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BIOENGINEERING, FOOD, AND NATURAL PRODUCTS

Fumed Silica Activated Subtilisin Carlsberg in Hexane in a Packed-Bed Reactor

Peter H. Pfromm and Mary E. Rezac
Dept. of Chemical Engineering, Kansas State University, Manhattan, KS 66506

Kerstin Würges and Peter Czermak
University of Applied Sciences Giessen, Giessen, Germany

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Enzymatic catalysis in organic solvents can enable applications, reactivities, and selectivities that are not accessible in water. The use of enzymes for catalysis in organic solvents has been somewhat hampered by their relatively low-catalytic activity. A new preparation of the enzyme subtilisin Carlsberg (SC) with fumed silica as support (FS) has been developed. This preparation matches and sometimes exceeds the activity of some of the best reported preparations of the same enzyme for use in solvents. The usefulness of our preparation in a packed-bed reactor is explored here. The catalytic characteristics of our preparation containing 5 wt % of enzyme/95 wt % FS in a packed-bed reactor for a model transesterification in hexane is determined. Mass-transfer limitations are explored, and a comparison to batch results is given. The activity of our preparation after prolonged storage as a packed bed is investigated. Our immobilized enzyme preparation may be a step towards more economical enzymatic catalysis in organic solvents. © 2006 American Institute of Chemical Engineers *AIChE J,* 53: 237–242, 2007
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Introduction

The catalytic activity of some enzymes in single-phase nonaqueous solvents has been reported some time ago, and the seminal work by Klibanov and coworkers opened the field of nonaqueous enzymology.[1–3] An excellent review and outlook for enzymatic catalysis in solvents is available.[4]

Enzymes are renewable catalysts, and they exhibit a wide array of attractive reactivities and selectivities in nonaqueous solvents[2,3,5–9] that are often not accessible in water. The thermal and storage stability of enzymes can be significantly enhanced in nonaqueous solvents.[3,5,10] Other advantages include the option to label some products as "natural" which is not possible with chemical catalysis,[11,12] and reduced concerns with catalyst residues. The relatively low-catalytic activity of many enzymes in nonaqueous solvents, however, is of concern in regard to industrial applications.[13–15]

Enzymes are generally not soluble in nonaqueous solvents that do not denature the enzyme, and immobilization of enzymes on a solid support is often applied when enzymes are used in nonaqueous solvents.[16] Some of the most successful attempts to date to obtain highly active enzyme preparations for use in nonaqueous solvents involve immobilization on porous polymeric beads,[17] or lyophilization with salts like potassium chloride, often termed salt activation.[18–20] Hypotheses have been put forward that the type of salt is of significance for salt activation. Others have hypothesized that overcoming mass-transfer limitations by finely dividing the enzyme on a solid surface is the main mechanism of the apparent enzyme activation.[21–23]

We have recently reported on the significant activation of subtilisin Carlsberg for use in hexane by colyophilization of

Correspondence concerning this article should be addressed to P. H. Pfromm at phromm@ksu.edu
Current address of K. Würges: Forschungszentrum Jülich, Jülich, Germany.

the enzyme from the aqueous phase with fumed silica (FS).[21] Fumed silica is a fractal amorphous material, and is made as a combustion aerosol where nucleation and growth of primary particles (several nanometers in diameter) is followed by sintering of these particles to form agglomerates (overall dimensions up to the micrometer range).[24] Virgin fumed silica physically appears as extremely low-density white flocs somewhat resembling fresh powder snow in size, consistency, and density. Fumed silica has been used for many years on a very significant scale, but the structure and chemistry are still under investigation.[25]

The fumed silica/enzyme preparation that we have developed is here tested in a tubular packed-bed reactor since this is often a preferred configuration for biocatalysts in the production of fine chemicals and pharmaceuticals.[26] We are using "packed bed" here in the traditional sense[27] of a tubular reactor filled with solid catalyst particles (in our case a biocatalyst supported on inert support particles). The focus here is the catalytic competency of the enzyme preparation when used as a packed bed. Properties of the packing beyond the superficial density, and the pressure drop were not investigated.

In summary, the work presented here documents the usefulness of a new activated enzyme preparation in form of a packed bed for catalysis in hexane. This preparation reaches or exceeds the activity shown previously for what is often termed "salt activation" of enzymes. Comparison is made to previous batch experiments.

Materials and Methods

Materials

Subtilisin Carlsberg (SC, EC 3.4.21.14; proteinase from *Bacillus licheniformis;* specific activity of 8 units/mg solid), fumed silica (purity 99.8%, specific surface area 258 m^2/g, particle diameter 7–50 nanometer, manufacturer's data), and nonadecane (puriss. p.a., internal standard for gas chromatography) were obtained from Sigma-Aldrich (St. Louis, MO).

The morphology of fumed silica can be described on several levels with increasing length scale.[28,29] Fumed silica consists fundamentally of solid (nonporous) primary amorphous silica particles formed by chemical reaction from silica compounds in a flame. The primary particles measure a few nanometers in diameter. During the manufacturing process, the primary particles then form necklace-like agglomerates with many primary particles in one "necklace." These largely linear but tortuous agglomerates entangle to form the very low-density white flocs that are visible to the naked eye when inspecting as-received fumed silica.

N-acetyl-*L*-phenylalanine ethyl ester (APEE, purity >99%) was obtained from Bachem California Inc. (Torrance, CA). KH_2PO_4 (purity >99%), hexane (optima grade, purity >99.9%) and anhydrous 1-propanol (purity >99.9%) were from Fisher Scientific (Pittsburgh, PA), and were of the highest grade commercially available. The solvents were stored over 3Å molecular sieves (4–8 mesh beads, Fisher Scientific) for at least 24 h prior to use. The enzyme preparations were prepared and stored at −20°C in 15 mL flat-bottom glass vials closed with Teflon-lined screw caps after lyophilization. Batch activity assays were performed in 15 mL Teflon screw capped round bottom test tubes. All glassware was low-extractable borosilicate glass (Fisher Scientific).

Analytical

To determine the amount of the enzymatically synthesized *N*-acetyl-*L*-phenylalanine propyl ester (APPE), 400 μL aliquots of the effluent from the packed-bed reactors were analyzed via gas chromatography (GC, 1 μL sample, Varian Model 3800, Varian Analytical Instruments, Sugar Land, TX; DB-5 capillary column, 30 m, 0.25 mm I.D., 0.25 μm film thickness; J&W Scientific Inc., Folsom, CA; helium carrier gas 1.3 mL/min, 1/400 split, injection/detection at 250°C, linear column temperature ramp 150–210°C at 8°C/min; alternatively, a Hewlett Packard HP6890 gas chromatograph with similar parameters was used). The analytical procedures for batch reactions were similar, and are reported in detail elsewhere.[21] Samples from batch reactions were first centrifuged to remove enzyme preparation and aliquots of the supernatant were then analyzed as described earlier. The standard deviation for quadruple analysis of the samples for each datapoint in Figure 4 is less than the size of the plot symbols shown. The same is true for representative samples analyzed multiple times for Figure 3.

Fumed silica/enzyme preparations

Details on our preparation can be found elsewhere,[21] and an overview will be given here. As-received SC was dissolved in a 10 mM potassium phosphate buffer (pH 7.8, room-temperature). As-received fumed silica was added to reach 0 to 95 wt % of silica relative to the total final enzyme preparation mass. The aqueous enzyme/silica/buffer mixtures were sonicated for 10 min in a water bath. A 3 mL aliquot per sample was then frozen in a 15 mL glass vial by immersing in liquid nitrogen for 20 min. The enzyme preparations were then lyophilized for 72 h (48 h primary drying, 24 h secondary drying, VirTis model 10-MR-TR; Gardiner, NY).

For the packed columns we chose 95 wt % of silica since our previous work showed that the catalytic activity of the preparation per gram of enzyme is to a large extent maximized at this composition.[21]

Two different batches of enzyme preparation were used for the work presented here. The data in Figure 3 was obtained with one enzyme preparation batch (A), while the data in Figure 4 and Figure 5 were obtained with a second batch (B) prepared independently.

Column packing and operation

The packed bed reactors were always operated in a temperature controlled air bath (Figure 1, 30/−0.2°C) with a 300 mL sample bomb as reactant reservoir. The hydrostatic pressure to force liquid through the bed was provided by compressed nitrogen (Linweld, Manhattan, KS). The nitrogen pressure was set between 30 and 180 psi depending on the bed height and the flow rate of the reactant solution (0.27–2.05 mL/min).

The enzyme/fumed silica preparations appeared as a free-flowing white powder after lyophilization. Sections of 1/4″ stainless steel tubing (ID = 4.6 mm; packed bed lengths 29–220 mm) were used as housings for the packed beds (Figure 2). The beds were packed by manual compaction of the enzyme preparation using a stainless steel rod (OD = 4.5 mm) onto a glass wool plug resting on a metal frit installed at one end of the stainless steel tubing section. Another glass wool plug and

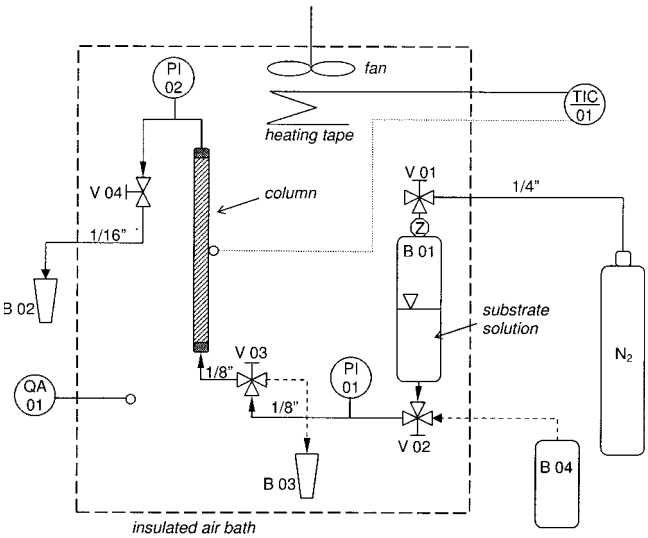

Figure 1. Test setup for catalysis by FS/SC preparations as packed beds. Substrate solution is pumped through the column by pressure applied from a compressed nitrogen cylinder.

The column, all tubing and valves, and the substrate solution reservoir B01 were stainless steel. Samples were taken at B02 and B03. B04 is a wash bottle containing the substrate solution to be loaded into B01 prior to an experiment. A combustible gas detector was used to monitor for hexane leaks (QA01).

frit was then used to close the column. Consistent superficial densities of the packed beds of 0.22 mg enzyme preparation/mm^3 for all bed heights indicated that the packing procedure was reproducible. The bed-void volume was about 90% based on the mass of enzyme preparation that was used to prepare the bed, the overall volume of the bed after packing, and the bulk density of amorphous silica (taken as 2.2 g/cm^3), neglecting the volume occupied by the enzyme residing on the surface of the silica. The packed columns were rinsed with hexane and stored capped and filled with hexane at -20°C.

The substrate concentration was always $[S]_0 = 36.31$ mM APEE in the hexane fed to the beds. The flow rate through the beds was determined volumetrically within less then 2% of the reported value. Steady state was assumed after about twice the average residence time of feed solution in the packed bed had elapsed.

Results and Discussion

The purpose of this work is to confirm results from previous batch tests for a novel enzyme preparation in a continuous packed-bed reactor. Plug flow with negligible axial dispersion can be safely assumed here.[30] Lortie and Pelletier have investigated the plug flow assumption in immobilized enzyme reactors and they conclude.[30] "The results obtained in this study explain the findings of previous works (...) stating that it is not necessary to take the axial dispersion into account to describe an immobilized enzyme fixed-bed reactor at steady state."

We shall estimate the Peclet number (Pe_a) in the axial direction, representing the ratio of the rate of transport by convection relative to the rate of transport by diffusion,[31] to further support the assumption that plug flow is a good approximation. The overall average fluid velocity V in our tubular packed-bed reactor (neglecting the about 10 vol % of the packed bed occupied by fumed silica, which is a worst case "minimum" assumption in regard to Pe, see below) is

$$V = (1.4\ \text{cm}^3/\text{s})/0.166\ \text{cm}^2 = 8.4\ \text{cm/s} \quad (1)$$

Diffusion coefficients D in liquids are generally on the order of 10^{-5} cm^2/s.[32] For the shortest of our reactors (worst case) with a length $L = 2.9$ cm we can estimate

$$\text{Pe}_a = V\,L/D = 8.4\ \text{cm/s}\ 2.9\ \text{cm}/1 * 10^{-5}\ \text{cm}^2/\text{s} = 2.4 * 10^6 \quad (2)$$

This estimate indicates clearly that dispersion is not important, in accord with Lortie and Pelletier's conclusion.[30]

The Michaelis-Menten parameters K_m and V_{max} for the catalyst preparation used here (95 wt % FS/5 wt % SC), were determined previously in batch reactions (5 mL) where external mass transfer was assumed negligible,[21] and found to be $K_m = 14.6$ mM and $V_{max} = 116.4$ μM/min mg$_{enz}$. These values can only be matched by packed-bed reactors when mass-transfer limitations are negligible and the substrate concentration is sufficient (see below).[33] To determine when mass transfer limitations become negligible one can increase the flow rate through the packed bed in steps, and observe the rate of reaction achieved.[34,35] The performance of the batch reactions should be approached at high-flow rates in the packed bed.

The packed beds were operated at 36.31 mM substrate concentration $[S]$ in the feed which exceeds K_m derived from batch experiments substantially. If the conversion in a single pass through the bed is not excessive then zero-order reaction in $[S]$ can be used as a good assumption, and the packed-bed performance should approach the batch reaction data reported previously.

Determination of mass-transfer limits

The native fumed silica particles are essentially non-porous so that external mass transfer in the hydrodynamic boundary layers around the particles and particle agglomerates, but not diffusion limitations in the particles is of concern. Since the reaction rate v with which substrate is converted into product is strongly dependent on the actual substrate concentration, that is available to the enzyme, the stagnant-boundary layer between bulk solution, and the silica surfaces where the enzyme resides should be minimized.[34] This minimizes diffusional limitations that can lead to low-apparent reaction rates. Conversions were calculated by analyzing the effluent from the bed at some time t (at steady state conditions) for the substrate

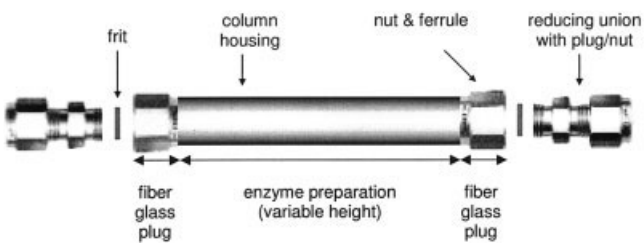

Figure 2. Packed column.

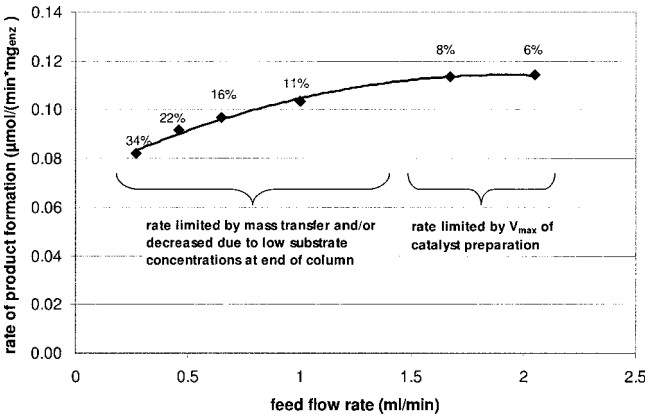

Figure 3. Rates of product formation and % conversion in a packed-bed reactor (bed height 220 mm, 0.8018 g enzyme preparation, enzyme preparation batch A).

Mass-transfer limitations are considered negligible above about 1.4 mL/min based on this data. The line was added to guide the eye.

concentration $[S]_t$, and calculating the conversion according to

$$\%\text{Conversion} = \left(\frac{[S]_0 - [S]_1}{[S]_0}\right) * 100 \qquad (3)$$

Figure 3 shows the steady-state conversions and the rates of product formation at different feed flow rates for a packed bed (bed height h = 220 mm, dia. 4.6 mm, enzyme preparation mass 0.8018 g). A feed flow rate above about 1.4 mL/min appears sufficient to avoid mass-transfer limitations, and the possibility of a rate of reaction higher than zero-order since no significant further increase of the rate of product formation with increased flow rate is observed. The conversion had decreased to near 7% when mass-transfer limitations became negligible. At this relatively low-overall conversion the reaction will be assumed here to take place with an essentially constant order of zero along the packed bed since the product formation approaches a constant value as the feed flow rate is increased.

We conclude that mass-transfer limitations can be excluded at flow rates above about 1.4 mL/min, similar to results reported elsewhere.[36] All further reactions were performed at flow rates above 1.4 mL/min to investigate the behavior of the enzyme preparation with negligible mass-transfer limitations.

Influence of catalyst bed height on substrate conversion

The steady-state conversions and the rates of product formation for different packed-bed heights at a constant feed flow rate of 1.44 mL/min are shown in Figure 4. It is important to that all data in Figure 4 and Figure 5 was obtained using the same batch B of enzyme preparation. This batch B was prepared independently from the enzyme preparation used for Figure 3 (Batch A). Data from Figure 3 can, therefore, only be used for qualitative comparison with the data in Figures 4 and 5. However, since the rate of product formation is higher for the batch B used for Figures 4 and 5 than for batch A

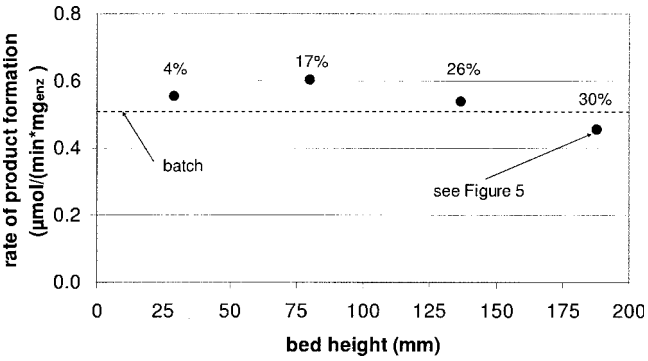

Figure 4. Rates of product formation and % conversion for different bed heights.

The rates for the packed beds are generally on the order of the value expected from a batch reaction with an enzyme preparation that was prepared using the same procedures (feed flow rate 1.44 ± 0.03 mL/min, enzyme preparation batch B, 30°C, substrate concentration $[S]_0 = 36.31$ mM).

(Figure 3), the assumption of absence of mass-transfer limitations is still valid.

The relatively constant substrate conversion over the whole length of the columns confirms that the reaction is zero-order. The rate for a packed-bed height of 188 mm decreases somewhat to about 0.43 μmol/(min mg_{enz}) when compared to the average value of the three other bed heights. This may be due to typical issues with small-scale packed beds like bypassing or channeling in the bed,[37] nonuniform packing, bed compression, and so on. A linear regression ($R^2 = 0.97$) gives an average conversion of 0.17% per mm packed-bed depth.

A catalyst preparation prepared using the same procedure that was used to manufacture the packing for the columns was tested in our previous work[21] in batch reactions at identical initial substrate concentration as in the packed beds. The specific rate of product formation in the batch experiments was 0.511 μmol/(min*mg_{enz}) and was, thus, within 10% of the continuous packed-bed results (Figure 4). The use of our preparation in form of a packed column did apparently not signifi-

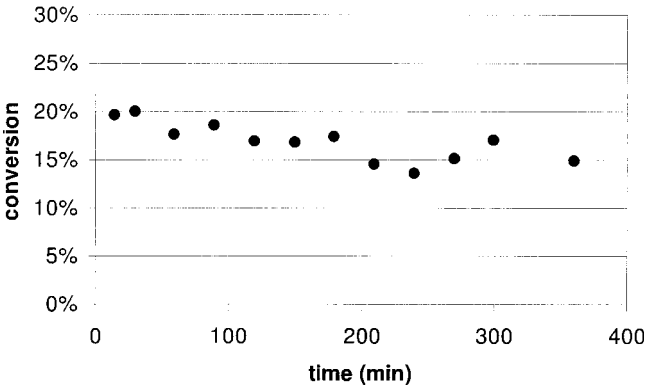

Figure 5. Packed-bed conversion after storage under hexane for 15 days at −20°C (bed height 188 mm, same bed as 188 mm in Figure 4; feed flow rate 1.3 ± 0.02 ml/min).

The performance after prolonged storage is diminished from the initial value of 30% conversion in Figure 4.

cantly diminish the reactivity. We conclude that fumed silica may be a useful support to activate enzymes in solvents, such as hexane when packed-bed reactors are to be employed. In regard to the variability of the catalytic performance of the enzyme preparations from batch to batch one might consider that the results of the packed-bed reactors investigated here, and the previous preparations investigated in batch experiments agree to a significant extent.

Operational stability of packed bed reactor over period of 6 h

The operational stability of the packed bed reactor using the column with a bed height of 188 mm (see Figure 4) was studied during 6 h of continuous operation with a feed containing 36.31 m*M* APEE in hexane (30 ± 0.2°C; 1.3 ± 0.02 mL/min). The column had been rinsed with pure dry hexane (stored over 3 Å mol. sieves for at least 24 h), closed and stored at −20°C for 15 days after the previous tests reported in Figure 4. Figure 5 shows that the conversion decreased about 41% compared to before storage when a conversion of 30% was observed. There is also a slight downward trend from about 20% to 15% conversion (Figure 5) during this test. This loss in conversion is comparable to results in the literature.[38] The reasons could be compression of the catalyst bed causing less favorable flow conditions, or loss of enzyme activity. Nonetheless, it is encouraging to see that substantial activity remained after prolonged storage.

Concluding Remarks

The outstanding catalytic performance of a novel enzyme preparation (95 wt % fumed silica and 5 wt % subtilisin Carlsberg) previously investigated in batch experiments was confirmed here in a continuous packed-bed reactor. The regimes of mass-transfer limitation were explored. One packed bed was characterized, stored for an extended time, and then tested again. This bed showed reduced activity after storage but the resilience to this test is still encouraging for practical applications.

The path forward should certainly include that the promising results for this enzyme preparation should be extended to other enzymes and reactions to show a more general applicability. The mechanical properties of the packed beds should be investigated to move towards larger reactors. The loading of the fumed silica support with the biocatalyst also should be balanced against the reactor pressure drop (possibly modified by adding an inert packing material), reaction kinetics, and stability over time to find an optimum for the use of the valuable catalyst.

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