Research Article

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Bioproduction of *cis*-(1S,2R)-indandiol, a chiral pharmaceutical intermediate, using a solid–liquid two-phase partitioning bioreactor for enhanced removal of inhibitors

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Abstract

BACKGROUND: A solid-liquid two-phase partitioning bioreactor (TPPB) was used in the biotransformation of indene to *cis*-(1S,2R)-indandiol by *Pseudomonas putida* 421-5 (ATCC 55687). Metered substrate feeding in single-phase operation, or delivery from an immiscible liquid, have previously been employed to regulate the exposure of the biocatalyst to inhibitory concentrations of the substrate. In contrast, the solid-liquid platform provided *in situ* substrate addition (ISSA) as well as simultaneous *in situ* product removal (ISPR) as a means of overcoming substrate and product toxicity. Three different modes of operation were compared for their effects on the performance of this biotransformation: single-phase, fed-batch operation was carried out as a benchmark in 2.75 L aqueous medium, and subsequently with the inclusion of either 700 g liquid silicone oil or 700 g solid polymer beads.

RESULTS: Biphasic modes achieved a 3-fold productivity improvement with respect to single-phase (30 to 90 mg L^{-1} h^{-1}), and solid-liquid productivity was similar to liquid-liquid operation while achieving more extensive removal of inhibitory compounds resulting in a slightly higher product titer (1.29 vs 1.16 g L^{-1}). The operability of the reactor was improved by the phase stability of the solid polymer beads relative to immiscible organic solvents, preventing emulsion formation and facilitating analytics.

CONCLUSION: Solid polymer beads replaced the immiscible liquid auxiliary phase for substrate delivery while performing simultaneous inhibitory molecule sequestration.

Keywords: indandiol; indene bioconversion; two-phase partitioning bioreactor; polymer beads; *Pseudomonas putida*

INTRODUCTION

Applications of whole cell-based biotransformations are increasingly targeting the production of high-value compounds, and often impart a specific chirality to their products due to the inherent 'handedness' of many biological processes.[1] An example of the bioproduction of a chiral pharmaceutical intermediate is the conversion of indene to *cis*-(1S,2R)-indandiol, which possesses the stereochemical configuration required in the synthesis of Crixivan® (indinavir sulfate), an HIV protease inhibitor from Merck and Co. Inc. The biological process is a potentially viable alternative to the synthetic approach, in which chiral resolution of the required enantiomer represents the most difficult step in the pathway.[2]

Pseudomonas putida F1 is among several organisms known to express toluene dioxygenase (TDO), which oxygenates toluene to *cis*-toluene dihydridiol, and can also asymmetrically convert indene in the presence of toluene to cis-indandiol favouring the (1S,2R) stereochemical configuration. Mutagenesis and screening studies by Merck researchers produced an isolate, *P. putida* 421-5 (ATCC 55 687), which expresses TDO inducible with indene alone.[3] The biocatalytic system shown in Fig. 1 illustrates the reactions, with the significant accumulation of an inhibitory by-product, 1-indenol, adding complications to achieving high product titers.[3]

P. putida 421-5 (ATCC55687) produces the desired enantiomer in a 2 : 1 ratio, corresponding to an enantiomeric excess of approximately 30%.[4] The 'ee' is fixed by the enzyme system in operation, such that 'ee' is not a performance metric appropriate for evaluating a bioproduction platform, but one that could be used when comparing cells.[5] Instead, yield, overall product concentration, specific productivity and overall volumetric productivity are more representative metrics of a production platform's performance. Low product yields reported for this system in the range of 0.2 are a result of by-product formation and kinetic resolution of the desired enantiomer through selective degradation of the (1R,2S) enantiomer.[2,3] Up to 97% of indene mass has been accounted for, including evaporation losses in another study investigating feeding strategies.[6]

The bioconversion of indene has previously been accomplished by controlled substrate feeding or by employing a two-phase

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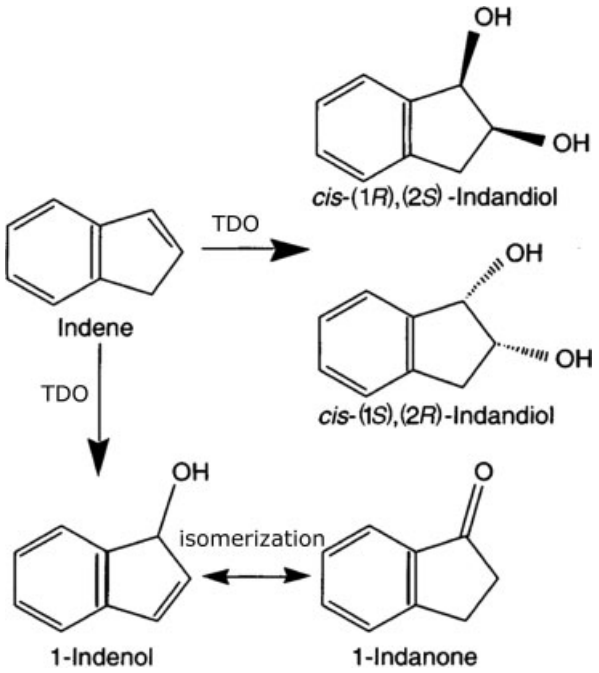

Figure 1. Indene bioconversion by *P. putida* TDO enzyme system showing indene conversion to detected products and by-products, adapted from Connors *et al.*[3] The monoxygenation product, 1-indenol, is a result of 'improper fit' of the non-natural substrate, indene, in the dioxygenase active site. 1-indenol undergoes slow, spontaneous isomerization to 1-indanone.

partitioning bioreactor (TPPB) platform, in which an immiscible carrier liquid such as silicone oil or soybean oil sequesters the hydrophobic compound, maintaining sub-inhibitory aqueous concentrations as a result of the distribution coefficient.[7] Biocatalytic consumption of substrate from the aqueous phase causes additional substrate to diffuse from the carrier phase, maintaining the concentration equilibrium between phases, and accomplishing *in situ* substrate addition (ISSA) at the rate of biocatalytic demand.[8] This process, driven thermodynamically by the concentration gradient, is bi-directional, and can be similarly exploited to remove inhibitory products as they are formed as in *in situ* product removal (ISPR).[9]

The substrate and desired product, indene and *cis*-indandiol, are known to be inhibitory to the biocatalyst at concentrations of 5 and 15 g L^{-1}, respectively. The by-products 1-indenol and its isomer, 1-indanone are the most inhibitory compounds (toxic at 1.5 and 3 g L^{-1}, respectively) with only 1-indenol accumulating significantly in this system.[7] The use of a hydrophobic carrier liquid for improving this biotransformation has focused exclusively on substrate delivery until this point; however, the by-product 1-indenol is more inhibitory than indene with a toxic concentration of 3 g L^{-1}, and could be proactively targeted to reduce biocatalyst inhibition and improve performance.[10]

Two-liquid-phase TPPBs have commonly used a rationally-selected immiscible liquid phase with affinity for the substrate and compatibility towards the biocatalyst.[11] The simultaneous requirements of biocompatibility, non-bioavailability and good partitioning, with biocompatibility being an absolute requirement, often results in a compromise in partitioning performance.[12] Recently, absorbent polymer beads have replaced immiscible liquids acting as the sequestering phase in otherwise identically-operated systems, with reports of significant improvements.[13,14] Solid polymer beads have been shown to significantly improve the operability of TPPBs compared with immiscible liquids because they are universally biocompatible and do not suffer from emulsion formation in the presence of biomass.[15] Association of the cell membrane with a dispersed immiscible liquid phase results in an emulsion which traps a significant portion of the biomass away from the aqueous phase, negatively impacting productivity as well as analytical procedures.[15–17] The presence of silicone oil complicates the quantification of products distributed between the two phases, which has been identified as a major hurdle in this bioproduction system.[5]

The bioconversion under investigation suffers inhibition from both substrate and products.[7] Using solid polymer beads having affinity towards the biotransformation products in place of silicone oil permits simultaneous *in situ* substrate addition (ISSA) and *in situ* product removal (ISPR), and is expected to improve performance by removing inhibitory products from the aqueous phase which would otherwise inhibit the biotransformation.

EXPERIMENTAL

Chemicals

Unless otherwise stated, all compounds were purchased from Sigma-Aldrich or Fisher Scientific Canada. Indene used for analytical standards was 98% pure, and >90% pure for bioconversions. (1,2)-indandiol was purchased from Wako Chemicals, Richmond, VA. Polymers used are described in Table 1.

Microorganism

The indene-converting organism, *P. putida* 421-5 (ATCC 55 687), isolated by Merck researchers, was purchased from ATCC and used for all biotransformations. The organism converts indene to indandiol during its growth phase, unlike several indandiol-producing strains used in other studies, which convert indene during the stationary phase. The choice of this organism was based on its commercial availability and its high specific productivity, which was thought to be a challenge for the solid–liquid two-phase partitioning bioreactor system from a system performance standpoint: a high rate of production requires an equally rapid mass flux rate between the two phases in order to meet the biocatalyst's metabolic demands. The chiral distribution of the product is assumed to have no impact on the diffusion processes operating in the two-phase systems because the polymers lack any chiral character.

Analytics

Cell concentrations were determined by measuring optical density at 600 nm with a Biochrom Ultrospec 3000 UV/Vis spectrophotometer using a calibration curve relating optical density to cell dry weight (CDW). It was not possible to quantify biomass in the silicone oil run due to emulsion formation affecting optical density measurements.

High performance liquid chromatography (HPLC) analysis of bioconversion was performed by separation on a Varian Pursuit C8 5 μm 4.6 × 250 mm column and detection on a Varian ProStar 325 UV/Vis at 220 nm, as described by Connors *et al.*[3] and using an injection volume of 20 μL. Under these conditions, retention times for *cis*-indandiol, 1-indenol, 1-indanone, and indene were 9.6, 13.9, 14.9, and 21.6 min, respectively. Concentrations were determined by area count calibrations generated with known analytical standards, with the exception of 1-indenol, which is

Table 1. Properties of evaluated polymers

Name	Composition	Glass transition temperature (°C)[reference]	Supplier
Hytrel® 8206	poly(butylene terephthalate)-polyether	−59[18]	DuPont
Hytrel® 3548	poly(butylene terephthalate)-polyether	−40[a]	DuPont
Elvax® 40 W	ethylene/vinyl acetate	−17[19]	DuPont
Kraton® D4150K	styrene/butadiene block copolymer	−80[b]	Kraton Performance Polymers Inc.
PEBAX® 4033	polyether block amide	−65[b]	Arkema Canada Inc.
Silicone rubber	poly(dimethylsiloxane)	N/A	Mastercraft
Silicone oil (5 cSt)	poly(dimethylsiloxane)	N/A	Sigma-Aldrich

[a] E.I. du Pont de Nemours and Company http://www2.dupont.com/Plastics/en_US/assets/downloads/processing/H81091.pdf.
[b] Automated Creations Inc. http://www.matweb.com/.

unavailable commercially. The initial consumption of glucose was monitored using the DNS assay[20] and refractive index HPLC, but a biotransformation product interfered with these methods at later times. Other studies on this system have used more sophisticated methods to measure glucose.[10]

1-indenol analysis

A large HPLC peak was produced in accordance with literature reports of 1-indenol's presumed formation as a monooxygenation by-product, and eluted 1 min before its close relative 1-indanone.[3] The identity of 1-indenol in this system was confirmed in another study by nuclear magnetic resonance (NMR) and mass spectrometry (MS).[4] To further substantiate the identity of this peak in our study, the octanol–water distribution coefficient (log *P*) values of (1,2)-indandiol and 1-indanone were predicted by the group contribution method with the Molspiration miLog*P* 2.2 tool as 0.47 and 1.72, respectively (Ertl P. (http://www.molinspiration.com/cgi-bin/properties)). Indene's log *P* of 2.92 was obtained from published data.[21] The log *P* values were correlated with their observed retention times under identical reversed-phase HPLC analysis conditions.[22] The predicted log *P* value for 1-indenol of 1.39 was found to fit this linear trend ($R^2 = 0.99$). Area counts, expressed throughout as mAu*min L^{-1} for injection volumes of 20 µL, were used for quantification of 1-indenol concentration because this compound is not commercially available.

Indene biotransformation experiments

All experiments were conducted using a 5 L BioFlo III bioreactor (New Brunswick Scientific, Edison, NJ) filled with 2.4 L of medium K as described by Buckland *et al*.[2] The bioreactor was inoculated with a 24 h culture of *P. putida* 421-5 (ATCC 55 687) grown in 6 × 125 mL Erlenmeyer flasks each containing 40 mL TSB incubated at 180 rpm and 30 °C. The vessel was aerated at 0.6 vvm and agitated at 500–600 rpm with two Rushton turbines. Dissolved oxygen was monitored with a Broadley-James D100 series OxyProbe and remained above 40% air saturation. pH was maintained at 7.0 with 5 mol L^{-1} KOH using a Broadley-James FermProbe. Antifoam 204 was added dropwise as required. A sterile 50% glucose feed was initiated prior to glucose depletion at 55 mL h^{-1} (10 g L^{-1} h^{-1}) as described by Buckland *et al*.[2] using an Imed Gemini® gravity-fed intravenous pump.

Single-phase operation

As a benchmark for comparison with two-phase experiments, two single-phase, fed-batch biotransformations were performed using a medium amended for higher cell density to compensate for biocatalytic inactivation in an attempt to reduce indene toxicity by reducing the specific loading of inhibitors, which preferentially associate with the cells, as reported by Amanullah *et al*.[10] Sterile-filtered indene was fed hourly at a rate of 0.1–0.2 g L^{-1} h^{-1} to avoid its excessive accumulation or loss by air stripping, resulting in the cumulative addition of 5.6 g and 10 g of indene, respectively, after 12 h for each experiment. Samples were passed through a 0.2 µm filter prior to HPLC analysis.

Liquid–liquid two-phase operation

To reproduce the optimized two-liquid-phase operation described by Buckland *et al*.[2] as a basis for two-phase comparison, the bioreactor was run with the addition of 700 mL of silicone oil ($\rho = 0.99$, $\eta = 5$ cSt) with the addition of 30 g L^{-1} indene, giving a phase fraction of 25% (w/v). Bioreactor samples were extracted with 2 volumes of isopropanol by shaking at 180 rpm and 30 °C for 20 min. Samples were centrifuged to remove the cells and oil from the aqueous/alcohol phase and the supernatant was analyzed by HPLC.

Polymer selection

To compare the ability of different polymers to partition the various components of this biotransformation under realistic conditions, seven commercially-available polymers, including liquid silicone oil ($\eta = 5$ cSt), were evaluated for their affinity for the biotransformation products in the aqueous phase. All polymers evaluated occur as small spherical or oval beads approximately 2–3 mm in diameter, and have a glass transition temperature (T_g) well below the operating temperature of the biotransformation. This property is thought to be important for polymer chain mobility, ensuring that the polymeric matrix permits the diffusion of large molecules, for which a rigid glassy structure would be impermeable.[15]

The by-product, 1-indenol, has been identified as one of the most inhibitory compounds and is also the major bioconversion product in this system.[7] For this reason, 1-indenol affinity was chosen as the basis for polymer selection. Because 1-indenol is not commercially available, spent biotransformation medium containing (1,2)-indandiol (250 mg L^{-1}), 1-indenol (450 mAu*min L^{-1}), and 1-indanone (50 mg L^{-1}) was centrifuged and passed through a 0.2 µm filter to be used to evaluate the polymers. Aliquots of 10 mL were placed in 20 mL scintillation vials, containing varying masses of each polymer from 0.5 to 2.5 g in 0.5 g increments. The vials were agitated in a shaker at 30 °C and 180 rpm overnight to establish equilibrium conditions. Concentrations

were measured by HPLC at equilibrium and compared with a control lacking polymer beads, and concentrations in the polymers were calculated by mass balance. The ratio of the concentration in the polymer (g kg^{-1}) to aqueous concentration (g L^{-1}) at equilibrium gave the distribution coefficient of each compound for each polymer in the concentration range.

Prior to the 1-indenol affinity evaluation, seven commodity polymers had been previously evaluated for affinity towards 1-indanone due to its structural similarity to 1-indenol and its commercial availability; however, 1-indenol was determined to be a more appropriate test compound for reasons given above.

Because the delivery of indene from a polymer first requires its loading into the polymer phase, the partitioning behaviour of Hytrel® for indene was investigated. To determine the stability of the polymers when in prolonged contact with the aromatic solvent, 5 g of polymer beads were immersed in 5 mL of indene for several days.

Polymer loading and washing

The beads were immersed in 700 mL isopropanol with the addition of 50 g indene and equilibrated overnight to give an estimated indene concentration of 50 g kg^{-1} in the polymer beads using the equation:[23]

$$S_{polymer} = \frac{M_{indene}}{M_{polymer} + M_{liquid}/K_{S/L}} \quad (1)$$

where $S_{polymer}$ is the concentration of indene in the polymer (g kg^{-1}), M_{indene} is the mass of indene (g), M_{liquid} is the mass of isopropanol in the system (kg), and $K_{S/L}$ is the distribution coefficient for indene between the solid polymer and the liquid solvent.

The loading solution was decanted from the beads through a sterilized stainless steel screen and washed with five volumes of sterile water because residual isopropanol within the beads was found to strongly inhibit the culture. A target aqueous isopropanol concentration of below 1% (v/v) in the reactor medium was found to not adversely affect the culture and was confirmed using refractive index HPLC. Indene removal as a result of the washing procedure was found to be approximately 25%, resulting in an indene concentration in the polymer of approximately 33 g L^{-1} based on Hytrel®'s density of 1.16 g kg^{-1}.

Solid–liquid two-phase operation

The bioreactor was run with the addition of 700 g Hytrel® 8206 ($\rho = 1.16$) polymer beads loaded with 33 g L^{-1} indene, giving a solid phase fraction of 25% (w/v). Bioreactor medium samples were periodically withdrawn and filtered for HPLC analysis or extracted with two volumes of isopropanol before centrifugation and HPLC analysis of the supernatant. Contents of the polymer beads in the reactor were analyzed by removing 20 beads (approximately 0.4 g), briefly rinsing their surface with water, then placing them in a 20 mL scintillation vial containing 10 mL isopropanol and allowing to equilibrate at 180 rpm and 30 °C for at least 4 h before HPLC analysis. It was found that >90% of sorbed material is recovered in the first round of extraction and >90% of the remainder upon a second extraction. A negligible amount of material was extracted in the third round of extraction, demonstrating the simplicity of product recovery from absorbent polymer beads. Other studies have demonstrated the reusability of the polymer beads after this extraction procedure.[13,14,24]

Figure 2. Photograph comparing swelling behavior in different grades of Hytrel® when exposed to indene: 3548 swelling and cracking on left, 8206 unaffected on right. Unexposed beads in foreground for comparison with paper clip for scale.

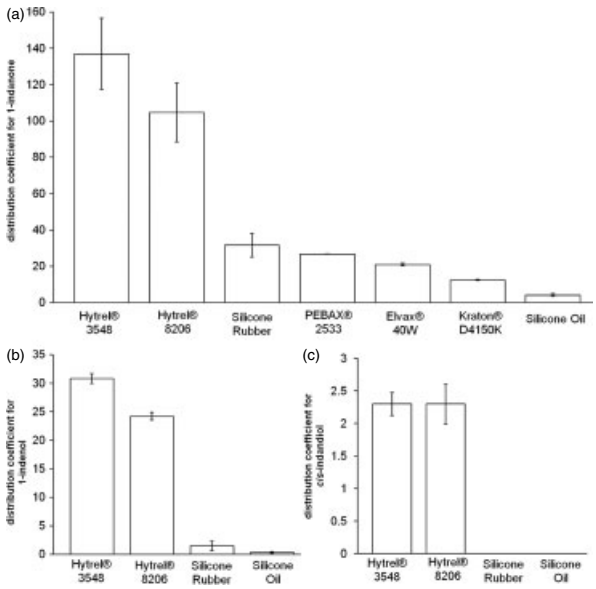

Figure 3. Polymer distribution coefficients for biotransformation products: 1-indanone (a), 1-indenol (b) and cis-indandiol (c). Distribution coefficient is expressed as the concentration in the polymer (g kg^{-1}) divided by the concentration in the aqueous phase (g L^{-1}). Error bars represent one standard deviation from the mean value of five samples.

Concentrations in the polymer samples were calculated by mass balance of the extracted compounds assuming negligible losses due to volatility. Overall concentrations in the reactor were determined by summation of polymer-sorbed and aqueous-phase masses, then dividing by the entire working volume represented by the combined aqueous and polymer volumes.

RESULTS AND DISCUSSION

Polymer selection

Both grades of Hytrel® were found to have a distribution coefficient for indene in isopropanol of approximately 2. Excessive swelling and fission of the 3548 grade of Hytrel® and absence of swelling in 8206 with prolonged contact with indene, illustrated in Fig. 2, led to the selection of 8206 for biotransformation experiments despite its slightly lower affinity for the biotransformation products (Fig. 3). Communication with DuPont indicated that the 8206

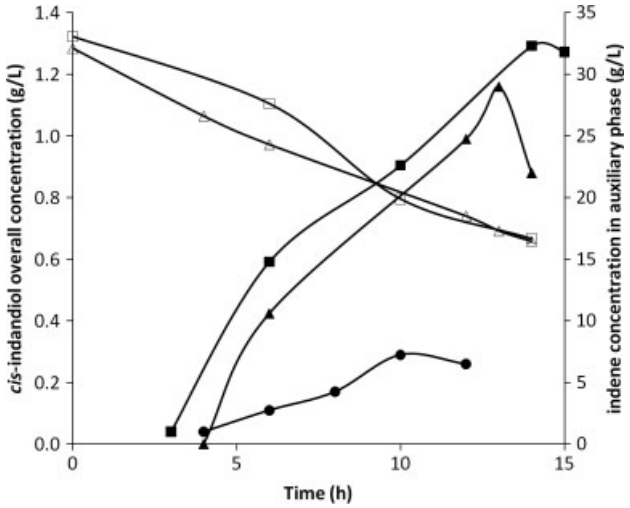

Figure 4. Time course plot of overall product concentration (closed symbols, left axis) and substrate concentration in auxiliary phase (open symbols, right axis) under liquid–liquid (triangles) and solid–liquid (squares) operation. Single-phase, high substrate feed operation (circles) lacks an auxiliary phase.

grade, lacking a crystallinity disruptor, may make it more solvent resistant than 3548, which contains a disruptor to increase softness.

Both grades of Hytrel® showed significantly higher affinity towards 1-indanone than the other polymers (Fig. 3(a)). The distribution coefficient of Hytrel® for 1-indenol was found to be approximately 65-fold that of silicone oil (Fig. 3(b)), indicating that there is potential for improving by-product removal from this biotransformation by selecting from a range of commodity polymers. Commodity polymers cost much less than silicone oil at approximately \$5 per kg[25] compared with \$300 per kg (Sigma-Aldrich Co. (http://www.sigmaaldrich.com)), greatly reducing the cost of an otherwise identical system.

The partitioning behavior of (1,2)-indandiol between the polymer and aqueous phases was much less extensive than with the other compounds, probably a result of its relative hydrophilicity due to the presence of two hydroxyl groups. There was no detectable uptake of (1,2)-indandiol by any polymer except Hytrel®, which had a distribution coefficient of approximately 2 for each grade (Fig. 3(c)). This suggests that the desired product remains almost entirely in the aqueous phase when silicone oil is employed as the auxiliary phase.

Bioconversion experiments

Product and by-product accumulation began 3–4 h post-inoculation and lasted for approximately 8–9 h in every run (Fig. 4). The lag in product accumulation was probably a period of enzyme induction upon exposure to indene in the bioreactor, as the TDO system would not have been induced in the inoculation flasks lacking indene. Accumulated products appeared as chromatogram peaks corresponding to the conversion of indene to 1-indenol, *cis*-indandiol, and 1-indanone, illustrated in Fig. 1. Degradation products were not detected during the time course of the experiments, and indene concentrations in the aqueous phase remained very low throughout the production period in each experiment, demonstrating its higher affinity for the auxiliary phase and the biocatalyst than for the aqueous phase. The difference in productivity between single-phase and two-phase experiments, despite similar substrate availability, highlights the benefits of an auxiliary phase in alleviating inhibition arising from the use of an extremely hydrophobic substrate.

The earlier end point in the two-liquid experiment may be a result of biocatalyst inactivation with the accumulation of higher aqueous concentrations of 1-indenol, which was more suppressed when using the solid polymer. Indene and *cis*-indandiol did not accumulate to inhibitory concentrations in the medium. A nutrient deficiency was suspected but it was found that additional nutrient supplementation provided no change in growth or productivity in other experiments (data not shown). The extent and duration of each bioconversion used to calculate the performance metrics presented in Table 2 were based on the point of maximum product titer, as enzymatic degradation of the undesired enantiomer occurs rapidly with a consequent decrease in overall product titer.[2]

Single-phase operation

The single-phase, fed-batch runs were the least productive mode of operation evaluated (Table 2). This was probably a result of indene's rapid and direct exposure to the biocatalyst causing inhibition, with higher feed rates resulting in lower biomass concentration. Without an auxiliary phase to sequester indene, it preferentially associates with the hydrophobic cell membrane resulting in cellular stress response and eventual membrane failure.[7] A brown pigmentation was more intense in the experiment with the highest cumulative indene addition than with either lower indene feeding or the more productive biphasic experiments. The inhibitory by-product 1-indenol did not accumulate to inhibitory concentrations in single-phase experiments.

Liquid–liquid operation

The reactor operated using silicone oil achieved a similar final titer and volumetric productivity to that using polymer beads (Table 2). These are in agreement with reported volumetric productivities for other indene-converting systems; however, there is no reported specific productivity for a system using this biocatalyst in the presence of silicone oil. Although product titer has been reported to reach 2 g L^{-1} at the 23 L scale, the lack of biomass quantification makes comparisons of performance difficult.[2]

The presence of silicone oil complicated analytical procedures, requiring a phase separation step after whole-broth extraction, eliminating the possibility of determining relative concentrations between the two phases. However, maximum 1-indenol and 1-indanone concentrations in the medium were estimated at 1720 mAu*min L^{-1} and 300 mg L^{-1}, respectively, based on the concentration in the whole-broth extract. Optical density measurements were also confounded by the emulsion formed during agitation and aeration, so it was not possible to quantify biomass despite efforts to resuspend the cells in fresh medium.

Solid–liquid operation

The reactor operated using polymer beads achieved a similar and slightly higher overall product concentration to that of the silicone oil system (Table 2). Operation of the bioreactor using polymer beads simplified analytical procedures: direct aqueous sampling as well as the extraction of polymer-sorbed compounds enabled the separate analysis of each phase. Biomass concentration was quantified without complications and enabled the determination of specific productivity, an important performance metric. Polymer beads were examined microscopically and found to have no microbial attachment to their surface. Electron microscopy of bead

Table 2. Comparison of single-phase, two-liquid, and solid–liquid productivity. Overall product titer calculated by dividing total product mass in both phases by total working volume

	Operational mode (Auxiliary phase volume) Total volume			
Performance metric	Single-phase Low indene feed 2.75 L	Single-phase High indene feed 2.75 L	Liquid–liquid (0.7 L) 3.45 L	Solid–liquid (0.6 L) 3.36 L
Substrate consumed (g) (overall)	5.6	10.0	9.4	13.5
Final product titer (g L^{-1}) (overall)	0.14	0.29	1.16	1.29
Time to completion (h)	13	12	13	14
Biomass concentration (g L^{-1}) (aqueous)	3.8	2.5	Biomass not quantifiable	2.7
Molar yield (indandiol/indene)	0.08	0.09	0.51	0.40
Specific productivity ($mg_{product}$ g^{-1}_{cells} h^{-1})	1.0	4.2	Biomass not quantifiable	11.8
Overall volumetric productivity ($mg_{product}$ L^{-1} h^{-1})	10	29	89	92

surfaces has also previously confirmed the absence of biofilm on polymer beads.[24]

The polymer phase successfully removed 1-indenol, maintaining concentrations below 864 mAu*min L^{-1} in the medium throughout the experiment while sequestering approximately 12 460 mAu*min L^{-1}. Similarly, the polymer phase absorbed a total of 689 mg of 1-indanone by the end of the experiment, and maintained very low aqueous concentrations below 50 mg L^{-1} throughout. It is not possible to directly compare aqueous concentrations to the silicone oil experiment for reasons previously mentioned.

The mass distribution of *cis*-indandiol at the end of the solid–liquid runs was found to be approximately two-thirds remaining in the aqueous phase and one-third absorbed by the solid polymer, corresponding to the observed distribution coefficient in which the product concentration in the polymer (2 g kg^{-1}) was twice that in the aqueous phase (1 g L^{-1}) of the solid–liquid system, resulting in an overall concentration of 1.29 g L^{-1}. While not an ideal case with respect to product separation and recovery, this is a demonstration of ISPR operating simultaneously with ISSA from a single auxiliary phase, and there is potential to improve product absorption using a second type of polymer.

Two-phase comparison

The molar yield was higher in each two-phase experiment than in single-phase (Table 2). Yield was calculated from the observed change in indene in the auxiliary phase for the two-phase runs, and the total amount of indene added for the single-phase runs because of its absence from the aqueous phase and near-complete association with the biomass. The substrate concentration in the auxiliary phase was similar but not identical between the liquid–liquid and solid–liquid systems, and productivity was similar. Indene was oversupplied and incompletely consumed in both two-phase experiments such that substrate availability does not appear to be a limiting factor in these systems.

While yield values are higher than literature reports, they represent the combination of both enantiomers, with literature reports specifying *cis*-(1S,2R)-indandiol.[2] If the enantiomeric excess is assumed to be approximately 30% for this organism as reported in literature, the corresponding yield of the specific enantiomer would indeed be lower and similar to reported literature values of 0.2. Substrate loss due to evaporation was probably more severe under single-phase operation than two-phase operation[2], and may negatively affect single-phase yield coefficients.

The slightly higher product yield in the two-liquid system relative to the solid–liquid system may be the result of enhanced removal of 1-indenol with respect to *cis*-indandiol by the polymer phase. Buckland *et al.*[2] investigated the presence of each product inhibiting its own formation, and found that excess 1-indenol shunted production towards relatively more *cis*-indandiol and vice versa. A reduced 1-indenol concentration in the aqueous phase could negatively affect the *cis*-indandiol yield coefficient as more 1-indenol is produced in response to lower feedback inhibition.

Alternatively, the difference in yield may be a feature of the solid polymer's higher affinity towards the product: as kinetic resolution of the undesired enantiomer occurs rapidly towards the end of the biotransformation, the portion of substrate retained in the solid auxiliary phase would be protected and yield would remain higher than with a liquid auxiliary phase having poor affinity and leaving the vast majority available for degradation. This hypothesis is supported by the apparently large difference in degradation rate at the end of the two-phase experiments. While not ideal for this system in terms of the desired enantiomer, this feature would be very useful where product degradation was a significant barrier.

The production kinetics are very similar between the two biphasic systems, suggesting that there was sufficient interfacial area available for substrate mass transport between the solid and aqueous phases to enable similar productivity to a liquid–liquid system having a larger interfacial area permitting near-instantaneous equilibrium.[23] If interfacial mass transfer were found to be a limiting factor with respect to product removal, the specific surface area of the polymer beads could easily be increased by size reduction, reducing the diffusion path length.[25] Additionally, in such situations, a criterion for the selection of solid polymer phases may be diffusivity: the rate at which a target molecule permeates and diffuses through the polymeric matrix.

Using solid polymer beads in place of silicone oil for indene delivery accomplished *in situ* substrate addition with simultaneous product removal, and provided significant operational and cost benefits. Volumetric productivity under solid–liquid operation was similar to liquid–liquid operation, presumably due to the polymer's affinity for the major inhibitory by-product of this biotransformation.

Potential improvements to this biotransformation may arise from opposite ends of process development: biocatalyst modifications to improve yield, volumetric productivity, and 'ee' through enzyme modification or by manipulating gene expression for constitutive production will improve productivity, but would require considerable effort and expense. Identifying materials with superior affinity and selectivity for the desired product and inhibitory by-products could also significantly enhance performance by

allowing more complete conversion of substrate to product by alleviating inhibition and achieving preliminary separation and product recovery. An improved biocatalyst would also benefit from advances in ISPR materials.

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