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ORIGINAL PAPER

Production of 4-valerolactone by an equilibrium-limited transformation in a partitioning bioreactor: impact of absorptive polymer properties

Julian T. Dafoe · Andrew J. Daugulis

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Abstract The biotransformation of levulinic acid to 4-valerolactone (4VL) is pH-dependent and equilibrium limited, distinct from the more common irreversible biotransformations that are constrained by product toxicity or biocatalyst inhibition. Our processing strategy for this system was to selectively remove the product, 4VL, which is in equilibrium with its precursor, 4-hydroxyvalerate (4HV), to pull the reaction to a greater extent of conversion. 4VL is challenging to separate from the aqueous phase due to its water miscibility, necessitating the use of water-absorbing polymers to provide affinity toward the hydrophilic product. Manipulating the composition of copolymers, thereby varying the architecture of polymer chains, conferred drastically different extents of water absorption and caused different biotransformation outcomes. A custom-synthesized random copolymer designed to maximize the proportion of material with affinity for the solute had high water uptake, which resulted in the poor selectivity for the target molecule relative to its precursor. Conversely, a moderately water-absorbing commercial segmented block copolymer, Hytrel® 8206, demonstrated selectivity toward 4VL relative to its precursor, 4HV, and increased 4VL production by approximately 30 % by shifting the equilibrium toward the product. This work has shown that water absorption is an important, previously neglected criterion in evaluating polymer affinity and selectivity toward hydrophilic target molecules.

J. T. Dafoe · A. J. Daugulis (✉)
Department of Chemical Engineering, Queen's University, Dupuis Hall, 19 Division St., Kingston, ON K7L 3N6, Canada
e-mail: andrew.daugulis@chee.queensu.ca

J. T. Dafoe
e-mail: julian.dafoe@chee.queensu.ca

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Introduction

The production of 4-valerolactone (4VL) from renewable levulinic acid has received increasing attention as a potential "green" platform chemical and transportation fuel, yet investigations have focused almost exclusively on thermochemical processes requiring the use of solvents, harsh conditions and expensive catalysts [1–3]. Although engineered biocatalysts operating under mild aqueous conditions could provide alternative routes to such building blocks molecules, the yield and productivity of biological systems are generally lower than chemical catalysis due to substrate and/or product inhibition which can range from simple feedback inhibition to toxic cellular deactivation [4]. The bioproduction of 4VL is characterized by a different limiting feature, however, as it is an equilibrium-governed biotransformation whose performance could potentially be improved by selectively removing the product, 4VL, from the aqueous phase, thereby pulling the equilibrium reaction to greater conversion. Recently, a hybrid chemo-enzymatic approach using an ion-exchange resin catalyst, followed by two isolated enzyme-catalyzed steps in a solvent-based membrane reactor, rapidly produced enantiomerically pure 4VL to a high yield on a small scale [5]. This approach, while extremely rapid and specific, suffers from additional costs of the specialized resin, isolated enzymes, cofactors, solvents and their distillation for re-use, which may be justifiable in the production of a high-value product, but limits applicability in processes intended to produce commodity products.

Aiming to minimize the additional costs and maintain simple operation, in this study we investigate the effect of

applying inexpensive polymer extractants to the whole-cell biotransformation of levulinic acid to 4-valerolactone by engineered *Pseudomonas putida* KT2440. This system operates via the action of two recombinant enzymes to first form 4-hydroxyvalerate (4HV), a hydroxyacid intermediate, which is subsequently converted to its lactone form in the extracellular medium by a paraoxonase (PON1) enzyme attached to the outer cell membrane surface [6]. The 4HV–4VL equilibrium reaction catalyzed by PON1 proceeds in either direction, governed by the relative concentrations of hydroxyacid and lactone in the medium, based on their relative stability at a given pH [7, 8]. The equilibrium position favors 4HV at neutral pH, but shifts approximately fivefold toward 4VL at pH 6 [6]. Because the PON1 enzyme is located on the membrane surface, the equilibrium position is adjustable by manipulating medium pH, whereas an intracellular enzyme would be inaccessible to pH manipulation [6]. While changing pH from 7 to 6 shifts the equilibrium position toward 4VL, we are proposing to selectively remove 4VL from the aqueous phase to further shift this equilibrium and bring about a greater extent of conversion.

The nature of a target molecule determines potential strategies for extraction. Hydrophobic, relatively apolar target molecules are easily extracted by immiscible solvents that are tolerated by the biocatalyst [9], or by adsorption on macroporous resins via hydrophobic interactions [10, 11]. Conversely, relatively hydrophilic target molecules have been separated using aqueous two-phase systems (ATPS), consisting of combinations of polymers, salts, ionic liquids, or surfactants which phase-separate under specific operating conditions, yielding a phase rich in the target molecule. These approaches require expensive components and are limited to biocompatible materials [12, 13].

A unique strategy, which we have adopted in this investigation, is the use of absorbent polymers featuring a soft, amorphous polymer network. These materials are phase-stable, inexpensive, and can range from hydrophobic rubbers to hydrophilic gels containing mostly water, depending on their composition. Using absorbent polymers obviates the requirement for biocompatibility screening, as the polymer phase is inert with respect to the biocatalyst, and the application is straightforward, requiring no process modifications aside from the addition of the polymer. The mechanism of solute uptake by amorphous polymers above their glass transition temperature (T_g) is via absorption, identical to solvent extraction, with relative concentrations described by partition coefficients and the equilibrium position not being surface area-dependent [14]. This is distinct from surface adsorption described by sorption isotherms which are commonly seen in the use of macroporous resins having high T_gs [15].

Previously, commercially available absorptive polymers were used for in situ product removal (ISPR) of relatively hydrophobic target molecules from fermentation medium with excellent productivity enhancements [16–18]; however, many important target molecules are hydrophilic as judged by the octanol–water partition coefficient (4VL $\mathrm{Log}K_{O/W} = -0.1$), limiting the extent to which they can be separated from water. The vast majority of absorptive polymers used in TPPB systems are segmented block copolymers containing amorphous poly(ether) soft segments with low T_g, which are mechanically stabilized by hard segments having high T_g or highly crystalline domains comprised of poly(ester), poly(amide), or poly(urethane). In this architecture, the hard segment, ostensibly inert with respect to target molecule absorption, is required only in the amount sufficient to mechanically stabilize the polymer, while the soft segment provides the necessary solute affinity for target molecule absorption [19]. It is therefore desirable to maximize the proportion of soft material available for absorption, while incorporating the minimum amount of inert hard material sufficient to preserve desirable physical properties.

The chemical composition of a copolymer's soft segment determines its affinity toward a target molecule. Hildebrand's solubility parameter (δ, $\mathrm{MPa}^{1/2}$) describes the compatibility of chemical species according to regular solution theory, and materials with similar δ values will generally be miscible [19]. Solubility parameters are tabulated for many polymers and small molecules, and poly(ethylene oxide) has the highest solubility parameter ($\delta \approx 20\ \mathrm{MPa}^{1/2}$) within the poly(ether) series, is closest to that of 4VL ($\delta \approx 22\ \mathrm{MPa}^{1/2}$) [20], and therefore should provide mutual affinity. Polymer δ values can be increased via higher polymer polarity, but this increases internal polar interactions at the expense of polymer chain mobility, and thereby reduces target molecule absorption by creating a tighter network [19]. To circumvent this, a polymer's polarity may be increased while maintaining flexible chain architecture through plasticization by absorbed water [19]. Water's exceptionally high solubility parameter ($\delta \approx 48\ \mathrm{MPa}^{1/2}$), due to its strong hydrogen bonds, precludes meaningful comparisons of its solubility parameter value with those of other compounds, however, the incorporation of water in a polymer may increase its overall solubility parameter, providing a more favorable environment for hydrophilic molecules [19]. An early study on improving polymers for the extraction of relatively hydrophilic target molecules in TPPBs identified water absorption as an important characteristic in conferring affinity toward polar solutes, however, the target molecule, phenol, was relatively hydrophobic ($\mathrm{Log}K_{O/W} = 1.5$), and water uptake in the polymers reached maxima of only about 10 wt% [21].

The effect of high water content within an absorbent polymer is not well understood in the context of TPPBs, as the presence of a continuous water phase within the material is expected to impose a tradeoff between target molecule affinity and selectivity [22]. The extent to which water exists as a polymer-associated, "bound" complex as opposed to a continuous "bulk" water phase depends on the polymer's composition and is not easily discerned [23]. Instead, water uptake is generally, and superficially, described as the percent of total mass gained upon swelling. Therefore, although water can improve affinity toward hydrophilic solutes, it may also cause poor selectivity relative to other hydrophilic solutes by providing an expanded network which is more available for permeation of all solutes.

In this work, the biotransformation of levulinic acid to 4VL serves as a model equilibrium-limited biocatalytic system for equilibrium pulling via product removal using absorbent polymers. We believe that this work reports the first attempt to use absorbent polymers to pull an equilibrium-governed biocatalytic system, despite demonstrations of equilibrium shifting by extraction using adsorbent porous resins or molecularly imprinted polymers, which operate via surface interaction mechanisms, in contrast to the solution-diffusion mechanism occurring within absorbent materials [8, 24, 25].

To achieve this objective, polymer composition, specifically the amount and type of stabilization provided to the polymer network, was tailored to provide different architectures, and the effect of these different architectures on water uptake was investigated in a range of polymers. One commercial, hydrophilic poly(ether)-poly(ester) segmented block copolymer, Hytrel® 8206, was compared to two custom-made, random copolymers, crosslinked poly(ethylene glycol diacrylate) (XLPEGDA) and *N*,*N*′-*m*-phenylenedimaleimide-crosslinked poly(ethylene oxide) (BMI-PEO). These latter polymers are comprised of poly(ethylene oxide) (PEO) as the active material based on affinity (similar solubility parameter) toward the hydrophilic target molecule 4VL, and differ in their architecture (nature of network stabilization) that would affect physical properties and degree of water uptake.

Materials and methods

Chemicals

All chemicals were from Sigma-Aldrich (Canada) or Fisher (Canada) with the exception of the polymers. Hytrel® 8206, a segmented block copolymer of poly(ether) and poly(ester) of proprietary composition, with water uptake of 30 %, was from DuPont (Canada). Poly(ethylene oxide) (PEO), 5×10^6 g/mol and polyethylene glycol diacrylate (PEGDA), 258 g/mol, the starting materials for the custom-synthesized polymers, BMI-PEO and XLPEGDA, were from Scientific Polymer Products (New York). Because it is not commercially available, the 4-hydroxyvalerate analytical standard was prepared by saponification of a 100 mM 4VL solution assuming equimolar conversion [26].

Microorganism

Pseudomonas putida KT2440 harboring the genes tesB and PON1 for this biotransformation was kindly supplied by Dr. Kristala Prather of the Massachusetts Institute of Technology, Cambridge, MA, USA. The culture was grown for 24 h in Lysogeny Broth (LB) supplemented with 10 mg/L tetracycline and 20 mg/L gentamycin for plasmid maintenance, and frozen in 1 mL aliquots containing 10 % (v/v) glycerol at −80 °C.

Polymer synthesis

XLPEGDA

This copolymer was synthesized to provide a PEO-containing network, providing similar 4VL affinity to the commercial Hytrel® 8206, while exhibiting a range of water uptake due to varying network stabilization, to compare its affect on affinity. XLPEGDA, comprised of 258 g/mol polyethylene glycol diacrylate, was crosslinked with 0.1 wt% 1-hydroxycyclohexyl phenyl ketone (HCPK) photoinitiator relative to prepolymer mass, by UV irradiation in a covered glass mold (2 mm × 75 mm × 25 mm) with a mirror surface for 120 s at 5 mW/cm^2. Varying amounts of water (0, 20, or 40 %) were included in the prepolymer solution to obtain different crosslink degrees while maintaining an identical chemical composition, giving a range of materials with different water uptake levels. The materials' designations corresponding to the weight percent of PEGDA present in the prepolymer solution [22].

BMI-PEO

A second copolymer was synthesized to provide a range of materials having the maximum feasible amount of "active", soft PEO content available for solute absorption (i.e. maximizing affinity), and minimizing the presence of inert material in the copolymer. BMI-PEO was synthesized using 250 g high MW poly(ethylene oxide) which was solution coated with 0.04 wt% dicumyl peroxide dissolved in acetone and dried overnight. 1, 3, or 5 % *N*,*N*′-*m*-phenylenedimaleimide (BMI) was added then blended in a

slurry and cured at 160 °C for 30 min in a press. The polymer was cut into cubes of about 3 mm. The materials' designations correspond to the weight percent of BMI added to the prepolymer slurry.

Water uptake

As all polymers were expected to have moderate to considerable water uptake, triplicate samples of each polymer (ca. 1 g) were dried in an oven at 60 °C for 24 h and weighed on an analytical balance. The polymer samples were immersed in an excess volume of high-purity water or bioreactor medium as indicated in text, and shaken in 50 mL centrifuge tubes at 180 RPM at room temperature, periodically separated from the liquid, dried, and weighed to determine the extent of water uptake before being returned to the liquid until there was no significant change in mass at successive times. Water uptake was determined by Eq. 1:

$$\%\,\text{water uptake} = \frac{M_{\text{wet}} - M_{\text{dry}}}{M_{\text{dry}}} * 100. \quad (1)$$

Polymer partition coefficients

Polymer partition coefficients toward the target molecule, 4VL, were determined in triplicate using a known mass of hydrated polymer, pre-swollen for 24 h in high-purity water, and added to a vial containing 10 mL of 3 g/L 4VL in high-purity water. Control vials without polymer were treated identically and the mass of absorbed 4VL was determined by mass balance calculation. The use of high-purity water eliminated potential osmotic effects on phase ratios due to solutes. The partition coefficient of 4HV was not determined in high-purity water because its preparation required significant use of salts, which are known to skew partition coefficient values [27], and would not provide results comparable to experiments performed using high-purity water.

Partition coefficients for 4HV and 4VL were calculated in the bioreactor experiments using the concentration of the target molecule in the polymer, determined by solute desorption in high-purity water, and the concentration measured in the aqueous phase. The polymer mass was corrected by calculating its swollen mass in the reactor, and an aqueous phase density of 1,000 kg/m^3 was assumed to simplify comparisons with high-purity water.

Biotransformations

The biotransformation of levulinate to 4VL proceeded in four periods: growth period, 4HV accumulation period, 4VL production period, and ISPR period. The ISPR period is the focus of this investigation, and the experiments were conducted in the same manner except for the type of polymer that was added during the ISPR period.

Cells were grown from frozen stock for 24 h at 32 °C and 180 RPM in six 50 mL shake flasks containing LB as described above. The bioreactor (BioFlo III, 5L vessel, New Brunswick Scientific, Edison, NJ, USA) contained 2.6 L Terrific Broth (TB): 12 g/L tryptone; 24 g/L yeast extract; 1.1 g/L KH_2PO_4; 4.7 g/L K_2HPO_4; 0.48 g/L $MgSO_4{\cdot}7H_2O$, which was amended with antibiotics as above, and 1 mL/L of trace mineral solution, containing 22 g/L $CaCl_2$ and 0.1 g/L ferric ammonium citrate. Glucose was autoclaved separately as a 500 g/L solution and 120 mL was added prior to inoculation, giving 20 g/L. Isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM working concentration) was added for gene induction.

Aeration at 1.3 VVM during cell growth, was reduced to 0.3 VVM during stationary phase to minimize foaming. Dissolved oxygen (DO) was maintained above 20 % of saturation. Antifoam 204 (Sigma-Aldrich) was added as required. pH was automatically controlled with 5 M KOH and 5 M H_2SO_4.

Cell growth period and 4HV accumulation period

The cells were grown at pH 7.0, 32 °C and 800 RPM during the first 8 h until cell density peaked near 14 g/L CDW and DO rose rapidly, corresponding to the depletion of glucose. At the onset of stationary phase, agitation was reduced to 650 RPM and substrate feeds were initiated. Glucose (500 g/L) was fed at 1 g/L/h and levulinate (400 g/L, neutralized to pH 7 with 6 M KOH and sterile-filtered) was fed at 3.5 g/L/h initially to obtain a high concentration and then adjusted according to the consumption rate to maintain a level between 10 and 20 g/L throughout the 4HV accumulation phase.

4VL accumulation period

When 4HV plateaued, the levulinate feed was stopped and the glucose feed rate was cut by half. The pH of the medium was adjusted to 6.0 using bioreactor control to shift the pH-dependent equilibrium further toward 4VL, and agitation was reduced in response to lower oxygen demand.

ISPR (polymer absorption) period

After 24 h of 4HV conversion to 4VL at pH 6.0, the rate of 4VL accumulation slowed as the system approached an equilibrium between the relative concentrations of 4HV and 4VL. The ISPR period was then initiated by adding a mass of polymer beads to the bioreactor to remove 4VL

from the medium and renew the driving force for conversion for an additional 24 h. In the BMI-PEO experiment, 240 g of dry BMI-PEO pellets were added to the bioreactor. In the Hytrel® experiment, 930 g of dry Hytrel® 8206 pellets were added. The mass of dry polymer added was determined based on measured equilibrium water uptake in the bioreactor medium to provide a similar swollen mass of polymer in each experiment. Dry polymers were added because pre-swollen polymers of drastically different water contents would skew aqueous concentrations to varying degrees and introduce unacceptable errors.

Analytics

Biomass was monitored by optical density at 600 nm and correlated to cell dry weight (CDW, g/L). Glucose was monitored using the DNS assay [28]. Bioreactor medium samples were centrifuged at 16 kG for 5 min, and 100 μL of a 10 wt% H_2SO_4 solution was added to 900 μL supernatant and centrifuged again at 16 kG for 5 min to coagulate proteins and ensure protonation of all analytes. Polymer samples were periodically removed from the bioreactor after the initiation of ISPR, their surface rinsed briefly with high-purity water, then placed in 5 mL high-purity water for 24 h to extract absorbed compounds. This desorption procedure was repeated twice for each polymer sample to ensure complete extraction, as there were no detectable desorbed compounds in the third desorption. Desorption samples were acidified prior to analysis in the same manner as the bioreactor medium samples. Liquid samples were passed through a 0.2 μm Nylon syringe filter prior to injecting 100 μL through a 20 μL sample loop. Bioreactor medium samples were run both undiluted and diluted 1:10 in high-purity water. The mobile phase was high-purity water with 0.1 % H_3PO_4 and 5 % acetonitrile at 1 mL/min. Separation was performed on an Agilent Zorbax SB-Aq 3.5 μm 4.6 × 150 mm column at 65 °C, with detection using a Varian 356 Refractive Index detector at 35 °C.

Results and discussion

Polymer extractant characteristics

We chose to use inexpensive absorbent polymers as the sequestering phase because they can provide a range of water uptake capacities by means of different architectures and compositions. The mechanism of solute absorption, via diffusion among mobile chain segments, is distinct from fluid diffusion and surface interactions within the rigid fluid-filled pores of adsorbent resins [29], and similar to the polymer solution interactions occurring in ATPS, where the polymer acts as a solvent. Absorbent polymers differ from ATPS in their phase stability as solids or gels, and do not undergo phase-forming transitions in response specific operating conditions, simplifying their implementation.

These characteristics of absorbent polymers place them in a unique, intermediate class between ATPS and adsorbent resins, offering a favorable combination of properties from both types of extractant, with the most attractive features being low cost [30], and ease of implementation. The term "hydrogel" is often used to describe polymers exhibiting significant water uptake, however this term lacks concrete boundaries and may include any polymer exhibiting water uptake [15].

Water uptake experiments

These experiments were conducted to compare the effect of the different copolymer architectures on water uptake, with the same active component in both synthesized polymers being poly(ethylene oxide) (PEO), chosen because of its affinity toward the water-miscible target molecule, 4VL based on their similar solubility parameters. The copolymers had either minimal random crosslinking (BMI-PEO), extensive random crosslinking (XLPEGDA), or segmented block architecture (Hytrel®). The equilibrium water uptake by the synthesized and commercial polymers in high-purity water are shown by the bars in Fig. 1a, b. The 10- to 20-fold difference in water uptake between the polymers is therefore due to the differences in their architecture, i.e. stabilization provided by the inert hard segments (Hytrel®) or crosslinks holding the network together (XLPEGDA and BMI-PEO), preventing the complete dissolution of the water-soluble PEO chains. The distance between crosslinks or hard domain regions has two effects: it determines the length of the poly(ether) chains which are able to move freely and absorb solutes, and in the same fashion also provides a "looser" network corresponding to higher water uptake. The two custom-synthesized copolymers were prepared by randomly crosslinking PEO-containing prepolymers, the difference being the relative amount of crosslinker, which determines the synthesized copolymers' architecture, and therefore influences its water absorption. BMI-PEO, containing only 1–5 wt% inert material (BMI) forming the crosslinks between PEO chains, is highly available for water permeation, and caused this material to absorb the most water. Conversely, XLPEGDA contains terminal acrylate groups comprising approximately 75 % of the prepolymer's mass which become chemically crosslinked to a high extent with adjacent acrylate groups, resulting in a more constrained network that absorbs less water. The inclusion of increasing amounts of water in the prepolymer solution as a diluent increases the distance between adjacent acrylate groups, forcing some to react

intramolecularly instead of intermolecularly, and creating "wasted crosslinks" which form a progressively looser network that is more amenable to water absorption [22].

The commercial copolymer, Hytrel® 8206, also contains significant hydrophilic poly(ether) content based on its 30 % water absorption, which was the basis for its selection in this study [31]. Although its proprietary composition precludes direct comparison of component monomers with the prepared polymers, its segmented block copolymer architecture, consisting of approximately 50 wt% inert, hard poly(ester) material, more extensively stabilizes this material against water infiltration than BMI-PEO, and is intermediate among the grades of XLPEGDA, shown in Fig. 1b.

The architecture of a copolymer not only affects its ability to take up water, but also affects the physical properties of the swollen material. Of the three materials investigated, only BMI-PEO and Hytrel® had physical properties considered acceptable for use in the high shear environment of a bioreactor. All grades of XLPEGDA were brittle solids when dry, but absorbed water to become a mucous-like material with poor physical integrity, likely a result of excessive intramolecular crosslinks rather than intermolecular ones, causing poor network integrity despite containing a large proportion of crosslinking material. Conversely, Hytrel® 8206, and all three grades of the BMI-PEO material despite having much higher water uptake, remained tough when swollen, due to their inert stabilizing components being largely intermolecularly bound, providing a more extensively stabilized network than XLPEGDA's significant intramolecular bonds. Physical toughness is an important consideration in sequestering phase material selection, as retrieval of the intact polymer is important for product recovery and polymer re-use. For this reason, only the 1 % BMI-PEO, having the highest amount of active material for affinity (PEO), and Hytrel® 8206, were used in subsequent bioreactor experiments.

Partition coefficient experiments

Polymer composition

The PC values of the lab-synthesized polymers, BMI-PEO and XLPEGDA, and the commercial polymer, Hytrel® 8206, in high-purity water toward the target molecule, 4VL, are shown in Fig. 1a, b. The swollen BMI-PEO polymers all had a partition coefficient of approximately 1.0, indicating a nearly equal concentration of 4VL in the polymer and aqueous phases. This value is logical as the material's composition is mostly water when hydrated, such that PEO itself, which possesses affinity for 4VL, is a minor contributor to solute uptake because of its relatively small quantity in the swollen material. Higher 4VL PC values with ever-increasing water uptake within the XLPEGDA and BMI-PEO grades (Fig. 1) suggest that water within the polymer expands the network, making it available for permeation and providing an aqueous environment for the hydrophilic target molecule, 4VL. Also, this trend shows that water is performing a substantial role in 4VL uptake because the PEO and crosslinker composition in XLPEGDA remains constant through the range aside from water content.

Hytrel® 8206 had a lower PC and also lower water absorption than some of the synthesized polymers because this commercial block copolymer is composed of ~50 wt% hard, inert material, which restricts the polymer's ability to absorb water and is not available for solute uptake. Nevertheless, Hytrel® 8206 absorbs 30 % water which may also provide an expanded network for permeation of hydrophilic target molecules in a similar way to the trend seen within the synthesized polymers.

Effect of water uptake on partition coefficients

The calculation of partition coefficients using mass balance requires accurate accounting of each phase's mass at

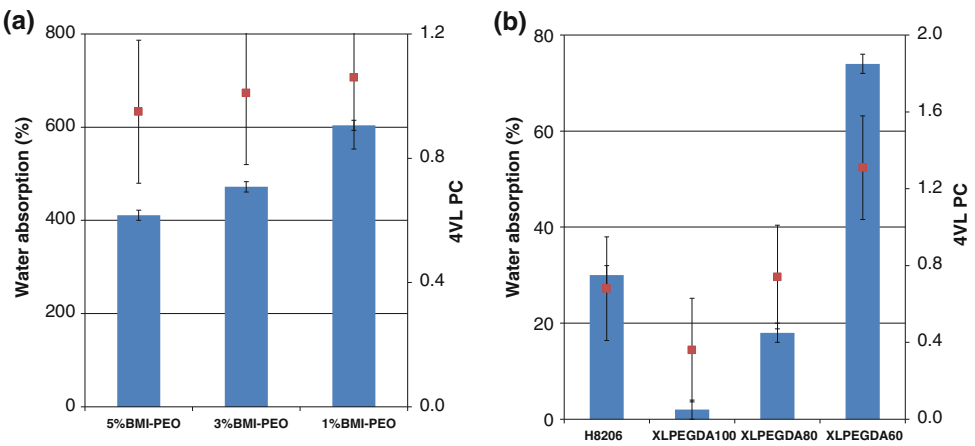

Fig. 1 Water absorption (*bars*) and 4VL partition coefficient values (*filled squares*) in **a** BMI-PEO or **b** Hytrel® 8206 and XLPEGDA

equilibrium (i.e. after water uptake). Virtually all prior studies evaluating polymers for TPPB applications have not considered, or accounted for, water uptake by polymers, and have unknowingly treated hydrophilic polymers the same as hydrophobic ones [32–34]. A sequestering phase which absorbs a large proportion of water affects such calculations and interpretations of partition coefficients, with the calculated PC value being artificially higher when water uptake and consequent changes in phase volumes are neglected. This effect is greatly exaggerated in highly water-absorbing polymers, but the effect is still significant even with modest amounts of water (e.g. Hytrel 8206® at 30 %).

By calculating the PC values using polymer samples which were pre-swollen for 24 h in high-purity water, potential changes between initial and equilibrium phase masses and volumes were eliminated. This approach assigns the entire bulk hydrated polymer mass as the sequestering phase, physically separated from the cell-containing aqueous phase, and the volumes of both phases remain unchanged throughout the experiment due to minimal water flux between the phases. The PC results using this approach are lower and more realistic than if only the dry polymer mass were incorrectly accounted for in the calculation; for example, the PC value of 1.0 calculated for 4VL in BMI-PEO would give a value of nearly 8.0 if water uptake were neglected. Because this new approach accounts for water uptake by the polymer, it enables a realistic comparison of polymers having widely different water absorptions which would otherwise give incorrect PC values if water uptake was neglected, and should be adopted in future investigations comparing polymer-target molecule affinity in aqueous systems to ensure comparison of polymers on an equal basis.

Biotransformations

Biotransformation experiments, employing two different polymers during the ISPR period, were performed to permit comparison on an equal basis, such that their affinity for 4VL and level of water absorption, due to their distinct composition and architecture, were responsible for the differences seen. Although biocatalytic systems are inherently variable, the experiments resulted in very similar time courses, divided into periods of cell growth, 4HV accumulation and conversion to 4VL at pH 6.0 for 24 h, followed by 4VL recovery using the polymers for an additional 24 h. It is during the final, ISPR period that our approach of absorbing 4VL to pull the equilibrium reaction toward additional 4VL production was implemented, and allowed comparison of the polymers' affinity toward 4VL and water uptake for their impact on the biotransformation outcome.

Figure 2 shows a typical time course plot of biomass, 4HV and 4VL concentrations before and after pH shifting, prior to initiating ISPR. 4HV accumulation was approximately linear throughout each experiment until its plateau at approximately 18 g/L, indicating that controlling the levulinate feed rate maintained constant biocatalytic activity. As expected, conversion to 4VL was low prior to pH shifting, with 4 % converted (4VL concentration divided by the sum of 4HV and 4VL concentrations) at pH 7.0. Conversion increased to approximately 27 % at pH 6.0, with 4VL concentration rising to approximately 4 g/L. The difference in 4HV/4VL concentration ratios at pH 7.0 and 6.0 clearly demonstrates the pH-dependent nature of the reaction, with the equilibrium position, the feature of this model biotransformation, substantially favoring 4VL at lower pH. These results are similar to but slightly lower than the 33 % conversion initially reported for this biocatalytic pathway; in our hands, this system was unable to produce equivalent titers of 27 g/L 4HV and 8 g/L 4VL [6].

ISPR was initiated after the lactonization reaction had occurred for 24 h at pH 6.0, when the system was approaching its final equilibrium 4HV/4VL ratio, to definitively demonstrate the differential action of the polymers. Alternatively, a polymer could be introduced either at the beginning of fed-batch operation, or upon pH shifting and the same effect would be achieved without the time required to reach equilibrium a second time.

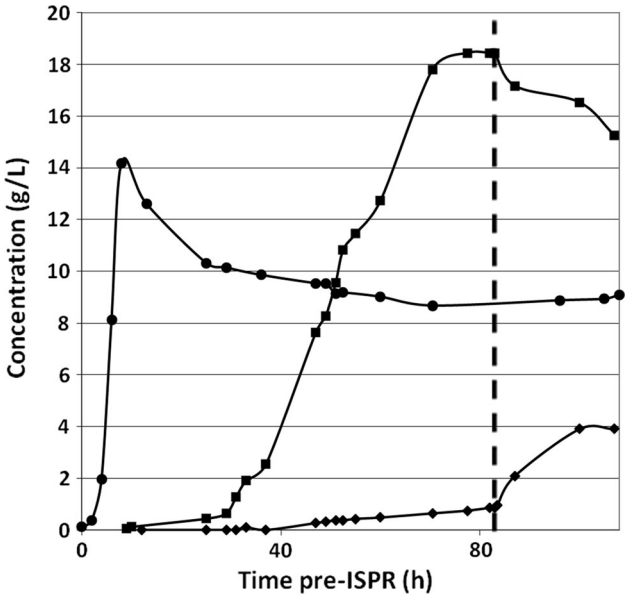

Fig. 2 Plot of biomass (*filled circles*), 4HV accumulation (*filled squares*) and 4VL (*filled diamonds*) during growth, 4HV accumulation, and 4VL accumulation phases. The *dashed line* indicates shifting pH to 6.0 to induce lactonization of 4HV to 4VL

Overall 4VL mass throughout the ISPR period was calculated using each polymer's swollen mass in the reactor at equilibrium, adding the change in volume to the polymer and subtracting it from the aqueous phase. Neglecting the polymer's dynamic swelling behavior has no effect on the calculated 4VL concentration at equilibrium. The polymers were added to the bioreactor dry, at a mass determined by the water uptake experiment performed in the bioreactor medium to give an equivalent swollen mass, to demonstrate the differential action of an equivalent wet mass of polymers. The addition of a pre-swollen polymer would dilute the bioreactor medium, skewing concentrations to different extents, potentially masking the effect of product absorption. This case is distinct from using pre-swollen polymers in the PC experiments, and medium uptake was accounted for prior to polymer addition.

BMI-PEO experiment

In the experiment with BMI-PEO, shown in Table 1, 240 g of dry polymer swelled by 375 % in the bioreactor medium, less than the 600 % in high-purity water. The difference in uptake between these two fluids is likely due to the presence of solutes in the medium, which decrease water activity and, consequently, its availability to hydrate the polymer chains. The swelling occurred at the expense of aqueous volume in the reactor, and increased the measured cell density, as cells were not absorbed by the polymer. However, aqueous concentrations of both 4HV and 4VL remained relatively stable, and the PC value of BMI-PEO toward 4VL in the reactor was similar to the value measured in high-purity water. The volumetric 4VL titer in the BMI-PEO experiment was lower relative to pre-ISPR because the additional volume occupied by the polymer was not offset by enhanced 4VL production. Synthesizing a polymer with a composition having maximum available PEO content for solute absorption gave an architecture which permitted significant water absorption, yet hampered selectivity, as discussed below.

Hytrel® 8206 experiment

In the experiment using Hytrel® 8206, summarized in Table 1, 930 g of dry polymer swelled by 24 % in the bioreactor. This modest water uptake also occurred at the expense of aqueous reactor volume; however, the selectivity toward 4VL relative to 4HV in this polymer was sufficient to shift the equilibrium by removing 4VL, due to its lower water uptake restricting the nonspecific permeation of all aqueous solutes. The mass of 4VL in the bioreactor using Hytrel® 8206 for ISPR increased by approximately 30 %, and the overall concentration of 4VL increased by 12 % on a volumetric basis, demonstrating that the polymer had a net beneficial effect which compensated for the additional volume it occupied. This represents the first successful report of equilibrium shifting of a biocatalytic reaction via product removal using an absorbent polymer.

Polymer selectivity

The production of additional 4VL with the introduction of a sequestering phase requires selective absorption of 4VL relative to 4HV, calculated by the ratio of partition coefficients observed in the reactor. Table 1 shows the variation in selectivity between the polymers which caused the observed differences. The vast majority of the hydroxyacid precursor, 4HV ($pKa = 4.6$), is deprotonated at the pH of this system and carries a negative charge, such that any significant uptake of this charged compound would be a result of non-selective water absorption by the hydrated polymer and would cause a loss of driving force for lactonization. Non-aqueous, non-ionic sequestering phases are known to selectively absorb neutral chemical species and reject charged compounds, and affinity can be augmented by the presence of charged solutes in the aqueous phase via the "salt effect" [27].

While the proportion of soft PEO available for solute permeation was maximized in the composition of BMI-PEO to promote 4VL affinity based on similar solubility

Table 1 Performance comparison of both polymer ISPR experiments

Performance metric	1,139 g BMI-PEO	1,153 g Hytrel® 8206
Dry polymer mass (g)	240	930
Total reactor volume (3L aqueous + dry polymer)	3.2 L	3.8 L
4VL PC in reactor	0.91	1.50
4HV PC in reactor	0.48	0.12
4VL/4HV selectivity	1.9	12.5
Volumetric 4VL improvement (g/L) from ISPR	−6 %	12 %
Mass 4VL improvement (g) from ISPR	0 %	30 %

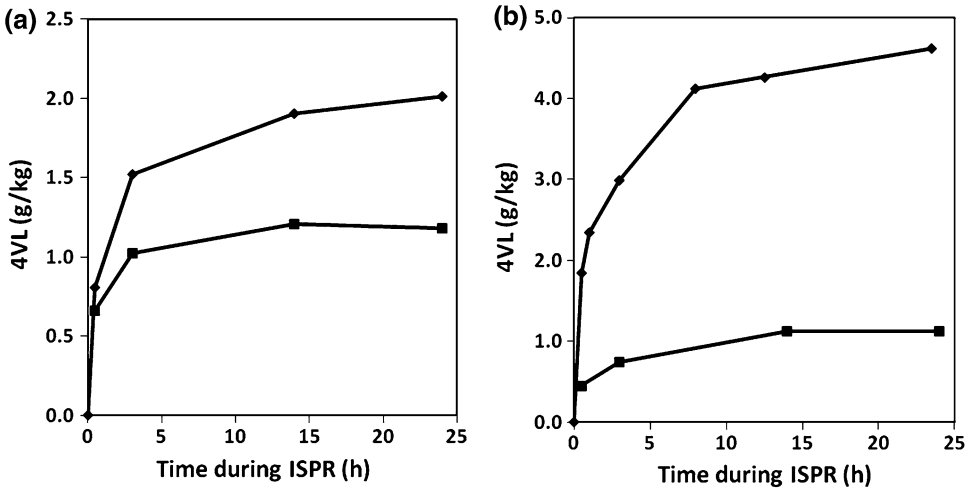

Fig. 3 4VL (*filled diamonds*) and 4HV (*filled squares*) accumulation in polymers during ISPR (g/kg) in **a** BMI-PEO, **b** Hytrel® 8206

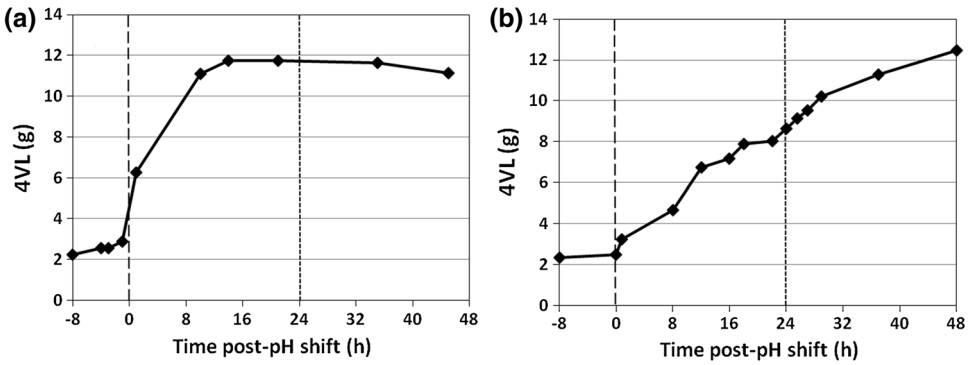

Fig. 4 Overall 4VL mass in the bioreactor vs. time normalized to onset of the pH shift. The first (*dashed*) line indicates the pH shift from 7.0 to 6.0, and the second (*dotted*) line indicates the addition of the polymer **a** 240 g BMI-PEO, **b** 930 g Hytrel® 8206

parameter values, this gave a loose architecture which allowed significant water uptake. The water uptake by BMI-PEO compromised selectivity by allowing diffusion of solutes (both charged and neutral) between both phases, shown by its relative uptake of both 4VL and 4HV in Fig. 3a. Consequently, BMI-PEO did not appreciably shift the equilibrium toward 4VL production, thereby hampering the effect of ISPR-driven equilibrium pulling, and produced no additional 4VL, shown in Fig. 4a. In contrast, the segmented block architecture of Hytrel® 8206 provided more robust network stabilization, allowing only modest water absorption, and maintaining sufficient selectivity toward 4VL relative to 4HV, illustrated in Fig. 3b. This selective removal of 4VL shifted the reaction equilibrium, producing 30 % more 4VL, shown in Fig. 4b. Furthermore, the partition coefficient of Hytrel® 8206 toward 4VL in the reactor was found to increase significantly relative to the value measured in high-purity water, a result of solutes in the medium which decreased water activity and drove more 4VL into the polymer, demonstrating Hytrel's selectivity in contrast to BMI-PEO's non-selective absorption of all solutes. The results indicate that a tradeoff exists between affinity and selectivity due to water uptake by polymers, such that minimizing inert content to maximize affinity must be balanced against selectivity losses imposed by water uptake, a property determined by polymer architecture.

Conclusions

Although this model biotransformation system clearly presents a practical challenge in product extraction due to the target molecule's affinity for water, this investigation has shown for the first time that using a selective, absorptive polymer phase can pull a pH-dependent equilibrium biocatalytic reaction by removing the product, thereby increasing production. This approach is simpler, less expensive, and operates on a different mechanism than conventional separations employing adsorbents having high specific surface areas, or those using ATPS which require tuning of specific operating conditions to trigger phase separation. A relationship between increasing water uptake and higher target molecule affinity was observed in the range of synthesized polymers, but selectivity was hampered by this high water uptake. In contrast, a moderately water-absorbing, commercial segmented block copolymer shifted the equilibrium due to its greater

selectivity toward 4VL and increased production by 30 %. In TPPB systems involving hydrophilic target molecules such as this one, water content may be a necessary feature of selected polymers, but should be controlled through polymer architecture, for example by employing block copolymers for the advantages demonstrated in their physical properties and selectivity. Future work aims to characterize the effect of water, determining at which point absorbed water begins counteracting the improvement to affinity by reducing selectivity in TPPBs.

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