (a reaction product) can lower the pH value of the reaction mixture, CALB activity was tested in OATS mixtures with varying pH. As the reaction pH was reduced from 8.2 to 4.8, the conversions observed after two hours decreased from 55% to 40%,respectively. So to attain the highest possible reaction rates in OATS, the pH should be carefully controlled.

Having demonstrated improved reaction rates in dioxane–aqueous mixtures that were nearly saturated with substrate and the absence of adverse effects on CALB resulting from pH changes during separation, the feasibility of implementing a process with dioxane–OATS mixtures and CO_2 for separation and recycling of the homogeneous biocatalyst was tested in 40:60 dioxane/buffer (*v*/*v*) mixtures containing PEA. After two hours of reaction, CO_2 was added to separate the mixture. The organic layer was decanted under pressure, and after CO_2 was removed from the cell, the next cycle began by adding buffer solution and dioxane containing PEA (300 mM) to the remaining reaction mixture. The cycle time, from the beginning of one reaction to the next, averaged three hours. Two trials of six consecutive reactions and separations were conducted. In each trial, the reaction mixture remaining in the cell was left overnight after the fourth separation, and the fifth reaction was initiated the following morning by adding more buffer and PEA (300 mM) in dioxane. Exact experimental details can be found in the Supporting Information.

The observed conversion for each two-hour reaction of both trials is shown in Figure 4. Over the concentration ranges

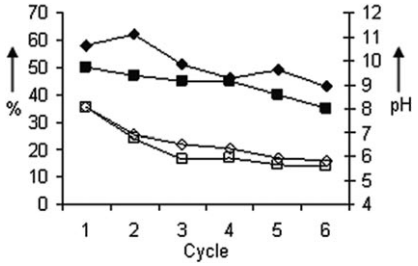


Figure 4. Conversions (◆, ■) and initial pH (◇, □) of recycled OATS reactions for trial A (◆) and trial B (■).

seen during the reactions, the CALB-catalyzed reaction in 40% dioxane mixtures is first order with respect to PEA concentration, with an apparent rate constant of 0.0067 min^{-1}. This indicates that the enzyme kinetics are not substrate saturated at these concentrations (see the Supporting Information). With the given rate constant, a conversion of 55% is predicted for two hours of reaction, and the observed conversions for the first reaction of both trials are very close to this value (58% and 54%, respectively). conversion of 100% is observed in both trials when the reaction mixture was allowed to stand overnight between reactions 4 and 5. An overall process conversion (total moles of 2PE formed/total moles of PEA added) of 61% was obtained for both runs.

The conversion in each trial decreased by 10 to 15 percent by the sixth reaction, suggesting deactivation of CALB. However, since some enzyme is removed as the reactor contents are sampled, the total enzyme concentration in the reactor will be diluted as new solution is added to begin new cycles. This dilution accounts for 11 of the 15 percent of conversion lost by the sixth reaction. Furthermore, the pH of the reaction mixture decreased from 8 to 5.5 over six cycles (Figure 4). This was most likely owing to residual CO_2 that remains saturated in the OATS mixture following the initial separation and accumulation of acetate in the aqueous phase during successive runs. As discussed earlier, CALB reaction rates decrease by up to 20% when reducing the pH from 8 to 5; thus, the pH drift likely explains the remaining observed conversion loss, and better pH control should reduce this effect.

The concentration of PEA and 2PE in the separated organic and aqueous layers was measured as well as the concentration of water in the organic layer. The organic layer, in agreement with previous results, contained approximately 4 wt% water for all cycles.[6] The distribution of PEA and 2PE between the separated organic and aqueous phases in each cycle was measured in both trials and is shown in Figure 5.

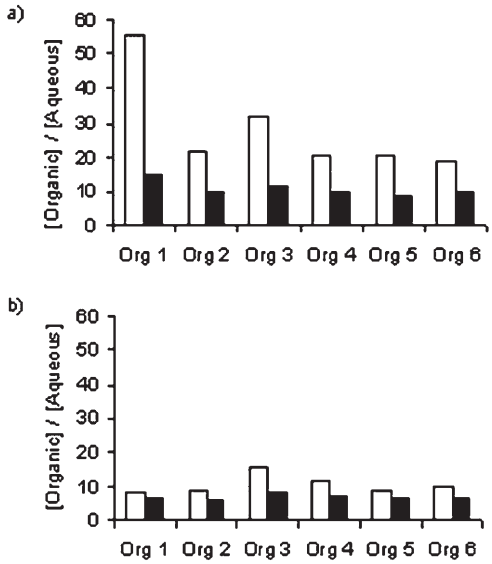


Figure 5. OATS distribution coefficients for recycling experiments beginning with 72 mM (A) and 52 mM PEA (B).

Distribution coefficients in trial A ranged between 20 and 55 for PEA and between 11 and 15 for the more hydrophilic 2PE. Trial B showed lower distribution coefficients for both PEA (8–16) and 2PE (6–9). A higher starting concentration of PEA was used in trial A (72 mM) as compared to trial B (56 mM); so it appears that the distributions may be concentration dependent. As 72 mM is above the solubility of PEA in 40% dioxane mixtures, it is likely that excess (insoluble) PEA partitions to the organic phase might explain the larger distribution coefficients for trial A. This and other factors

contributing to variability are currently being investigated. Regardless, the observed distribution coefficients are favorable and show that the separated organic phase can be used to extract product from the reaction mixture: we recovered 80% of the 2PE product in the organic layer for both trials.

By addressing concerns of reactivity, enzyme stability in separated media, and favorable partitioning, a biocatalytic OATS scheme for processing hydrophobic substrates with product separation and biocatalyst recycling has been developed. Given a cycle time of three hours, 56 mM starting concentration for PEA in buffer–dioxane (40%) OATS, and an average of 50% conversion per cycle, we calculated a volumetric productivity, expressed by the space–time yield, of 27.4 $g L^{-1} d^{-1}$. Even with an equivalent residence time of 2 h in the reactor and assuming a best case scenario of complete conversion, the space–time yield in a PEA-saturated (4 mM) dioxane (5%) mixture cannot exceed 5.9 $g L^{-1} d^{-1}$. This is almost five-times less than that observed in the OATS system. The space–time yield in pure buffer will be even lower as the substrate is nearly insoluble. Furthermore, we find that the enzyme can be recycled with very little activity loss between cycles. The activity loss observed is entirely due to dilution of the enzyme and pH decrease of the reaction medium. A larger reaction volume, decreased sampling, and improved pH control should minimize activity loss even further. Even better product recoveries could be obtained by using a reaction with more hydrophobic products.

Biocatalytic OATS reaction–separation schemes fulfill an identified need[14] to develop new options to meet current challenges in biochemical synthesis. By integrating reaction and separation, simpler and simultaneously more efficient processes with a reduced physical footprint can be designed. The work here shows, for the first time, that biocatalysis in OATS is feasible and can be an effective option for designing biocatalytic processes, especially when hydrophobic substrates are involved. This also opens the door to combining OATS with other biocatalysts and their array of unique chemistries to efficiently synthesize chiral products.

Experimental Section

Candida antarctica lipase B (SOL-101) was a kind gift from Biocatalytics (Pasadena, CA) and was diluted 100 times by volume with 150 mM sodium phosphate buffer solution (NaH_2PO_4; pH 7.12) before addition to reactions. Supercritical fluid chromatography (purity > 99.9999%) grade CO_2 was purchased from Airgas (Radnor, PA) and used without further purification.

OATS reaction mixtures containing the desired amount of dioxane (99%, Sigma), PEA (99%, Alfa Aesar), and 150 mM sodium phosphate were prepared and 1:100 diluted CALB was added in a ratio of 0.5 mL enzyme per 9.5 mL OATS mixture. 0.25 mL samples were removed periodically and immediately mixed 1:1 with a mixture of 1:1 glacial acetic acid/dioxane to quench the reaction. Reaction progress was followed by measuring the PEA and 2PE content of samples by using an Agilent GC-FID with a DB17 column (Agilent model 6890).

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