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Production of (*S*)-γ-fluoroleucine ethyl ester by enzyme mediated dynamic kinetic resolution: Comparison of batch and fed batch stirred tank processes to a packed bed column reactor

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Abstract

Immobilized *Candida antarctica* lipase B has been shown to form the pharmaceutically important (*S*)-γ-fluoroleucine ethyl ester intermediate from the ring opening of an azlactone with ethanol, providing 79% yield and 78% enantiomeric excess (ee) for a stirred tank batch process demonstrated at multi kilogram scale. A kinetic analysis of the reaction system was defined which included the selective enzyme mediated dynamic kinetic ethanolysis, the background hydrolysis to acid and the non selective ethanolysis to racemic ester. This was used to generate a kinetic model which made excellent predictions of the reaction behavior. This kinetic model was then used to develop two new optimized processes. A fed batch stirred tank process was developed that increased productivity four fold compared to the original batch process, while maintaining a similar product ee value. Additionally, a plug flow column reactor process was developed that significantly improved productivity *via* a 20 fold reduction in the enzyme deactivation rate observed in the batch processes. As a result, the plug flow reactor generated a 20 fold reduction in enzyme to substrate ratio and an increased product yield (>90%) and ee (86%) over the initial batch process, producing an effective and economical process for large-scale production.

Keywords: Biocatalysis; Column reactor; Mathematical modeling; Enzyme; Packed bed; Azlactone ethanolysis

1. Introduction

The asymmetric synthesis of fluorinated amino acids is a proven technology for the production of potential pharmaceutical intermediates that have a wide array of physiological functions, including enzyme inhibitors, receptor antagonists, and lipophilicity enhancing agents (Bey, 1978; Filler et al., 1993; Gelb et al., 1990; Kollonitsch, 1982; Welch and Eswarakrishan, 1991). Dynamic kinetic resolution (Noyori et al., 1995; Ward, 1995) ring opening of azlactones has been demonstrated as an effective way of introducing stereochemistry in amino acid intermediates. Enzyme catalysts have been used to successfully perform the selective ring opening of azlactones in dynamic kinetic fashion. Porcine pancreatic lipase and lipase from *Aspergillus niger* catalyze the hydrolysis of 2-phenyl-oxazolin-5-ones with excellent selectivity >99% (Gu et al., 1992). *Pseudomonas cepacia* lipase has been demonstrated to catalyze the enantioselective methanolysis of several 4-substituted oxazolin-5-ones (Crich et al., 1993). A variety of factors must be controlled in these reaction systems to obtain highly selective, highly productive processes. A comprehensive screen of enzyme catalysts, reaction solvents, nucleophilic alcohols, and base additives is crucial to identifying a highly selective reaction system (Bevinakatti et al., 1992; Turner and Winterman, 1995). Additionally, competing reactions, such as non-selective background hydrolysis (Crich et al., 1993) and non-selective nucleophilic alcohol addition can result in decreased product yield and ee.

A 1:1 (enzyme to substrate) ratio of Novozymes-435 immobilized *Candida antarctica* lipase B (CalB) in methyl tert-butyl ether (MTBE) at 21 °C with a catalytic amount of triethylamine

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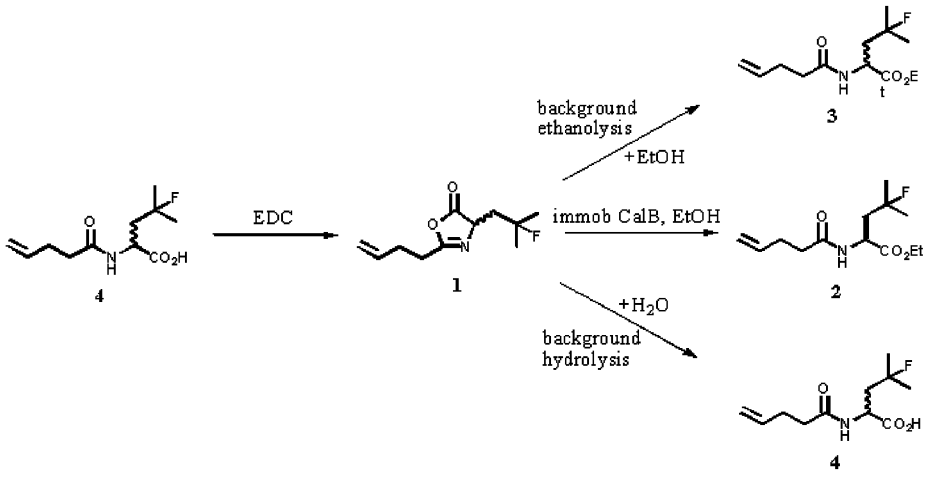

Scheme 1. Azlactone formation from pentenamide acid and conversion to ester product.

(Et_3N) has been used by Limanto et al. (2005) to form the (*S*)-γ-fluoroleucine ethyl ester **2** from the ring opening of azlactone **1** with EtOH, providing 79% yield and 78% ee (Scheme 1). This provided enantiomerically enriched *N*-protected γ-fluoroleucine ethyl ester. The *N*-pentenoyl group was then removed with *N*, *N*′-dibromodimethylhydantoin in the presence of trifluoroacetic acid to afford the (*S*)-γ-fluoroleucine ethyl ester. The system operates dynamic kinetically, with spontaneous racemization of the azlactone via enol tautomerization, allowing the yield of (*S*)-γ-fluoroleucine ethyl ester to be greater than 50%. A screen of several nucleophilic alcohols, reactions solvents, and base additives showed no significant improvement in selectivity or yield. A 1:1 ratio of enzyme to substrate was necessary as reducing the enzyme level led to decreased product yield and ee (Limanto et al., 2005).

This paper outlines the optimization work carried out to define a manufacturing process for the synthesis of the (*S*)-γ-fluoroleucine ethyl ester **2** intermediate. The 1:1 ratio of enzyme to substrate used for the initial batch process needed to be reduced at least three fold, while maintaining product yield and ee, in order to produce an economically feasible process. This work describes the development of a kinetic model that defined the key process optimization parameters to maximize productivity. The model was then used to develop robust processes that included stirred tank fed batch and column plug flow reactors, which significantly increased the productivity of the process when compared to the initial stirred tank batch process.

2. Experimental

2.1. Preparation of azlactone

The azlactone substrate for the enzymatic ring opening was synthesized by adding 1.2 M equivalents of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) purchased from Sigma-Aldrich (St. Louis, MO) to a 140 g/L solution of pentenamide acid **4** in tetrahydrofuran (THF) (Limanto et al., 2005). The reaction was aged at room temperature for 30 min. Upon completion, 1 × volume of DI water was added to quench the reaction and 1 × volume of methyl tert-butyl ether (MTBE) was added to extract the azlactone product into the organic phase. The aqueous phase was then extracted with 0.5 × volume of MTBE and the combined organic phase was washed with 1 × volume saturated sodium bicarbonate solution to remove any residual acid. Distillation of the organic phase with 5 × volumes of MTBE lowered the water concentration of the resulting solution to < 200 ppm by Karl Fischer (KF) analysis. The solution was then concentrated to an oil by vacuum distillation.

2.2. Biotransformation reactions

2.2.1. Stirred tank batch reaction

Batch reactions were run in two sizes of temperature controlled stirred tank reactors. Thirty milliliter Mettler-Toledo (Columbus, OH) MultiMax reactors and 250 mL Infors (Bottmingen, Switzerland) Sixfors reactors were used. Agitation was carried out with overhead stirring via a pitched blade impeller and was sufficient to suspend the immobilized enzyme resin (Novozyme-435 immobilized *Candida antarctica* lipase B). Batch reactions were carried out in MTBE with the following reaction component concentrations: 80 g/L azlactone, 86 g/L EtOH, 7.6 g/L Et_3N, and 80 g/L immobilized enzyme.

2.2.2. Stirred tank fed batch reactions

Fed batch reactions were carried out in the same vessels as the batch reactions using the same initial conditions. Substrate additions of 80 g/L azlactone and 17.2 g/L EtOH (i.e. 80 g azlactone and 17.2 g of EtOH for a 1 L reaction) were added directly to the reaction vessel when conversion reached > 90%. In total, three substrate additions were made after the conversion reached > 90%. After the final addition, the reaction was allowed to proceed to completion. *In situ* mid IR monitoring was accomplished using the Mettler-Toledo (Columbus, OH) ReactIR 1000. The azlactone was monitored at 1780–1863 cm^{-1} and the product ester was monitored at 1716–1777 cm^{-1}. This

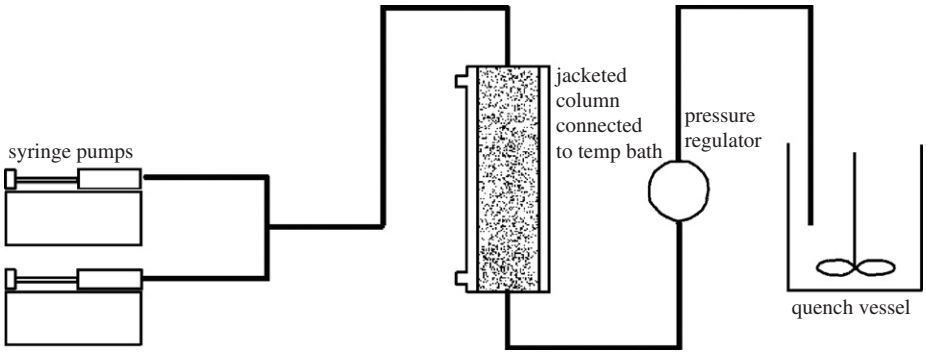

Fig 1. Schematic of column reactor setup.

allowed for real time analysis of the extent of conversion and aided in the determination of when to add substrate shots.

2.2.3. Plug flow column reactor

Column reactions were run in either a Whatman (Brentford, UK) stainless steel analytical column 50 mm long with a 4.5 mm inner diameter (capable of holding 0.2 g enzyme) or a Kontes (Vineland, NJ) jacketed glass column 100 mm long with a 10 mm inner diameter (capable of holding 1 g enzyme). The immobilized enzyme was pre-swelled in MTBE (to about twice their initial volume) and then packed in the column under gravity. Two feed solutions were then made: 160 g/L substrate azlactone feed in MTBE and a second feed containing 172 g/L ethanol and 15.2 g/L Et_3N in MTBE.

A schematic of the column reactor setup is shown in Fig. 1. The two solutions were fed via syringe pumps at equal rates (combined flow of 10 mL/h-g catalyst) and mixed just before entering the top of the column. The column effluent was fed through a back pressure regulator set at 20 psi to prevent the MTBE from boiling at elevated temperatures, and was sent to a quench vessel containing 1 N H_2SO_4. The acid quench converted any remaining unconverted azlactone to acid so background ethanolysis could not degrade the product ee value.

2.3. Experimental determination of rates for the kinetic model

2.3.1. Rates of reactions vs. temperature

The effect of temperature on reaction rates of ethanolysis and hydrolysis was studied by running batch reactions at 10 mL scale under standard conditions (Section 2.2.1) with magnetic stirring and temperature controlled at 22, 31, 40, and 55 °C. These reactions were then repeated under the same conditions and temperatures, but without enzyme in order to determine the background rates of hydrolysis and ethanolysis. Samples were taken (Section 2.4) every 10 min for the first hour of reaction time, and the initial reaction rates for enzymatic ethanolysis, background ethanolysis and background hydrolysis were determined.

2.3.2. Reaction rates vs. substrate concentrations

The effect of substrate concentration on reaction rates of ethanolysis and hydrolysis was studied. Batch reactions were run under the same conditions as those listed in Section 2.3.1, but reactions were run with the following azlactone concentrations: 0, 8, 17, 28, and 80 g/L. A second set of batch reactions was run with an azlactone concentration of 80 g/L but with EtOH concentrations of 8.6, 25.8, and 86 g/L. Samples were taken (Section 2.4) over the first hour of reaction time and the initial reaction rates for enzymatic ethanolysis, background ethanolysis and background hydrolysis were determined.

2.3.3. Enzyme deactivation rate

Experiments were set up to study the effects of agitation on enzyme stability independently of the impact of reaction components. Batch reactions (Section 2.2.1) were set up at 22, 31, 40, and 55 °C in MTBE with the following conditions: 80 g/L immobilized enzyme, with and without 86 g/L EtOH, with and without 7.6 g/L Et_3N. Eight identical reactions were set up for each condition. Four of the eight reactions were stirred at sufficient overhead agitation to suspend the immobilized enzyme (300 rpm). The other four reactions were stirred extremely slowly (60 rpm), insufficient agitation to suspend the immobilized enzyme. Eighty gram per liter of azlactone substrate was added to the first of each set of four reactions after 1 h. Substrate was charged to the second of each set of four reactions after 2 h, to the third of each set after 3 h, and to the fourth of each set after 4 h. After the substrate addition to the slowly mixing vessels, the agitation was increased until it was sufficient to suspend the immobilized enzyme (300 rpm). In this way, the enzyme was pre-incubated for a period of time (1, 2, 3, and 4 h) in the reaction system at varying temperatures (22, 31, 40, and 55 °C), agitation, and reaction components (with Et_3N and EtOH, with Et_3N and without EtOH, without Et_3N and with EtOH, without Et_3N or EtOH) prior to the addition of azlactone substrate. The initial reaction rate was then determined for each reaction, and enzyme inactivation over time was calculated.

2.4. Analytical techniques

2.4.1. Reverse phase HPLC analysis for conversion

Analysis of the extent of bioconversion was carried out by isocratic reverse phase Agilent (Palo Alto, CA) HPLC using a Zorbax SB-C18 (75 mm × 4.6 mm) column and a 70% acetonitrile/30% water (containing 0.5% H_3PO_4) mobile phase at

1 mL/min and 25 °C. UV absorbance was monitored at 210 nm. The pentenamide acid, ester and azlactone were quantified using their characteristic retention times of 0.8, 1.0 and 1.3 min, respectively, during elution.

2.4.2. Normal phase HPLC analysis for product ee values

Product ester ee was determined by isocratic normal phase Agilent HPLC using a Chiralcel OD-H (250 mm × 4.6 mm) column and a 98% hexanes/2% isopropanol mobile phase at 1.75 mL/min and 25 °C. UV absorbance was monitored at 210 nm. The undesired (*R*)-ester and desired (*S*)-ester were quantified using their characteristic retention times of 10.3 and 21 min, respectively, during elution.

2.4.3. KF water concentration analysis

The water content of solutions was measured by Karl Fischer analysis using a Metrohm (Herisau, Switzerland) 756 KF Coulometer®. A 100 uL sample of the solution of interest was injected, and the water concentration was determined in ppm.

3. Results and discussion

3.1. Initial batch process

The initial batch process, using an enzyme to substrate ratio of 1:1, produced product ester with a yield of 79% and ee of 78% in less than 6 h. The level of undesired acid, formed via azlactone ring opening hydrolysis, was 17% (Fig. 2). Initial attempts at lowering the enzyme charge resulted in lower product yield and ee. This was due to two undesired reactions competing against the desired stereoselective enzymatic dynamic kinetic ring opening of the azlactone to the (*S*) ethyl ester product (Scheme 1). The first undesired reaction, the non-selective background ethanolysis ring opening of the azlactone to the racemic mix of (*R*) and (*S*) ethyl esters, leads to a reduction of the product ee value. The second undesired reaction, the formation of acid through background hydrolysis from the presence of water, reduces the product yield. Understanding the kinetics of these unwanted reactions in conjunction with enzyme deactivation was key to optimizing the process. This is now described in the following Section 3.2 for the development of the kinetic model.

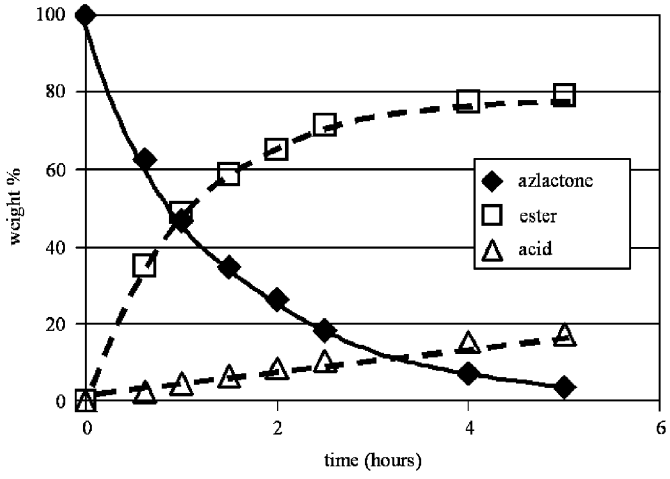

Fig 2. Typical reaction profile for the initial batch process conditions.

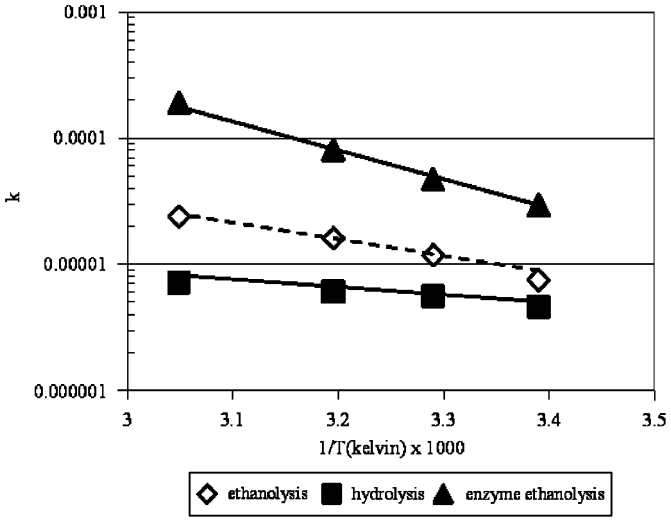

Fig 3. Reaction rates vs. temperature and Arrhenius plot.

Table 1
Table of kinetic parameters for each reaction

Reaction	Activation energy *E* (kJ/mol)	Preexponential factor *A*
Enzyme ethanolysis	45.9	16,500
Background ethanolysis	27.4	2.46
Background hydrolysis	10.2	0.002

3.2. Kinetic analysis of the reaction system

An analysis of the reaction system kinetics was conducted in order to develop a mathematical model for the purpose of aiding process development. Reaction component concentrations, temperature, and enzyme deactivation were studied. This data was then used to create a mathematical model that could predict reaction product yields and enantioselectivities based on input variables.

3.2.1. Effect of temperature on the rates of reaction

The effect of temperature on reaction rates was studied (Section 2.3.1) for each of the three reactions: non-selective ester formation via background ethanolysis, acid formation via background hydrolysis, and enzyme mediated selective ester formation under baseline concentrations. Fig. 3 shows the effect of a range of temperatures (22–55 °C) on the reaction rates in the form of an Arrhenius plot.

Based on this data, the activation energy, *E*, and preexponential factor, *A*, were determined for each of the reactions. These values are listed in Table 1 and were used to model the reaction system. As the Arrhenius plots are not

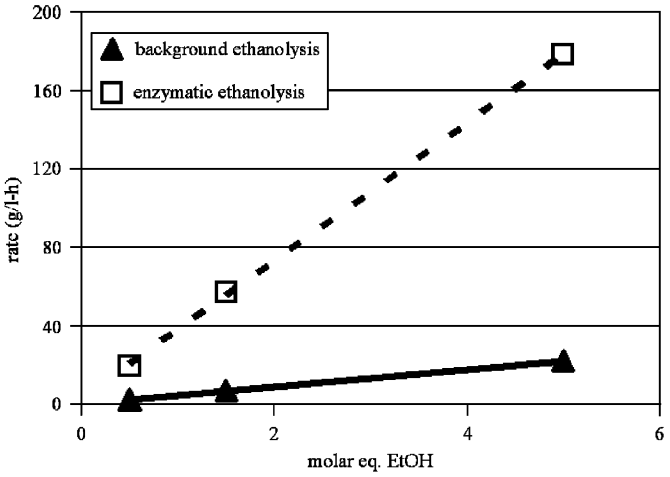

Fig 4. Background and enzymatic ethanolysis rates vs. EtOH concentration at 55 °C with 80 g/L azlactone and 7.6 g/L Et_3N in MTBE.

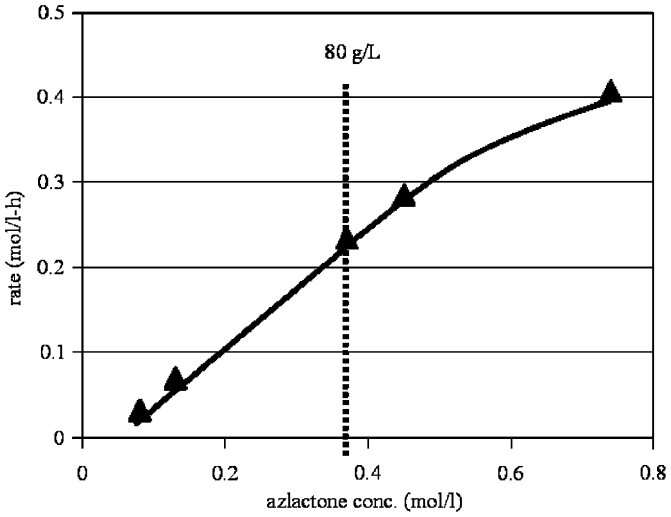

Fig 5. Ethanolysis rate vs. azlactone concentration at 30 °C with 86 g/L EtOH, 7.6 g/L Et_3N and 80 g/L CalB.

perfectly parallel, but instead diverge with increasing temperature (or decreasing $1/T$), the reaction rates of the three reactions are not affected equally across this temperature range. Therefore, the rate of the desired enzymatic reaction can be favored relative to the two non-specific reactions by increasing temperature. Additionally, the background hydrolysis rate was shown to be minimal compared to the rates of ethanolysis.

3.2.2. Effects of azlactone, ethanol and water concentration on the rates of reaction

The rate of hydrolysis of the azlactone was studied and found to be zero order dependent on water concentration over the concentration range studied. The azlactone substrate was incubated in the reaction system both with and without enzyme and exhibited a linear rate of hydrolysis over a 48-h period. The reaction solutions without enzyme contained < 200 ppm H_2O, while the reaction solutions with enzyme contained > 1000 ppm H_2O as measured by the KF method described in Section 2.4.4. This suggests that some water bound to the immobilized CalB enzyme prep was being stripped away from enzyme and going into the MTBE reaction solution. Despite the five fold difference in water concentration, no difference in hydrolysis rate was observed. Over the relevant concentrations of water, the rate of hydrolysis (r_h) can therefore be written as the following:

$$r_h = k_h[S], \tag{1}$$

where k_h and $[S]$ are the hydrolysis rate constant and the azlactone concentration, respectively. Again, according to the experimentally determined rate constants, the rate of hydrolysis is minimal, but will be included in the model to predict the amount of undesired hydrolyzed product that is formed.

The rates of enzymatic and background ethanolysis were studied. Both background and enzymatic ethanolysis were found to be linearly dependent (first order) on ethanol concentration. This was experimentally determined and shown in Fig. 4. The rates of background ethanolysis and enzymatic ethanolysis were also found to be linearly dependent (first order) on azlactone substrate concentration for the range of azlactone concentrations that are experienced throughout the reaction time course (0–80 g/L or 0–0.37 mol/L). Fig. 5 shows the rate of enzymatic ethanolysis of the azlactone vs. the molar azlactone concentration. Based on this rate data, the rates of enzymatic ethanolysis (r_{enz}) and background ethanolysis (r_e) take the following forms, respectively:

$$r_{enz} = k_{enz}[S][\text{EtOH}][\text{Enz}], \tag{2}$$

$$r_e = k_e[S][\text{EtOH}]. \tag{3}$$

Eqs. (2) and (3) are valid up to an azlactone concentration of 100 g/L.

3.2.3. Enzyme deactivation rate

A key component in the kinetic model of this reaction system was an understanding of the cause of enzyme deactivation observed in the initial stirred tank batch process. Enzyme deactivation leads to lower product yield and ee and limits the useful life of the enzyme. Limiting enzyme deactivation was an essential component in the development of an optimized production process.

The deactivation rate of the enzyme, k_d, in the batch process was determined to be 10% per hour at process conditions. An investigation of the cause of this rate of decline in enzyme activity showed it to be agitation dependent (Section 2.3.3). Reactions with agitation just sufficient to suspend the immobilized enzyme (300 rpm) gave a deactivation rate of 10% per hour compared to a rate of 0.5% per hour using extremely low agitation not sufficient to suspend the enzyme (60 rpm) at 55 °C. A series of stability studies in which the enzyme was incubated with each reaction component for periods of 1–4 h showed that enzyme deactivation due to reaction components was negligible (Section 2.3.3), and the primary cause for deactivation was agitation.

The effect of immobilized enzyme deactivation in various reactor geometries is a crucial factor in reactor engineering that

has been identified for some time (Regan et al., 1974; Sadana, 1978). Deactivation of immobilized CalB due to mechanical agitation has been demonstrated by Fishman et al. (2001). They observed attrition of enzyme particles and a loss of enzyme activity in immobilized CalB when run in a 100 mL batch reactor at a stir rate of 300 rpm. The batch system showed ∼9% loss in productivity between each cycle (60% conversion for cycle 1, 55% conversion for cycle 2, 50% conversion for cycle 3).

3.2.4. Kinetic model assumptions

The kinetic analysis data was used to make a kinetic model of the reaction system. The purpose of this model was to develop a better understanding of the complex reaction system in which multiple reactions are simultaneously taking place (enzymatic ethanolysis, background ethanolysis, background hydrolysis, and enzyme deactivation). The kinetic model was then used to guide process development to an optimized manufacturing process. The following assumptions were made for the development of the kinetic model. The rate of racemization of the azlactone substrate **1** was fast compared to the ethanolysis and hydrolysis rates and was therefore not a rate limiting step (this was also confirmed experimentally). The conversion of azlactone **1** to both the ethyl ester **3** and the pentenamide acid **4** was irreversible. The rate of enzyme deactivation was assumed to be an irreversible deactivation occurring in a single step and obeying first order kinetics (Torres et al., 2004).

3.2.5. Kinetic equations

Enzyme activity, [Enz], was determined over time using the following equation:

$$[\mathrm{Enz}](t) = [\mathrm{Enz}]_0 \mathrm{e}^{-k_d(t-t_0)}. \tag{4}$$

The deactivation rate, k_d, of the immobilized CalB in the batch reaction system was determined experimentally to be 10% per hour at 300 rpm and 0.5% per hour at 50 rpm, respectively.

The specific reaction rate constants were experimentally determined at various temperatures ranging from 22 to 55 °C (Section 3.2.1). From the rate constants, the activation energy, E, and preexponential factor, A, were calculated. The rate constant could then be determined for any temperature with the following equation:

$$k(T) = A\mathrm{e}^{-E/RT}. \tag{5}$$

The rate of disappearance of azlactone substrate **1**($-r_S$), formation of the desired S-ester **2** (r_{P1}), formation of the undesired R-ester **3** (r_{P2}), and formation of the undesired acid **4** (r_A) were modeled using the following equations, respectively:

$$-r_S = k_{\mathrm{enz}}[S][\mathrm{EtOH}][\mathrm{Enz}] + k_e[S][\mathrm{EtOH}] + k_h[S], \tag{6}$$

$$r_{P1} = k_{\mathrm{enz}}[S][\mathrm{EtOH}][\mathrm{Enz}] + \tfrac{1}{2}k_e[S][\mathrm{EtOH}], \tag{7}$$

$$r_{P2} = \tfrac{1}{2}k_e[S][\mathrm{EtOH}], \tag{8}$$

$$r_A = k_h[S]. \tag{9}$$

The rate of disappearance of azlactone substrate was simply derived from the sum of all the rates of reactions that consume azlactone (Eq. (6)). Making the assumption that the enzyme is perfectly selective, formation of the product was derived from the enzymatic reaction rate plus half of the non-specific reaction rate. If the enzyme was not perfectly selective and the model did not fit the experimental data, an additional enzymatic rate term would need to be added to Eq. (8). Directly measuring the selectivity of the enzyme was a challenge due to the nonselective background ethanolysis.

The kinetic model was built with these equations (Eqs. (4)–(9)), using an iterative calculation process. Fixed rate constants were calculated as a function of temperature based on the experimentally obtained activation energy and preexponential factor. The enzyme activity, [Enz], was solved for at $t = 0$ using Eqs. (4) and (5). The enzyme activity over the reaction course was found to be dependent only on time and the deactivation constant observed for a given reactor geometry. Therefore, the enzyme activity for each calculation was strictly dependent on the time increment chosen (5 min) and the iteration number. The calculated enzyme activity at each iteration was then used to estimate the next set of data points based on the component concentration. The initial azlactone substrate concentration, $[S]$, was set at 0.37 mol/L (80 g/L). The initial EtOH concentration, [EtOH], was set at 1.87 mol/L (86 g/L). The initial product concentrations, $[P_1]$, $[P_2]$, $[H]$, were set at 0 mol/L. Using the rate equations, new substrate and product concentrations were calculated for a small increment forward in time (5 min). The updated concentrations were then inserted back into the rate equations for another round of iteration. In this way, through iterative calculations, the model was generated. The calculations were carried out in Microsoft Excel. Because the rate equations (Eqs. (6)–(9)), are linear at a fixed temperature, the reaction progress could be predicted simply by plugging the component concentrations of the first iteration into the next, and so on.

Finally, the yield and ee of the ester product were calculated with the following equations, respectively:

$$\mathrm{yield} = \frac{[P_1] + [P_2]}{[P_1] + [P_2] + [S] + [A]}, \tag{10}$$

$$\mathrm{ee} = \frac{[P_1] - [P_2]}{[P_1] + [P_2]}. \tag{11}$$

3.2.6. Kinetic model fit to experimental batch reaction data

The kinetic model developed using the equations outlined in Section 3.2.5 was used to predict the yield and ee of product ester under various conditions of temperature and enzyme concentration and then compared to experimental data obtained by running a series of batch reactions. The kinetic model fit the experimental data very well for the batch system described in Section 3.1 at a range of temperatures and enzyme concentrations (Fig. 6). The excellent fit of the model to the data confirmed the assumption that the enzyme was perfectly selective.

3.3. Fed batch reaction system

Both the data in Fig. 6 and the kinetic model demonstrated that the enzyme to substrate ratio in the batch reaction

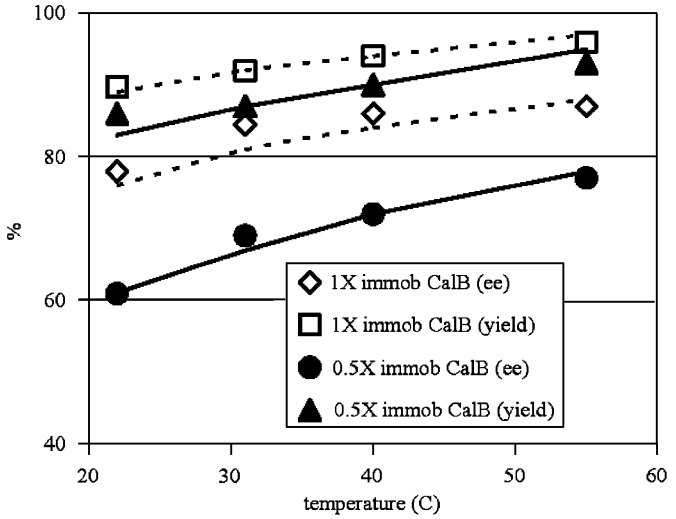

Fig 6. Product yield and ee of batch system vs. temperature, predicted (lines) and experimental (points). The calculated values used in the model to generate the predicted data were separate experiments from the experiments run to validate the model.

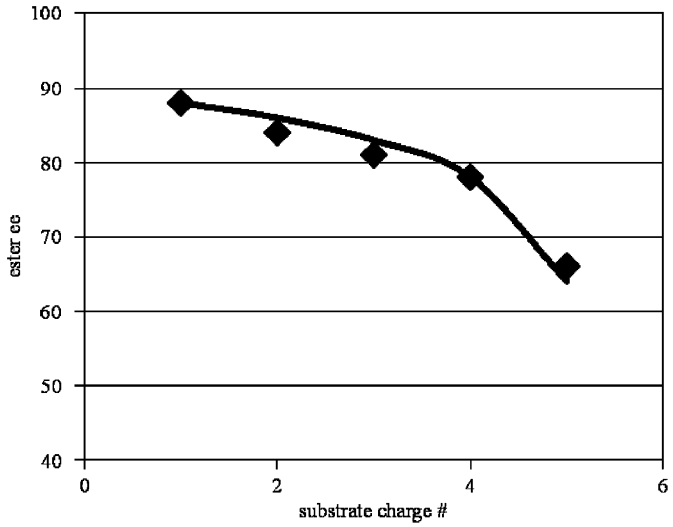

Fig 7. Predicted ee (line) vs. actual ee (points) for the fed batch system. The calculated values used in the model to generate the predicted data were separate experiments from the experiments run to validate the model.

system could not be reduced to meet the economic targets, as the reaction would suffer reduced ester yield and ee. The model also predicted that the highest yield and ee of the product ester would be achieved at elevated temperature. Based on this information, the optimal batch reaction system would be one in which substrate was fed to the reaction, so at all times the enzyme to substrate ratio was $\geqslant 1:1$.

The kinetic model was used to develop a fed batch reaction system in which multiple substrate additions of azlactone and EtOH were added when the extent of conversion exceeded 90%. Initial reaction component concentrations remained the same as the baseline batch process with reaction temperature raised to 50 °C (increasing the enzyme rate by greater than five fold and also increasing the enzyme rate relative to the background ethanolysis and hydrolysis rates). Each substrate charge consisted of 1 M equivalent of azlactone and EtOH (i.e. 80 g azlactone and 17.3 g of EtOH for a 1 L reaction). The model predicted that a total of four substrate charges could be converted while maintaining a product ee value of 78% and a yield $> 80\%$. An additional fifth substrate charge would result in an unacceptably low product ee value. The fed batch system charging strategy was run and the experimental product ee values obtained after each substrate charge matched the predicted values very well (Fig. 7). The enzyme inactivation rate of 10% per hour led to a decline of enzymatic reaction rate, and therefore the product ee values decreased over time as each substrate charge was added.

A fed batch system was therefore developed with a total of four substrate charges (including the initial 80 g/L substrate concentration). The reaction was complete in 6 h with a total product concentration of ~ 250 g/L. A typical reaction profile is shown in Fig. 8. The fed batch system successfully increased productivity by reducing the enzyme to substrate ratio by a factor of four to 1:4. Additionally, the fed batch system increased product yield to 84%, decreased acid formation from 17% in the baseline batch process to 6%, and maintained an acceptable product ee value at 78%.

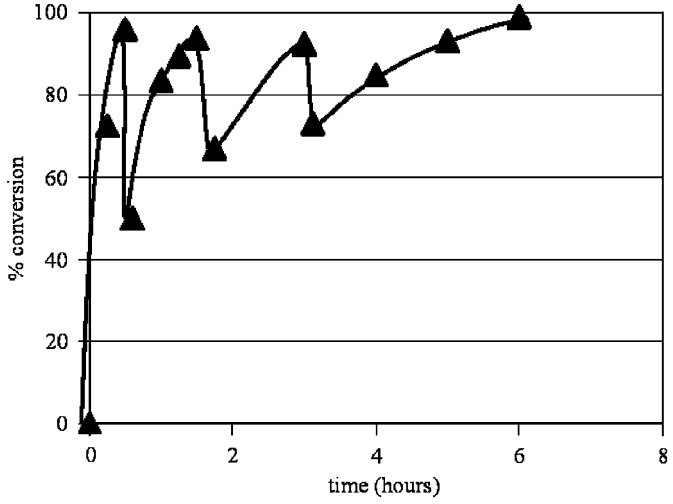

Fig 8. Fed batch process with substrate charging.

3.4. Plug flow column reactor system

The discovery that agitation was the primary cause of enzyme deactivation in the batch reaction systems led to the development of a plug flow column reactor. The deactivation constant, k_d, when the enzyme was incubated in the reaction system with low agitation (60 rpm) was experimentally determined to be 0.5% per hour, compared to 10% per hour for batch systems that were stirred with sufficient agitation to suspend the immobilized enzyme (300 rpm). Based on the concept that a column reactor would eliminate the shear effects from agitation and reduce the deactivation rate of the enzyme to 0.5% per hour, the kinetic model was revisited. With the rate of enzyme deactivation set at 0.5% per hour (experimentally

Table 2
Comparison of batch, fed batch and column processes (based on a 100 kg run)

	Enzyme to substrate ratio	Undesired acid (wt%)	Ester yield (wt%)	Ester ee	Reactor volume (L)
Batch	1:1	17	79	78	1250
Fed batch	1:4	6	84	78	383
Column	1: > 20	2	90	86	24

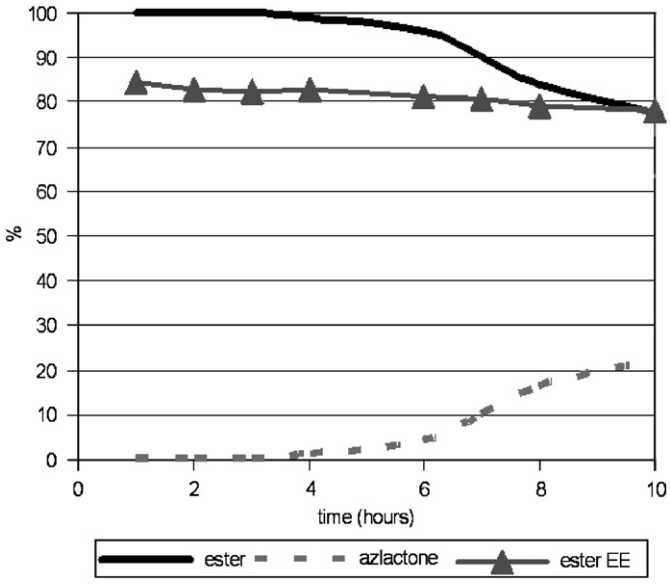

Fig 9. Column reactor pilot plant run.

confirmed in the column reactor system) and the temperature set at 55 °C, the model predicted a 20 fold increase in enzyme life and productivity (through the elimination of enzyme deactivation due to shear), an increase in ester yield to 93%, and an increase in the product ee value to 88%.

The column reactor was set up as a single pass process. Plug flow behavior was visibly observed by watching the substrate front move through the packed column. The flow rate was set to provide a residence time of 25 min, which was experimentally determined to provide optimal product ester yield and ee. The azlactone substrate feed was kept separate from the EtOH feed so that background ethanolysis would be eliminated until the solutions were mixed just before entering the column. The stream exiting the column was sent to a quench vessel to convert any un-reacted azlactone to acid, thereby preventing any residual azlactone from non-selectively forming the ethyl ester via background ethanolysis and degrading the product ee value.

The column process was demonstrated in a glass jacketed column packed with 1 g of CalB immobilized enzyme. The two feed solutions were pumped into the column at a combined flow rate of 10 mL/h for 25 h. At a column temperature of 55 °C, 20 g of azlactone were converted to product. Using the plug flow column reactor, ester yield was increased to 90% with a reduction in acid formation to 2%. Additionally, the product ester ee increased to 86%. Finally, the column reactor dramatically increased productivity by reducing the enzyme to substrate ratio by a factor of > 20 compared to the original baseline process. These numbers were closely predicted by the kinetic model.

The productivity of the column process compared to the initial batch and fed batch processes is shown in Table 2. The plug flow column reactor was demonstrated in the lab at batch sizes ranging from 0.8 to 20 g. Due to the ability of the kinetic model to predict reaction behavior precisely, no intermediate scale demonstration was required, and the column process was scaled up directly from 20 to 150 kg scale in the pilot plant. A plot of product and substrate concentration as well as product ee values of the column effluent is shown in Fig. 9. At all scales, the process performed similarly, proving this to be a robust and easily scalable manufacturing process. Scaling the process simply required keeping the residence time in the column consistent across all scales at 25 min.

4. Conclusion

This work has demonstrated the usefulness of creating a kinetic model to aid in process development, and is the first example of using kinetic modeling to predict enzymatic azlactone ethanolysis under various reaction conditions and reactor geometries. The kinetic model developed successfully modeled a highly complex reaction system that included: racemization of the substrate azlactone, selective enzymatic ethanolysis of azlactone to the desired ester product, background ethanolysis of azlactone to racemic ester, background hydrolysis of the starting material to acid, and enzyme deactivation throughout the course of the reaction. The model made good predictions for ester yield and ee for a variety of process types (batch, fed batch and column) and conditions, and significantly reduced process development time by predicting the best reaction conditions without the need to run every experiment. A parity plot of experimental actual data vs. model predicted data for all of the different reactor geometries and conditions run is shown in Fig. 10.

Experiments were run with the conditions that the kinetic model proposed would provide the highest product yield and ee using the lowest possible enzyme to substrate ratio. Using this methodology, a fed batch process was developed that exceeded economic targets (four fold reduction in enzyme loading vs. a target of three fold). The kinetic model suggested that the ideal reaction system would be an immobilized enzyme plug flow column reactor for the continuous production of the (*S*)-γ-fluoroleucine ethyl ester intermediate, as this reactor geometry would eliminate enzyme deactivation due to mechanical agitation. The confidence in the model also enabled the elimination of an intermediate scale demonstration, allowing us to scale directly from 20 g in the lab to 150 kg in the pilot plant. Other

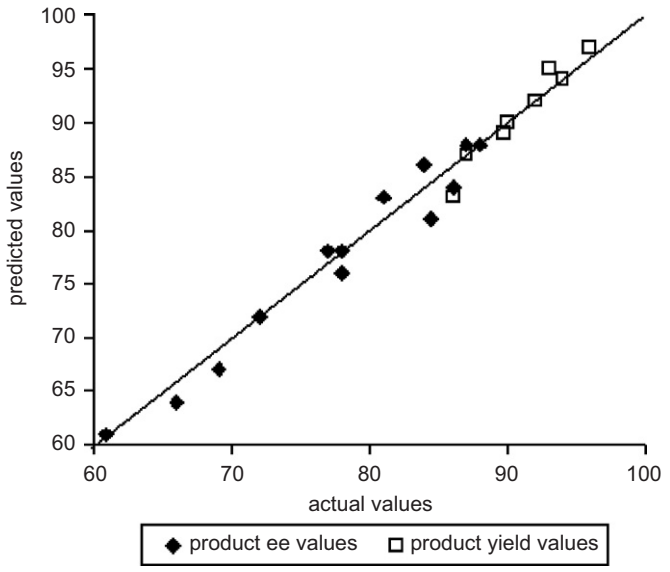

Fig 10. Parity plot of experimental actual data vs. model predicted data for different reactor geometries and conditions.

groups have also demonstrated the benefit of column reactors vs. batch reactors in reducing the deactivation rate of enzymes. Gusakov et al. (1985) showed that using a plug flow column reactor for the enzymatic hydrolysis of cellulose reduced the deactivation of the enzyme by their substrate when compared to the batch process. Fishman et al. (2001) found that a stirred tank reactor was unsuitable for their process due to attrition of enzyme particles and moved to an immobilized enzyme fixed-bed loop reactor, which reduced the enzyme deactivation significantly from 9% loss of productivity per cycle in the stirred tank batch reactor to 3% loss of productivity per cycle in the fixed bed reactor (yielding a three fold improvement in productivity).

Our plug flow reactor process, when compared to both the batch and fed batch processes, was shown to lower the enzyme to substrate ratio to $< 1{:}20$. Additionally, the continuous plug flow reactor produced ester product with greater yield and ee (90% yield, 86% ee) compared to the initial batch process (79% yield, 78% ee). Finally, the column reactor process has been shown to be a robust and easily scalable process. Column reactions ranging from 0.8 g azlactone scale to 150 kg azlactone scale have all exhibited similar profiles for product yield, product ester ee and enzyme inactivation rate. It should also be noted that 80% enzyme activity is retained at the end of the column reactor run (Fig. 9). Operating multiple catalyst beds that can be replenished as the enzyme deactivates, or re-injecting unconverted substrate stream and thereby, will lead to even higher productivities.

Notation

$[A]$	acid, mol/L
[Enz]	enzyme, g/L
[EtOH]	ethanol, mol/L
k_d	enzyme deactivation rate constant, %/h
k_e	background ethanolysis rate constant, L/mol h
k_{enz}	enzymatic ethanolysis rate constant, L^2/mol g h
k_h	background hydrolysis rate constant, 1/h
$[P_1]$	S-ester product, mol/L
$[P_2]$	R-ester product, mol/L
r	rate, mol/L h
R	ideal gas constant, 8.3144 J/mol K
$[S]$	substrate azlactone, mol/L
t	time, h
T	temperature, K

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