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Cartridge filter systems containing immobilized enzymes Part I. Concept and features

Steven M. Heilmann [a,*], Gary J. Drtina [b], Phillip D. Eitzman [c], Louis C. Haddad [d], Patrick L. Coleman [a], Fredrick W. Hyde [e], Todd W. Johnson [f], Jerald K. Rasmussen [a], Howell K. Smith II [a], Jie J. Liu [a], Robert T. Fitzsimons [a], Michael G. Williams [g], Stephanie J. Moeller [a], Masayuki M. Nakamura [a], Kelly J. Gibbens [a], Tara L. Buhl [a]

[a] *Corporate Research Materials Laboratory, 3M, 3M Center 201-2N-20, St. Paul, MN 55144-1000, United States*
[b] *Film & Materials Resource Division, 3M, 3M Center 236-1B-22, St. Paul, MN 55144-1000, United States*
[c] *Occupational Health and Environmental Services Division, 3M, 3M Center 235-2B-86, St. Paul, MN 55144-1000, United States*
[d] *Bioanalytical Technology Project, 3M, 3M Center 270-2A-08, St. Paul, MN 55144-1000, United States*
[e] *Corporate Research Analytical Laboratory, 3M, 3M Center 201-BW-11, St. Paul, MN 55144-1000, United States*
[f] *Filtration Laboratory, 3M, 60-1W-17, Mendota Heights, MN 55120, United States*
[g] *Medical Division, 3M, 3M Center 270-3N-02, St. Paul, MN 55144-1000, United States*

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Abstract

Immobilized enzyme particulates positioned on the upstream surface of a cartridge filter were shown to provide effective catalytic conversion of a recirculating solution of reactant to product. This arrangement provided for low-pressure drop operation and minimal pH change across the catalyst bed, and no measurable loss in catalytic function was observed over the course of tens of reaction cycles in the laboratory. Although 60 larger scale (100 L) reaction cycles were insufficient to conclusively differentiate the cartridge filter reaction system arrangement from slurry batch operation, isolation of the enzyme from mechanical and chemical stresses normally associated with slurry batch processing operations is expected to lead to enhanced catalyst lifetimes when conducting the 500–600 reaction cycles that are typically desired for industrial biocatalytic systems.

Keywords: Pig Liver Esterase; Penicillin G acylase; Cartridge filter reaction system; Immobilized enzyme; Biocatalysis

1. Introduction

Immobilized enzymes have been extensively utilized to conduct industrial scale synthetic reactions, often being employed in batch processing operations in which the immobilized enzyme particles are simply added to a stirred reactor [1]. There are at least two major problems associated with the slurry or batchwise utilization of an immobilized enzyme to conduct repetitive industrial syntheses. First, mechanical agitation required to provide adequate mass transfer of reactant to the heterogeneous catalytic site generally causes significant physical deterioration of the support particles. Initial catalyst separation operations that can be as simple as allowing the particles to settle and providing a means to essentially decant the supernatant become substantially more complex and costly as the catalyst particles fracture, eventually requiring centrifugation to separate catalyst from product solution. A second and equally problematic issue with many enzyme-catalyzed industrial reactions is pH control. Often an acidic or basic byproduct is created requiring addition of a pH correcting complementary basic or acidic solution to maintain a stable environment for the enzyme. Relatively high product solution concentrations are desired to facilitate product isolation, so typically very concentrated pH correcting solutions are utilized. When added to a heterogeneous reaction mixture and before homogenization can occur, extreme pH conditions can momentarily exist in the vicinity of a subset of the immobilized enzyme particles, causing irreversible damage and loss of catalytic function and activity.

* Corresponding author.
E-mail address: smheilmann@mmm.com (S.M. Heilmann).

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One of the most time-honored and effective methods of removing a particulate from a liquid stream is to utilize cartridge filters that exhibit significant particle removing capacities and the ability to rapidly process the stream. In one common arrangement, a filtering element of defined porosity is configured perpendicularly to the flow of the liquid stream in an arrangement known as direct flow filtration (also known as dead end filtration). This provides for deposition of the particles on the upstream surface of the filter element and a "clean" downstream filtrate.

It occurred to us that an arrangement of a shallow bed of immobilized enzyme particles positioned on the upstream surface of a cartridge filter could be very useful for conducting industrial scale synthetic operations. In contrast to conventional column or plug flow reactor arrangements of immobilized enzyme particles, a reactant solute flowing through a cartridge filter would be challenged by a catalytic column of particles having a very wide frontal surface area and shallow bed depth, and high conversion of reactant to product could conceivably be accomplished by multiple recirculation through the device. Configured in this fashion with the sensitive catalyst contained in a separate module and isolated from the mechanical and chemical stresses occurring in the reactor, catalyst separation issues and destructive changes in pH in the vicinity of the biocatalyst would be completely eliminated. A very thin column of particles would also allow for low pressure drop operation and cause small changes in pH to occur across the bed per pass which has been a source of instability and poor conversion with certain immobilized enzymes employed using reactor arrangements involving deeper catalyst beds [2]. A further advantage of the shallow bed depth is that low pressure drop operation could be maintained even with very small immobilized enzyme particles and their attending advantages in terms of high diffusion rates compared to larger macroporous particles. This proposed catalytic system should also scale fairly easily, as several multi-cartridge assemblies are presently available that offer a parallel arrangement of cartridge filters.

A highlight of our work describing 2-alkenyl azlactone technology has appeared [3] that contains brief mention of cartridge filters modified by inclusion of immobilized pig liver esterase (PLE). This report more fully examines the cartridge filter reaction system (CFRS) and discloses investigations that establish important process parameters for the system for conducting preparative organic reactions with both PLE and Penicillin G acylase (PGA). The synthetic potential of CFRS was shown to be essentially equivalent to slurry batch operation in terms of catalytic efficiency and conversion of reactant to product in a time efficient manner, while providing complete elimination of catalyst removal operations from product solutions as an inherent feature. The issue of greater longevity of an immobilized enzyme in CFRS due to its isolation from the chemical and mechanical rigors normally associated with stirred tank reactors was supported, but conclusive support for this latter performance feature awaits results from several hundred reaction cycles.

2. Experimental

2.1. Materials, equipment and methods

Synthesis conditions for Emphaze™ AB 1 Biosupport Medium, which is a macroporous, azlactone-functional polymer support has been reported [4]. As normally supplied by 3 M, the reactive support typically has an average particle diameter of about 60 μm. The Emphaze™ supports utilized in the present study consisted of fines from that manufacturing process which passed through a 38 μm sieve and had particle diameter sizes between 6 and 40 μm (mean = 21 μm). This size range was ideal for penetrating between the pleat tips of the cartridge filters and providing a more uniform layer of particles over the entire filter element surface area. The dispersion copolymer HEMA-30 was a terpolymer of trimethylolpropane trimethacrylate (50 pbw), 2-vinyl-4,4-dimethylazlactone (20 pbw) and 2-hydroxyethyl methacrylate (HEMA, 30 pbw) and was prepared according to a published procedure [5]. Eupergit™ C, pig liver esterase (PLE, EC 3.1.1.1), Penicillin G, potassium salt and 4-(2-hydroxyethyl)piperazine-1-propanesulfonic acid (EPPS) were purchased from Sigma. Phosphate buffered saline (PBS) was either purchased from Sigma or made directly from the reagents specified. Penicillin G acylase (PGA, EC 3.5.1.11) was obtained from Pharma Biotechnologie Hannover (Hannover, Germany). 1-Methoxy-2-propanol acetate (MPA), dimethyl 3-methylglutarate (DMMG), and triacetin were purchased from Aldrich. Cartridge filters were obtained from 3 M (Filtration Products, St. Paul, MN), and two models were utilized in the present study: (1) 3 M High Capacity Liquid Filter Cartridge Model 313B having an all polypropylene filter element with a 2 μm pore size rating, dimensions of 7.6 cm (diameter) × 25.4 cm (height), and 8361 cm^2 of filter element area; and (2) 3 M High Capacity Liquid Filter Cartridge Model 743B having an all polypropylene filter element with a 2 μm pore size rating, dimensions of 17.8 cm (diameter) × 101.6 cm (height), and 139,350 cm^2 of filter element area. The filter housing for the 25.4 cm units (Model PSCL) was purchased from Ametek Inc. (Sheboygan, WI), and a 3 M 740 Series Filter Housing was employed for the 101.6 cm cartridges. For 25.4 cm unit operation, a Millipore Peristaltic Pump (Model 802G230) and Norprene™ tubing were utilized for all particulate loading and synthetic operations conducted with flow rates of 1400 mL/min and higher. A smaller MasterFlex Model 7520-00 peristaltic pump was utilized for synthetic operations conducted with flow rates of 550 mL/min and lower. The pH stat arrangement consisted of a New Brunswick Scientific titrant pump Model pH 4000 (Edison, NJ), an Ingold pH Electrode (Model 9100232, Wilmington, MA), and a 100 mL burette containing the standard NaOH titrant. Homogenization of dispersion copolymer supports was conducted using a Willems Polytron Model PT 10-35 Tissue Homogenizer (available from Brinkman Instruments). GC Analyses were performed using a Hewlett-Packard Model 5880A Gas Chromatograph equipped with a DB-1 Column. Particle sizes were determined using a Coulter LS-100 Particle Size Analyzer.

2.2. Immobilized PLE and PGA materials

HEMA-30/PLE and Emphaze™/PGA materials were prepared according to literature procedures [6,7]. Parenthetical numerical percentages refer to percent enzyme coupled to the reactive support on a weight basis.

2.2.1. Emphaze™/PLE (3.4%)

Into a 5 L, three-necked, round-bottomed flasked equipped with a mechanical stirrer were charged sodium citrate dihydrate (588 g; 2.0 mol), sodium dihydrogen phosphate (6.7 g; 0.05 mol), disodium hydrogen phosphate (34.5 g; 0.24 mol) and 2.86 L of deionized water. The pH of the resulting solution was adjusted with a small quantity of 1.0 M NaOH to 7.4. PLE (7.70 g) was added and dissolved in the media. Emphaze™ particles (220 g) were added and stirring was maintained at room temperature for 24 h to ensure coupling. The mixture was filtered, the filtercake was washed with PBS (pH 7.4) (3 × 600 mL), and the moist filtercake was transferred to a sealed container and refrigerated until use.

2.2.2. Preparation of Eupergit™ C/PLE (3.4%)

A variation of a published procedure [8] was employed. Into a three-neck, round-bottomed flask equipped with a mechanical stirrer were added 2100 mL of 1 M NaH_2PO_4 at pH 7.5 and 7.70 g of PLE. Eupergit™ C (220 g) was added, and the mixture was stirred slowly at room temperature for 24 h. The mixture was filtered using a medium porosity sintered glass funnel, and the filtercake was washed successively with 2 × 1500 mL of NaCl (1 M) and sodium phosphate (0.1 M) at pH 7.5, followed by 3 × 1500 mL washes with PBS (pH 7.2). The moist filtercake was collected, stoppered well, and refrigerated until further use.

2.3. Cartridge filter loading procedures

2.3.1. HEMA-30/PLE support

Ten grams HEMA-30/PLE (2.2%) support (particle diameters ranging from 6 to 112 μm; obtained by treatment of the enzyme catalyst in 600 mL of 0.05 EPPS pH 8.0 buffer with the Tissue Homogenizer at medium speed for 5 min) were resuspended in the same buffer solution for the loading procedure. The cartridge loading apparatus consisted of a Millipore pump connected with Norprene™ tubing to an Ametek Water Housing, which contained a 3 M Model 313B cartridge filter. A 6 L beaker equipped with efficient magnetic stirring and 5 L of 0.05 M EPPS buffer (pH 8.0) was connected to the system by immersion of both inlet and outlet tubing ends beneath the surface of the liquid in the reservoir to minimize air bubble formation. The filter housing was transparent and allowed visual inspection of the cartridge filter. The pump was turned on with a flow rate of at least 4 L/min and ranged as high as 8 L/min. The purpose of the particle loading operation was to cause the particles to penetrate deeply within the pleats, and relatively high flow rates were employed for that purpose. Flow through the system was conducted for at least 15 min before the first addition of the HEMA-30/PLE slurry to ensure that the cartridge was adequately wetted. The initial pressure drop across the unloaded cartridge filter was 3 psi (0.02 MPa). Portions (100–150 mL) of the slurry were added, and the turbid reservoir mixture was stirred briskly and allowed to clarify before the next slurry addition. Loading required about 20 min to complete and generally corresponded to a pressure drop increase of about 5 psi (0.034 MPa), i.e., the final pressure drop across the loaded cartridge was 8 psi (0.055 MPa). When not in use, the liquid was aspirated from the housing, and the damp cartridge filter, housing and particulate contents were stored at 5 °C.

2.3.2. Emphaze™/PGA supports

An identical procedure to the above PLE operation was conducted with the PGA supports, except the reservoir buffer consisted of 0.01 M potassium dihydrogen phosphate and 0.10 M potassium chloride (pH 7.8).

2.4. Cartridge filter reaction system (CFRS) arrangement

The reaction system (depicted in Fig. 1) consisted of a particle-loaded catalytic filter housing module as described above, a 5.0 L round-bottomed flask reservoir, and a peristaltic

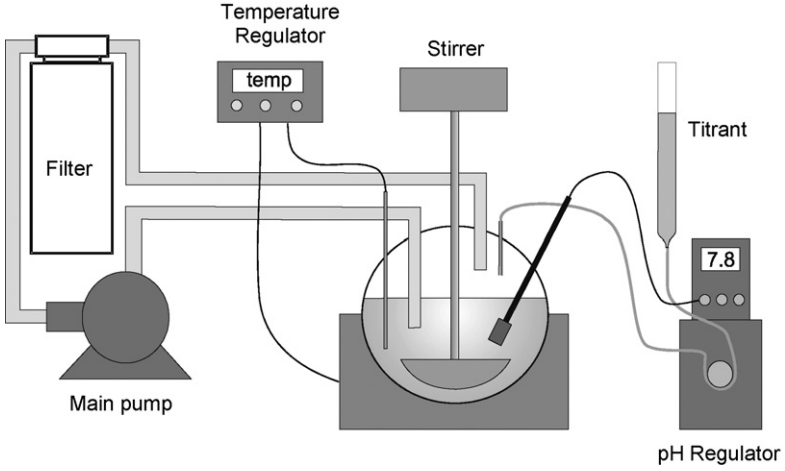

Fig. 1. Cartridge filter reaction system (CFRS).

pump with connecting Neoprene tubing. The reservoir was further configured with a mechanical stirrer, pH stat, and temperature controller. Progress of the desired reaction could be monitored from the volume of the standard base solution added by the pH stat to compensate for pH changes occurring during the reaction. The substrate was typically dissolved in a relatively low capacity buffer solution such as 0.01 M KH_2PO_4 also containing 0.10 M KCl at pH 7.8. In an alternative arrangement, pH control was maintained by simply employing excess buffer capacity to compensate for pH changes in the desired preparative reaction, and the progress of reaction was monitored by GC.

2.5. Synthetic reactions conducted using CFRS

2.5.1. Propylene glycol monomethyl ether acetate (PGMEA) in single-pass operation

A total of 16 L of a solution containing 0.10 potassium phosphate buffer (pH 7.2), 0.05 M PGMEA, and 28 mL of *N*,*N*-dimethylacetamide (as an internal standard for GC analysis) was prepared in a large vessel and passed through a horizontally pleated (3 M Model 313B; 25.4 cm × 7.6 cm) cartridge filter that contained 10 g of HEMA-30/PLE (2.2%) at various flow rates. The volume of the catalytic module including filter, housing and tubing was approximately 1280 mL, and, with each flow rate condition, 2000 mL of solution were passed before an "equilibrated" sample was obtained for GC analysis. Two peristaltic pumps were utilized in the study that provided a range of flow rates to be examined from 180 to 5825 mL/min.

2.5.2. PGMEA in recirculation CFRS

Employing a modified version of CFRS with no pH stat controller, 4 L of 0.10 M EPPS (pH 8.0) buffer, *N*,*N*-dimethylacetamide (7.02 mL; as an internal standard for GC analysis), and PGMEA (26.4 g; 0.20 mol) were added to the 5 L flask. Recirculation of the reservoir solution through the same catalytic module as in the single pass experiments was conducted at various flow rates, and aliquots (1 mL) were removed from the reservoir every 5 min for GC analysis to determine reaction rates.

2.5.3. Hydrolysis of Penicillin G using immobilized PGA in recirculation CFRS

Ten grams of the Emphaze™/PGA (4.0%) biocatalyst were loaded onto a 3 M Model 313B cartridge filter, and the filter assembly was connected to the apparatus pictured in Fig. 1. The round-bottomed flask contained 3 L of 0.010 M potassium phosphate and 0.10 M KCl at pH 7.8 and 28 °C. Penicillin G, potassium salt (60 g; 0.168 mol) was added to achieve a concentration of 2.00 wt% or 0.056 M. The flow rate utilized was approximately 4 L/min (accurately measured values at the approximate pump setting position actually ranged from 3700 to 4145 mL/min), and the sodium hydroxide titrant concentration was 0.75 N. When this system was utilized to conduct multiple experiments with the same biocatalytic cartridge filter, 8–10 L of buffer solution were used to rinse and clean the unit before the next experiment. For the comparison study of free PGA, slurry operation and CFRS, the same apparatus was utilized, and equal quantities of PGA, either free, immobilized in slurry, or in CFRS were employed.

2.5.4. Enantioselective hydrolysis of dimethyl methylglutarate (DMMG) using HEMA-30/PLE (2.2%) in recirculation CFRS

The CFRS arrangement of Fig. 1 was utilized with the cartridge filter containing 10 g of HEMA-30/PLE (2.2%). The reservoir contained 3.0 L of 0.01 M KH_2PO_4 and 0.10 M KCl at pH 7.8 initially at 22 °C. The pump was turned on at a flow rate of 4 L/min and the pH stat contained 1.00 M NaOH. Due to solubility reasons, the DMMG charge (96.2 g; 0.552 mol) was added to the stirred reservoir in four portions. Each portion was completely hydrolyzed before the next addition. No provisions for temperature control were applied to the system, and the temperature ranged from 22 °C for the first portion to 25.5 °C for the last. The total reaction time for the conversion of the 96.2 g was 144 min. The contents of the reservoir were pumped into a large separatory funnel, and the catalyst filter assembly was washed with 1700 mL of buffer solution, which was also added to the aqueous product solution. The solution was washed with ether (1 L) and acidified to pH 2.4 by addition of 46 mL of concentrated HCl. Extraction with ether (2 × 1500 mL), followed by continuous liquid–liquid extraction overnight, drying over magnesium sulfate, and removal of the ether at reduced pressure provided 80.8 g (91.5% yield) of methyl hydrogen 3-methylglutarate. The IR and NMR spectra matched those reported [11]; the %ee was not determined.

2.5.5. Repetitive hydrolysis of triacetin

2.5.5.1. Preparation of the CFRS catalytic unit. A large 3 M 743B Liquid Cartridge Filter was immersed in 80 L of PBS at pH 7.4 in a 100 L polyethylene container. A Tri-Clover centrifugal pump (model C216MD18T-S; Tri-Clover, Inc., Kenosha, WI) was attached to the downstream outlet of the filter, and pumping at a rate of 152 L/min was initiated with the return line from the pump immersed in the 100 L container as well. The Emphaze™/PLE (3.4%) catalyst was slurried in PBS and introduced portionwise to facilitate relatively uniform distribution of the particles across the entire filter element area. When all the particulate had been added, pumping was discontinued, the cartridge was lifted slowly above the surface of the aqueous solution to allow partial draining of the unit, and the cartridge filter was fitted into a 3 M 740 Series Filter Housing having 5.08 cm diameter inlet and outlet pipes. This unit was transported to the BioProcess Resource Center of the Biotechnology Institute at the University of Minnesota where the repetitive experiments were conducted under a research contract.

2.5.5.2. Repetitive hydrolyses using Emphaze™/PLE (3.4%) in CFRS. A 300 L DCI fermenter (DCI, Inc., St. Cloud, MN) equipped with a parabolic impeller (having the dimensions of 12.7 cm diameter, 7.6 cm height, and 6.5 cm depth); pH stat consisting of an Extech (model 402004) pH controller and transmitter (model 402006), a MasterFlex pump (Cole-Parmer), and a Broadly James gel-filled pH probe; and a 3 HP Baldor variable speed motor on a Ladish Tri-Flow 216 centrifugal

pump for recirculation flow were employed for these repetitive experiments. The filter housing containing the Emphaze™/PLE (3.4%) cartridge filter was connected to the fermenter using Tri-Clover sanitary fittings. PBS (pH 7.4, 100 L, also containing 1 mM EDTA) was introduced, stirring initiated at 100 rpm, and recirculation was established at 95 L/min. Triacetin (500 g; 2.29 mol) was added in one portion, and the time and rate of 1.0 M NaOH solution addition by the pH stat were recorded until 95% conversion of the hydrolysis of one of the acetoxy groups was completed. This procedure constituted run #1 with subsequent runs #2 and #3 in 1 day being conducted by adding fresh triacetin charges and examining the hydrolysis reactions. At the completion of the three runs, the contents of the fermenter were drained to waste. Sodium phosphate buffer (0.01 M; 40 L; pH 7.4; also containing 0.15 M NaCl) was added and re-circulated through the housing for 15 min before draining to waste. Subsequent to the first 15 runs in which some activity was being lost, the washing procedure after the three runs per day was modified such that sodium phosphate buffer (0.04 M, 100 L, pH 7.5; containing a reduced quantity of NaCl (0.08 M); and sodium benzoate (0.5 mg/mL) as an antimicrobial agent) was added to the fermenter and recirculated through the cartridge filter unit for 30 min, before draining to waste. The unit was allowed to stand at room temperature before the next day's reactions. When weekend storage was required, the cleaning procedure was conducted twice. A total of 60 reactions were conducted using the above procedure.

2.5.5.3. Repetitive hydrolyses using Eupergit™ C/PLE (3.4%) in slurry batch mode. These experiments were conducted in a 200 L stainless steel tank with agitation supplied using a Chemineer mixer (model ADP-10; Chemineer, Inc., Dayton, OH) having a 11.43 cm diameter impeller and operating at 150 rpm. The pH stat arrangement was the same as that employed with the cartridge filter. The PBS buffer solution (100 L) and Eupergit™ C/PLE (3.4%) (227 g) catalyst support were introduced, followed by the triacetin charge as above. After the completion of 95% hydrolysis of one of the acetoxy groups (the 1st run), 2nd and 3rd runs were conducted by adding additional triacetin charges and hydrolyzing one of the acetoxy groups. Workup involved allowing the particulate catalyst to gravity settle and removing the supernatant by aspiration through an in-line filter stick (180 mesh) using a Wilden diaphragm pump (Wilden Pump & Engineering Co., Grand Terrace, CA). When aspiration had been completed, sodium phosphate buffer solution (0.05 M; 40 L; pH 7.4) also containing NaCl (0.12 M) and 0.5 g/L sodium benzoate were added, and the beads were stirred and washed for 30 min, before settling and removal of the washing solution by aspiration. After all reactions had been completed for a day, the beads were covered with wash buffer in the reaction vessel at room temperature. A total of 60 hydrolysis reactions were examined.

3. Results and discussion

In considering the spatial relationship of an immobilized enzyme particulate and the filter element of a cartridge filter, an arrangement in which the particulate was positioned on the upstream surface seemed most attractive. This configuration was easily achieved by "wet" slurry loading the particulate onto the filter and presumably caused less interference with basic flow and pressure drop characteristics of the cartridge filter unit compared to having the particulate loaded within the filter element itself (as is common with many active carbon containing filters). Horizontally pleated surface filter cartridges in which the pleats are arranged perpendicularly to the length axis of the filter were preferred for two reasons:

(1) Substantially more (ca. 2×) frontal surface area was available than in corresponding vertically pleated units. This allowed for increased particle loading, maintenance of shallower bed depths, and reduced linear velocities of liquid flowing through the unit (and correspondingly less shearing stress on the particulate at a given flow rate).
(2) When the cartridge filter was employed in a vertical arrangement and the unit was not in use and under pressure, the horizontal pleats retained the particles better.

3.1. CFRS experiments conducted using immobilized PLE

3.1.1. Single-pass experiments

Before examining CFRS in a recirculation mode, the degree of one-pass conversion achieved at different flow rates (and correspondingly varying residence times in the catalytic module) was examined. A HEMA-30/PLE (2.2%) support was prepared and 10 g of the catalyst were loaded onto a 25.4 cm cartridge filter. Racemic propylene glycol methyl ether acetate (PGMEA) was selected as the reactant because of its relatively high water solubility. To our knowledge, this substrate had not been previously investigated using PLE, and the general hydrolytic reactivities of both free and immobilized [HEMA-30/PLE (2.2%)] PLE were examined employing analytical procedures described in our earlier report [6]. K_m values with PGMEA for free and immobilized PLE were determined by a standard double reciprocal plot method to be 3.2 and 2.7 mM, respectively, and the level of immobilized PLE activity was 51% compared to the free enzyme. The plot below in Fig. 2 provides results obtained employing one-pass flow rates from 180 to 5825 mL/min. The

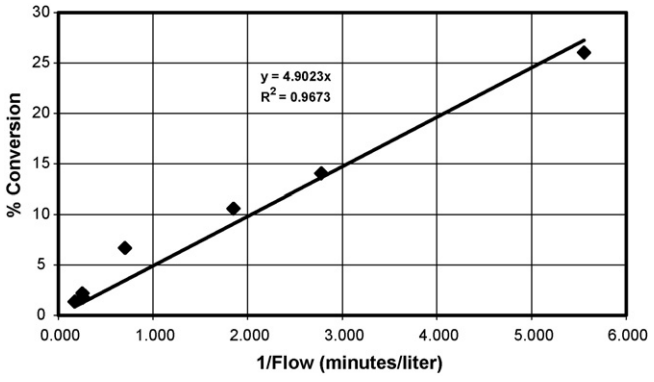

Fig. 2. Plot of hydrolysis conversion of PGMEA vs. flow rate^{-1} (residence time in min/L).

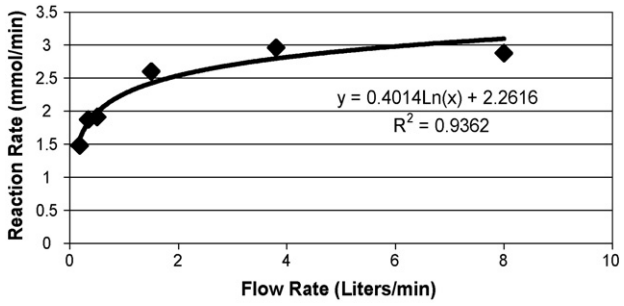

Fig. 3. Plot of PGMEA hydrolysis rate vs. recirculation flow rates.

data indicated a linear relationship between residence time (1/flow rate) in the filter housing and conversion of PGMEA and, as expected, the longer the reactant was exposed to the catalyst the greater the extent of reaction.

3.1.2. Recirculation CFRS experiments

PGMEA (0.05 M) was again examined as substrate—this time contained in 4 L of phosphate buffer in the reservoir with recirculation rates ranging from 175 to 8000 mL/min. A plot of reaction rates, i.e., the slopes of the individual reactions at a particular flow rate, and flow rates at 22 °C are shown in Fig. 3. The plot illustrated that a more complicated logarithmic relationship existed between reaction rate and flow rate with recirculation in CFRS. Clearly, however, the higher the flow rate the better in terms of overall conversion up to a plateau level that was achieved at flow rates of about 2–4 L/min or approximately recirculating half to the entire batch volume every minute.

3.1.3. CFRS over the course of multiple reactions

The hydrolysis of PGMEA was conducted repetitively employing the modified arrangement of CFRS with no pH stat. Reactions were conducted until 50% conversion had been achieved that approximated 100% conversion of the potentially more reactive enantiomer, although no attempt was made to assess the enantioselectivity of the reaction. A constant flow rate of 4 L/min was maintained, and no reduction in performance was observed over the course of ten reaction cycles, resulting in conversion of 100 mmol of acetate in about 35 min per cycle.

3.2. CFRS experiments conducted using immobilized PGA

Possible negative aspects of CFRS that could affect performance include:

(1) *Contact time between enzyme catalyst and reactant*: In slurry batch, substrate and catalyst are in constant contact. With CFRS, only a portion of the substrate, i.e., that actually circulating through the filter housing, is contacting the catalyst at a given time.
(2) *Access of the reactant to all the catalyst charge*: Because some of the particles may have loaded unevenly or in subsequently inaccessible regions of the filter element, only a portion of the catalyst charge may be effectively utilized in CFRS.
(3) *Catalyst as a layer having definite thickness in CFRS*: In CFRS the catalyst is present as a layer having a thickness (albeit on the order of a millimeter or less) and particles within the bed would experience reduced concentrations of substrate compared to more upstream surface positioned particles. Slurry operation, on the other hand, provides for essentially every particle to be exposed to the same substrate concentration at all times.

Recirculating at a rate equivalent to the entire batch volume per minute was examined as a way to mitigate these potential negative aspects with CFRS, and PGA was examined because that immobilized enzyme in slurry had been shown to essentially retain free enzyme activity at coupling levels of up to at least 8 wt% [7]. Therefore, it was possible to more directly compare the activities of the same lot of free enzyme, the immobilized enzyme in slurry operation, and the immobilized enzyme in a CFRS arrangement.

PGA was known to be susceptible to product inhibition [9], however, and it was decided to first conduct the CFRS hydrolysis *to completion* with Emphaze^{TM}/PGA (4.0%). Curvature in the latter stages of reaction (Fig. 4) indicated that inhibition factors were probably operating in the cartridge filter arrangement as well. Reasonable linearity in the plot was observed, however, up to about 50% conversion (or 84 of the 168 mmol) that allowed direct comparison of the various enzyme configurations.

Employing equivalent reaction conditions, equal quantities of PGA were examined as the soluble (or free) enzyme, immobilized onto the Emphaze^{TM} support employed as a slurry, and with the immobilized enzyme in CFRS to 45% conversion. Virtually identical reaction rates were observed with all of the enzyme configurations (Fig. 5). This indicated that incorporation of the biocatalyst in a cartridge filter arrangement had no deleterious effect on catalytic activity when the batch was recirculated essentially every minute. Potential negative concerns regarding contact time, catalyst utilization and bed depth were therefore unfounded.

After 19 preparative reuse experiments employing a variety of flow rate conditions had been conducted using the same PGA filter cartridge, a rate value of 2.46 mmol/min was observed on the 20th experiment with a measured flow rate of 4145 mL/min. Compared to the initial value of 2.21 mmol/min at 3700 mL/min,

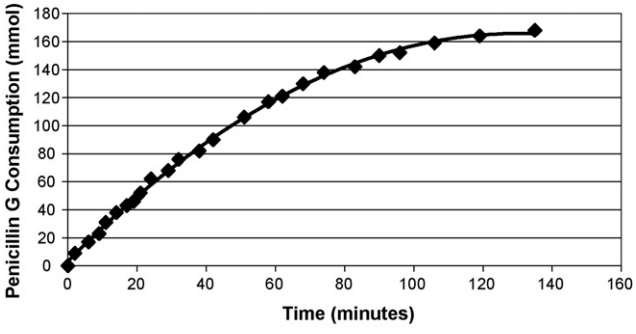

Fig. 4. Plot of Penicillin G consumed vs. time over the course of the complete reaction.

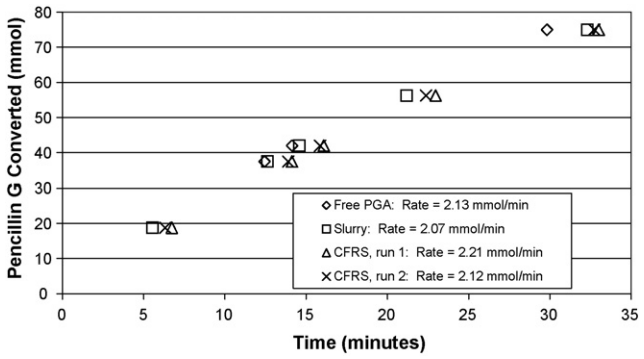

Fig. 5. Plot of Penicillin G consumption vs. time employing equal quantities of PGA in a free or soluble, immobilized slurry, and immobilized particulate in a cartridge filter configuration employing a flow rate of 3700 mL/min.

this indicated that there was no loss in catalytic activity over the course of those experiments.

3.3. Larger scale experiments conducted using CFRS

It was desired to assess the preparative capabilities of CFRS, and dimethyl 3-methylglutarate (DMMG) was chosen as substrate because soluble PLE had been reported to efficiently produce only the *R*-mono acid isomer [10]. A 25.4 cm × 7.6 cm cartridge filter unit packed with the HEMA-30/PLE (2.2%) biocatalyst was employed with a total reaction volume of 4 L and a recirculation rate of 4 L/min. Because the entire DMMG charge (100 g) exceeded the solubility limit for the diester in the aqueous buffer solvent, the substrate was added in 25 g portions. No precautions were made to control reaction temperature, and, since the hydrolysis was slightly exothermic, subsequent portions were hydrolyzed at slightly elevated temperatures causing reaction rates and the pH stat addition traces to be separated for each 25 g portion addition (Fig. 6). The overall result was that 96.2 g (552 mmol) of DMMG were hydrolyzed in less than 150 min, and the product was subsequently isolated in 91.5% yield. This result compared to literature reports of conversion of 5 g of DMMG in 3 days (98% yield) [10] and 15 g in 16 h (86% yield) [11] using soluble PLE. Product inhibition was generally not prominent in this reaction, except for the very last conversion stages of the last portion added in which a slight deviation from linearity was observed.

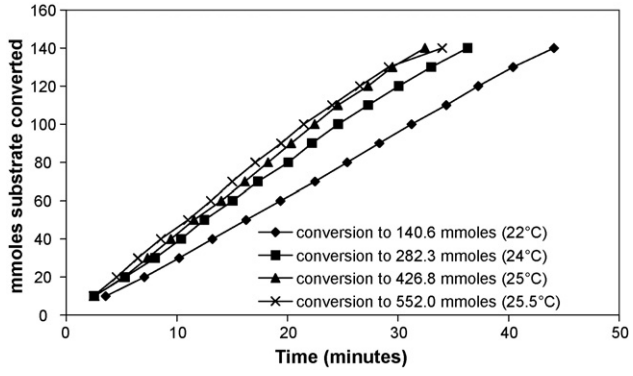

Fig. 6. CFRS [HEMA-30/PLE (2.2%)] conversion of DMMG.

In order to evaluate the applicability of CFRS as a manufacturing operation, a substantially larger-scale preparative experiment was examined by conducting a reaction using the larger 101.6 cm × 17.8 cm diameter (40 in. × 7 in.) cartridge filter containing an immobilized enzyme. These experiments were conducted in a 300 L fermentor at the Bioprocess Resource Center of the Biotechnology Institute of the University of Minnesota. In this initial study, reaction rates and number of reuses were of prime interest and not isolated yields of products. Hydrolysis was monitored using a pH stat, and reaction was conducted such that only one of the three acetoxy groups (in theory) of triacetin was hydrolyzed. In order to achieve a recirculation rate with the larger cartridge filter comparable to that utilized with the smaller ones, flow rates of about 25 gallons/min (95 L/min) were required.

An Emphaze^{TM}/PLE (3.4%) support was prepared, loaded onto a large cartridge filter, and examined as a catalyst for triacetin hydrolysis. As indicated in Fig. 7 below, within the first 15 reuses there was a slight loss of hydrolytic activity as indicated by increasing times to achieve 95% conversion. It was subsequently determined that hydrolysis of residual triacetin (or, more probably, partially hydrolyzed reaction products) in contact with the enzyme catalyst was apparently continuing in the CFRS unit during overnight storage causing a lowering of the pH, possibly into a range injurious to the enzyme. When improved rinsing conditions for the catalytic unit were employed (in runs 16–60), essentially no loss in catalytic activity was observed over the course of the next 45 reactions. The overall result was that 2.29 mol of triacetin were hydrolyzed in less than 20 min of recirculation operation with each reaction cycle for a total of 29.8 kg of substrate converted over the course of the 60 reaction cycles.

As a control and comparison experiment, the industry standard Eupergit^{TM} C support with 3.4% immobilized PLE was utilized in slurry batch mode. The data, also plotted in Fig. 7, indicate a significantly slower hydrolysis rate compared to the Emphaze^{TM}/PLE (3.4%) in CFRS. No definitive loss in activity was observed over the 60 reaction cycles, although the 95% conversion times may have been increasing from reactions 52

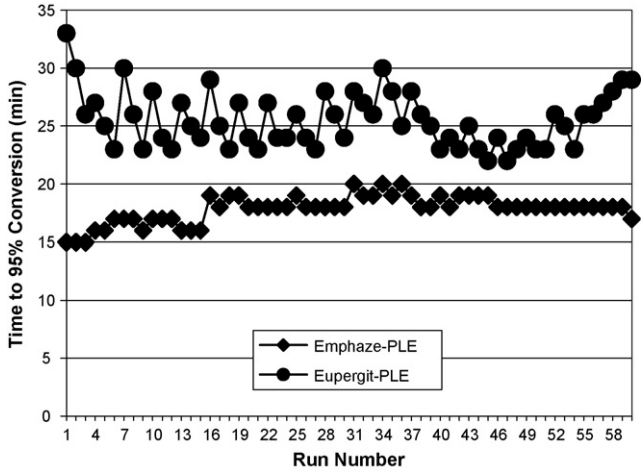

Fig. 7. Plot of the 95% conversion time for hydrolysis of one acetoxy group on triacetin using conventional slurry-batch with Eupergit^{TM}/PLE (3.4%) (upper trace) and CFSS with Emphaze^{TM}/PLE (3.4%) (lower trace).

and higher as indicated by the somewhat ascending trend in the data.

Evidently, this relatively small number of cycles was not enough to clearly distinguish CFRS and exemplify proposed advantages over slurry batch processes. It would be very desirable to conduct repetitive reactions over the course of 500–600 cycles. A somewhat surprising aspect of the slurry batch experiments was the increase in rates observed with runs 2 and 3 each day, especially in the early runs. While this is not completely understood, one possibility is that PLE immobilized onto Eupergit™ may simply be less discriminating in the selection of its substrates than when immobilized onto Emphaze™. Thus, when significant quantities of mono- and diacetoxy glycerol are present along with the additional triacetin charge, increased overall rates of hydrolysis would be observed with the Eupergit™ catalyst. This speculation will require additional investigation to confirm.

Authentic industrial manufacturing information regarding 6-aminopenicillanic acid (6-APA) is relatively scarce. One report [12], however, disclosed that 100 kg (550 L) of wet Eupergit™ C/PGA catalyst produced 23.65 metric tons of 6-APA over 5 months and 620 batch cycles. With appropriate assumptions, this corresponded to an average production rate of about 0.5 mol 6-APA/min over the life of the process. This rate was arrived at employing the following computations:

23.65 MT/620 cycles = 38 kg/cycle × 1 mol/216 g = 176 mol 6-APA/cycle.
5 months × 30 days/month/620 cycles × 24 h/day ≈ 6 h/cycle.
176 mol/cycle/360 min (6 h)/cycle ≈ 0.5 mol/min 6-APA production.

In our previous work with PGA [7], 20 wt% of the enzyme was coupled to Emphaze™ AB 1 with retention of 85% enzyme activity. The literature stirred tank reactor operations were conducted in a 2500 L (660 gal) reactor. Employing CFRS and a flow rate of about 400 gal/min (assuming 2/3 filling of the reactor) and 100 L of Emphaze™/PGA (20%) contained in 10 large cartridge filters with the same level of enzyme activity, a predicted production rate of about 4.25 mol/min should result. This rate was computed in the following manner:

100 L × 1000 g/8 L × 20% PGA × 85% activity = 212.5 g of active PGA.
212,500 mg × 20 U/mg × 10^{-6} mol/U min ≈ 4.25 mol/min 6-APA production with ca. 1/5 the quantity of immobilized PGA.

While it is appreciated that the projected rate with CFRS is an initial and not an average rate, it is anticipated that the stabilizing effects of CFRS listed above would enhance the lifetime and performance of the biocatalyst significantly.

4. Conclusions

At the outset of this investigation, a number of advantages seemed reasonable with the CFRS arrangement compared to slurry batch operation of an immobilized enzyme biocatalyst. These and conclusions obtained for each are as follows:

(1) *Elimination of the catalyst separation unit operation.* This is a significant and inherent advantage of CFRS. The biocatalyst is physically separated from the bulk of reactant and product solutions in the reservoir, and repetition of reaction simply involves processing the product solution, washing the biocatalyst module, and replenishing the reservoir with fresh reactant solution.
(2) *Significantly improved process economics in terms of increased biocatalyst lifetimes created by isolation from mechanical and chemical stresses normally associated with batch operations.* This concept was supported, though not definitively proven. Several hundred reuses of a reaction, e.g., hydrolysis of Penicillin G using PGA, would be required to really probe the economics of CFRS. With the 60 large-scale reactions conducted using CFRS, 124 micromol of the immobilized PLE hydrolyzed 136.8 mol of triacetin in 17.6 h with an average rate of 133 mmol/min. The overall turnover number for these reuses was more than 1.1 million—an impressive number and indicative of the tremendous catalytic power of enzymes. Additional concerns regarding possible deleterious effects of pH drop across the catalyst bed were found to be negligible due to the very shallow bed depth of the biocatalyst, as were any shearing effects of flowing water and resulting mechanical instability of the immobilized enzyme in the cartridge.
(3) *Use of high recirculation flow rates should provide comparable conversion rates to slurry batch processes.* Concerns about contact times between reactant and catalyst, utilization of only a portion of the catalyst charge, and catalyst bed depth differences between CFRS and slurry batch operations were largely laid to rest. With Emphaze™/PGA in CFRS, high conversions and reaction times that were comparable to slurry batch operations were achieved when recirculation rates were utilized that essentially exposed at least half (or, better, the entire volume) of the reactant through the biocatalytic module every minute.

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