CONTINUOUS ENZYMATIC REACTIVE CRYSTALLIZATION OF 
BETA-LACTAM ANTIBIOTICS

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Patrick R. Harris

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CONTINUOUS ENZYMATIC REACTIVE CRYSTALLIZATION OF BETA-LACTAM ANTIBIOTICS

Approved by:

Dr. Andreas S. Bommarius, Advisor
School of Chemical & Biomolecular Engineering
Georgia Institute of Technology

Dr. Julie A. Champion
School of Chemical & Biomolecular Engineering
Georgia Institute of Technology

Dr. Martha A. Grover
School of Chemical & Biomolecular Engineering
Georgia Institute of Technology

Dr. Mark P. Styczynski
School of Chemical & Biomolecular Engineering
Georgia Institute of Technology

Dr. Ronald W. Rousseau
School of Chemical & Biomolecular Engineering
Georgia Institute of Technology

Dr. Christopher L. Burcham
Executive Director, Synthetic Molecule Drug Development
Eli Lilly and Company

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To my lovely wife, Abbie
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## TABLE OF CONTENTS

ACKNOWLEDGEMENTS iv  
LIST OF TABLES viii  
LIST OF FIGURES x  
LIST OF SYMBOLS AND ABBREVIATIONS xxi  
SUMMARY xxvi  

### CHAPTER 1. Introduction 1  
1.1 Beta-lactam antibiotics 1  
1.2 Enzymatic beta-lactam synthesis 2  
1.3 Continuous manufacturing 3  
1.4 Reactive crystallization of amoxicillin and cephalexin 4

### CHAPTER 2. Kinetic Characterization of penicillin g acylase from E. coli for the synthesis of amoxicillin and cephalexin 6  
2.1 Introduction and Motivation 6  
2.2 Materials and Methods 10  
2.3 Results 15  
2.3.1 Determination of initial reaction rates for synthesis and hydrolysis of amoxicillin and cephalexin and precursors using Penicillin G Acylase from E. coli 15  
2.3.2 Kinetic model development, fitting, and parameter estimation for cephalexin synthesis 24  
2.3.3 Pareto optimization for homogenous continuously stirred reactor for cephalexin synthesis 27  
2.4 Conclusions 33

### CHAPTER 3. Amoxicillin trihydrate solubility and crystallization in presence of cosolutes 34  
3.1 Introduction 34  
3.2 Materials and Methods 35  
3.3 Determination of solubility of amoxicillin system components 40  
3.3.1 Effect of pH, temperature, and ionic strength 40  
3.3.2 Effect of cosolutes 45  
3.3.3 Raman spectroscopy to investigate aqueous phase cosolute interactions 48  
3.4 Amoxicillin crystallization 51  
3.4.1 Primary nucleation 51  
3.4.2 Modelling amoxicillin crystallization with and without presence of precursors 54  
3.5 Conclusions 60

### CHAPTER 4. Development and characterization of size-based enzyme separation for enzyme retention and product withdrawal in a continuous reactive crystallizer 62
# LIST OF TABLES

Table 2.1: Parameters from fitting to initial-rate data for cephalaxin synthesis, secondary hydrolysis, and primary hydrolysis using Equations (2-1) and (2-2) using data provided in Figure A.1 and Figure 2.2(A).  

Table 2.2: Parameters from the model fit for amoxicillin synthesis, secondary hydrolysis, and primary hydrolysis using Equations (1) and (2) using data provided in Supplemental Figure 2 and Figure 6.  

Table 3.1: Parameters of Apelbat equation fit to the data in Figure 3.1(A) by least-squares regression for the temperature dependence of amoxicillin solubility in water.  

Table 3.2: Summary of parameters used to model the solubility of amoxicillin, 4-HPGME, 4-HPG, and 6-APA. Model parameters for amoxicillin, 4-HPGME, and 4-HPG are from data in Figure 3.1(B-D) fit to Equation (3-2). The fraction in Equation (3-2) is inverted in the case of 4-HPGME solubility. aFrom [57]. bFrom [78].  

Table 3.3: Parameter bounds used in constrained parameter fitting for amoxicillin crystallization.  

Table 3.4: Parameter values for amoxicillin crystallization data fit to the crystallization model detailed. Error is 95% confidence intervals.  

Table 5.1: Equations used to calculate green chemistry metrics. The complexity of a process corresponds to the number of separate reaction steps.  

Table 5.2: Summary of initial pilot runs for system development and optimization. Runs are numbered chronologically based on the order that they were conducted.  

Table 5.3: Commercial enzyme immobilization supports tested for residual activity compared to soluble enzyme. Reactions were conducted with PGA-WT-loaded supports in 50 mmol/L cephalaxin in 50 mmol/L sodium phosphate buffer at pH 7.0 and 25°C. Residual activity was compared to wild-type in terms of specific activity (U/mg PGA).
Table 5.4 Green chemistry metrics for amoxicillin synthesis via various routes. Batch-PGA-RC and Cont-PGA-RC with and without raw material (RM) recovery data were collected in this work.  

Table 5.5 Green chemistry metrics for the continuous RC of cephalixin.  

Table 6.1 Summary of PAT evaluated for use in the continuous RC of cephalixin and amoxicillin. Bolded PAT are currently used in the process.
LIST OF FIGURES

Figure 2.1  Figure 2.1: Reaction network for the synthesis of cephalexin from 7-ADCA and PGME. Synthesis denotes the condensation of PGME and 7-ADCA to form the desired product. Primary hydrolysis denotes hydrolysis of PGME to form a byproduct. Secondary hydrolysis denotes hydrolysis of the desired product cephalexin to form a byproduct PG and 7-ADCA. For the amoxicillin system, 7-ADCA is replaced with 6-APA, PGME is replaced with 4-HPGME, and PG is replaced with 4-HPG. The crystal structure of PGA from E. coli (PDB code: 1GK9) is shown in the center of the figure.

Figure 2.2  Figure 2.2: (A) Initial-rate cephalexin synthesis selectivity for the wild-type, βF24A, and Assemblase® variants for 7-ADCA concentrations 2.5-50 mmol/L. Reaction conditions: pH = 7.0, CPGME = 100 mmol/L, CPGA = 500 nmol/L. (B) Initial-rate cephalexin synthesis selectivity for the wild-type for 7-ADCA concentrations 5-60 mmol/L. Each curve represents the same PGME concentration for each reaction. Reaction conditions: pH = 7.0, CPGA = 500 nmol/L.

Figure 2.3  Figure 2.3: Lineweaver-Burk plot for the inhibition of cephalexin hydrolysis by 7-ADCA for WT-PGA. \( v_{initial} \) represents the initial activity of cephalexin hydrolysis. Each data series represents different 7-ADCA concentrations and each solid line the best fit to Michaelis-Menten hydrolysis of cephalexin with a competitive inhibitor for each separate inhibitor concentration. Reaction conditions: pH = 7.0, CPGA = 300 nmol/L.

Figure 2.4  Figure 2.4: Initial selectivity and initial cephalexin synthesis rate versus total magnitude of substrate concentrations for cephalexin synthesis using wild-type PGA at a constant (PGME:7-ADCA) ratio of 2.5:1. Reaction conditions: pH = 7.0, CPGA = 500 nmol/L.

Figure 2.5  Figure 2.5: Initial rate amoxicillin synthesis selectivity for the wild-type, βF24A, and Assemblase® variants for 6-APA concentrations 25-150 mmol/L. Reaction conditions: pH = 6.2, C4-HPGME = 100 mmol/L, CPGA = 1.5 μmol/L.

Figure 2.6  Proposed Models 1, 2, and 3. Model 1: Model Proposed by Youshko and Svedas (Y-S); Model 2: Y-S Model with Competitive Inhibition by 7-ADCA; Model 3: Y-S Model with Random Binding of 7-ADCA and PGME. E corresponds to free enzyme, S the acyl-donor, Nu the nucleophilic beta-lactam.
nucleus, EA the acyl-enzyme complex, P the product antibiotic, P2 the byproduct. Note: All unshaded model parameters and reactions correspond to Model 1.

Figure 2.7 Parity plots for best fits of Models 1-3 for cephalexin reaction data. *(Bottom right)* Comparison of model fits by sums-of-squared errors (SSE) and Akaike Information criterion (AIC).

Figure 2.8 Pareto optimal fronts generated for the operation of a CSTR for the synthesis of cephalexin. (A) Fractional yield, defined as the fraction of PGME consumed which results in cephalexin, versus productivity for various PGME conversions. The bold curves show how the extremes of each pareto front change with respect to productivity and fractional yield. (B) PGME conversion versus productivity for various 7-ADCA conversions. CPGA = 5 μM, T = 25°C, pH = 7.0

Figure 3.1 (A) Effect of temperature on the solubility of amoxicillin at pH = 6.5; (B) Effect of pH value on the solubility of amoxicillin at 25°C; (C) Effect of pH value on the solubility of 4-HPGME at 25°C; (D) Effect of pH value on the solubility 4-HPG at 25°C. The solid line in Panel (A) is the fit to Equation (3-1). The solid lines on Panels (B-D) are fits to Equation (3-2). Error bars are one standard deviation

Figure 3.2 (A) 6-APA, 4-HPGME, and 4-HPG concentration versus amoxicillin solubility. Error bars are one standard deviation from three sample measurements. (B) Ionic strength due to ionized 4-HPGME, 6-APA, and NaCl versus amoxicillin solubility, calculated using the Henderson-Hasselbalch equation and Equation (3-3). pH = 6.5, T = 25°C. Solid lines are to guide the eye.

Figure 3.3 Amoxicillin solubility in various mixtures of 4-HPGME and 6-APA. Each curve corresponds to a single concentration of 6-APA, from left to right higher concentrations of 4-HPGME. pH = 6.5, T = 25°C.

Figure 3.4 In situ Raman spectra of water, amoxicillin, 4-HPGME, and a mixture of 4-HPGME and amoxicillin, all dissolved in water. (A) Full size spectra; (B) Spectra focused on peak at 852 cm⁻¹. pH = 6.5, T = 25°C, V = 200 mL. cAmoxicillin = 30 mmol/L, c4-HPGME = 30 mmol/L.

Figure 3.5 Primary nucleation studies for amoxicillin. (A) Working principle for Crystal16, from [97]; (B) Example of output data from Crystal16, laser transmission over time for various starting
supersaturation ratios; (C) Induction time versus supersaturation; (D) \( \ln t_{\text{ind}} \) versus \( (\ln S)^{-2} \). Line is data fit to Equation (3-5). \( T = 25^\circ C. \ V = 1 \text{ mL} \).

Figure 3.6 Batch crystallization of amoxicillin in water. (A) Concentration of amoxicillin in the liquid phase over time, with and without 100 mmol/L 6-APA present; (B) Examples of CSDs measured at end of crystallization via offline microscopy. Distinct CSDs are differentiate by color. Light blue and royal blue CSDs were in the presence of 100 mmol/L 6-APA and red and green CSDs were in pure water. \( T = 25^\circ C. \ \text{pH}_{\text{final}} = 6.5 \).

Figure 3.7 Example of model fit to the training set of liquid phase amoxicillin concentrations during amoxicillin crystallization.

Figure 4.1 Enzymatic synthesis of beta-lactam antibiotics amoxicillin and cephalixin catalyzed by PGA. 6-APA refers to 6-aminopenicillanic acid, 7-ADCA to 7-aminodesacetoxy-cephalosporanic acid, PGME to D-phenylglycine methyl ester, 4-HPGME to 4-hydroxy-D-phenylglycine methyl ester, and HPG to 4-hydroxy-D-phenylglycine. Crystallization can protect the antibiotic intermediate from the secondary hydrolysis reaction.

Figure 4.2 (A) Schematic representation of the carrier-crystal separation method proposed for continuous reactive crystallization using immobilized biocatalyst in a Mixed Suspension Mixed Product Removal Reactor (MSMPR) vessel. Inlet to the mill is withdrawn from the filter to avoid milling the carrier beads. Listed values correspond to those used in pH-induced continuous crystallization experiments (with no beads present) with intermittent milling described in the Methods section. A photo of an example 400 \( \mu \text{m} \) mesh used to make the filter is shown in the bottom right corner. (B) Optical microscopy of amoxicillin trihydrate crystals produced in pH-induced continuous crystallization experiments when no milling (left) or intermittent milling (right) is used. The scale bar in (B) applies to all four microscope images.

Figure 4.3 Impact of the enzyme carrier size and nucleophile concentration (6-APA or 7-ADCA) on the activity (top) and selectivity (bottom) of the synthesis reaction for amoxicillin (left) and cephalixin (right) systems.

Figure 4.4 Example of the process dynamics in pH-induced continuous crystallization experiments with intermittent milling from the initial batch-mode operation to steady state (feed concentration 45 mM, residence time 80 min, intermittent milling at 5000
RPM). (A) solution turbidity measured by the PVM probe, (B) crystal chord length counts measured by the FBRM probe, (C) MSMPR vessel pH controlled by the addition of 0.5 M HCl, (D) the total volume of the added acid for pH control. (E), (F), (G) in situ images captured by the PVM probe during the batch mode, continuous mode right after the first milling cycle, and at the steady state, respectively. The scale bar in (G) applies to (E) and (F) as well.

Figure 4.5 Efficiency of the slurry withdrawal when filters of 149 – 800 μm are applied to the outlet, at different withdrawal speeds for the AMX slurry made continuously with no milling. All values are normalized with respect to the solids density in the MSMPR vessel at steady state, ~11 mg/mL. (A), (B), and (C) panels correspond to withdrawal speeds of 5, 10, and 20 mL/min, while panel (D) provides a heatmap of all data presented in other panels. Using 5 mL/min and 800 μm filter results in significant settling of large crystals in the line preventing robust data acquisition (NaN).

Figure 4.6 Comparison of the CSD and means size for samples withdrawn from the crystallization process without milling using different filters. Panels (A) to (D) correspond to the crystal size distribution of the original MSMPR slurry at steady state and slurries withdrawn by 800, 300, and 149 μm filters, respectively, obtained by image processing. Panel (E) compares the number- and mass-based mean sizes for all filters. For all tests, withdrawal speed was set to 20 mL/min. NA1 and NA2 correspond to cases when no filter is placed on the outlet and 2.06 and 2.74 mm tubing were used. Particles smaller than 25 µm are excluded from the CSD analysis as they are likely to be unaffected by the filter.

Figure 4.7 Efficiency of the slurry withdrawal when filters of 149 – 800 μm are applied to the outlet, at different withdrawal speeds for the AMX slurry made with intermittent milling. All values are normalized with respect to the solids density in the MSMPR vessel at steady state, ~13.8 mg/mL. (A), (B), and (C) panels correspond to withdrawal speeds of 3, 5, and 10 mL/min, while panel (D) provides a heatmap of all data in those panels.

Figure 4.8 Comparison of the crystal size distribution and mean size for samples withdrawn from the crystallization process with intermittent milling using different filters. Panels (A) to (D) correspond to the crystal size distribution of the original MSMPR slurry at steady state, and slurries withdrawn by 800, 300, and 149 μm filters, respectively, obtained by image processing. Figure 4.8(E) compares the number- and mass-based mean sizes...
for all filters. For all tests, withdrawal speed was set to 10 mL/min. NA corresponds to the case when no filter is placed on the outlet. Particles smaller than 25 μm are excluded from the analysis as they are likely to be unaffected by the filter.

Figure 4.9 Efficiency of the slurry withdrawal for a slurry with 21.1 mg/mL solids density when filters of 149 – 800 μm are applied to the outlet, at withdrawal speed of 10 mL/min for the AMX slurry made with intermittent milling. All values are normalized with respect to the solids density in the MSMPR vessel, 21.1 mg/mL.

Figure 4.10 Impact of milling, slurry density, and pH cycle on the filtration rate of AMX crystals. (A) filtration rate for AMX slurries prepared in continuous crystallization with and without intermittent milling, with a subsequent pH cycle to redissolve and reduce the number of fines, and with a high slurry density (21.1 versus 13.8 mg/mL). (B) and (C) optical microscopy of AMX crystals prepared with intermittent milling and milling plus pH cycle. The scale bar applies to both (B) and (C).

Figure 5.1 (A) Enzymatic reaction and crystallization network for amoxicillin (with components for cephalexin in parenthesis). (B) Pilot plant apparatus (not shown, substrate recovery process). All probes were inserted into MSMPR-1. (C) Process diagram for pilot plant. MSMPR-1 and MSMPR-2 were operated in continuous mode, while downstream unit operations for product recovery, substrate recrystallization, and substrate recovery were operated in batch mode.

Figure 5.2 Simulations of continuous RC of cephalexin. (A) 7-ADCA conversion as a function of residence time and enzyme concentration. (B) Cephalexin volumetric productivity as a function of residence time and enzyme concentration. (C) 7-ADCA conversion as a function of residence time and enzyme concentration with regions of PG and 7-ADCA precipitation removed. (D) Cephalexin volumetric productivity as a function of residence time and enzyme concentration with regions of PG and 7-ADCA precipitation removed. Simulation conditions: \( \text{c}_{7-\text{ADCAfeed}} = 0.26 \text{ mol/L, c}_{\text{PGMEmfeed}} = 0.33 \text{ mol/L, T = 25°C, pH = 6.5.} \) (E-F) 7-ADCA conversion and cephalexin productivity during continuous RC of cephalexin with \( \text{c}_{7-\text{ADCAfeed}} = 0.30 \text{ mol/L, c}_{\text{PGMEmfeed}} = 0.48 \text{ mol/L and all other conditions the same as Panels (C-D).} \)

Figure 5.3 Concentration of cephalexin over the course of 28 hours for cephalexin hydrolysis with PGA-WT-loaded Immobead Cov-2 supports. \( c_{\text{Cephalexin, feed}} = 20 \text{ mmol/L in 100 mmol/L sodium} \)
phosphate pH 7.0, T = 25°C. V = 20 mL, Initial feed flowrate = 2 mL/min, manipulated to 1 mL/min at t = 13 h and to 2 mL/min at t = 22 h using a peristaltic pump. C_{PGA} = 1 \, \mu\text{mol/L}

Figure 5.4 Summary of results from Run 9. (A) Turbidity and solids production over time; (B) Liquid phase concentrations of all components measured by HPLC; (C) pH value over time, controlled using 2 mol/L NaOH; (D) \textit{In situ} microscopy of cephalaxin crystals on left (size of image is 1 mm$^2$), offline microscopy on right. (E) Produced samples of >99% purity cephalaxin monohydrate. V = 300 mL, F = 3 ml/min. T = 25°C, pH$_{setpoint}$ = 6.5.

Figure 5.5 Product purity analysis for Run 9. (A) Unwashed and washed filter cake and HPLC mass purity analysis; (B) Chiral purity analysis via optical rotation, comparison between collected product and standard (RIA, NJ); (C) HPLC chromatogram of collected cephalaxin product (washed); (D) TGA of multiple samples of collected cephalaxin. Dotted line corresponds to expected mass after loss of the water of hydration. Yellow point corresponds to mass loss after 24 hours oven drying at 50°C.

Figure 5.6 Relative molar concentrations of components in wash filtrate for cephalaxin system during Run 13. (A) Water, 100% ethanol, and 100% methanol as washing solvent. (B) Mother liquor component concentrations versus wash filtrate using 25-100% (v/v) ethanol in deionized water as washing solvent. For all experiments, 5 mL washing solvent/g cephalaxin was applied. All washing solvents were chilled to 4°C in an ice bath before use.

Figure 5.7 Residual activity of PGA-loaded Immobeads Cov-2 after 8 and 24 hours of process time in Run 9. Activity was assayed using 50 mmol/L cephalaxin hydrolysis, T = 25°C, pH = 7.0. Residual activity was calculated as a percentage of the initial activity of the PGA-loaded support prior to its use in Run 9.

Figure 5.8 Summary of results from Run 14, a continuous RC of cephalaxin monohydrate. (A) FBRM fines (<10μm) and mid-sized (10-100 μm) chords, pH, and cephalaxin production over the course of the pilot run; (B) Liquid phase concentrations of all components measured by HPLC; (C) Pilot plant apparatus for Run 14, complete with external wet milling loop; (D) Offline microscopy of crystals over time. Residence time from t = 0 to t = 400 minutes was 100 minutes. Residence time from t = 400 minutes to t = 1000 minutes was 150 minutes.
Figure 5.9 Summary of results from Run 15, the first continuous RC of amoxicillin trihydrate. (A) Process diagram of single MSMPR with external wet milling loop. Both product outlet and wet mill inlet travel through the size-based separator; (B) Liquid phase component concentrations over time measured by HPLC; (C) Offline micrograph of product crystals after 300 minutes process time; (D) FBRM counts, PVM turbidity, and productivity over time. T = 25°C, pH_{setpoint} = 6.5, V = 300 mL.

Figure 5.10 Summary results of Run 16, an experiment to test the effect of increasing the MSMPR residence time. (A) FBRM counts, PVM turbidity, and volumetric productivity over time; (B) Liquid phase concentrations of all components versus time, measured by HPLC of slurry filtrate; (C) 6-APA conversion over time (Red background data are after change in residence time). At t = 400 minutes, the residence time of the MSMPR was increased from 90 to 120 minutes. T = 25°C, pH_{setpoint} = 6.3, V = 300 mL.

Figure 5.11 Continuous pH-swing crystallization of amoxicillin trihydrate. Data represent liquid phase concentration of amoxicillin measured by HPLC. Feed conditions: 45 mmol/L amoxicillin, pH = 8.0, T = 4°C. First vessel conditions: T = 25°C, pH_{setpoint} = 6.3, V = 300 mL. Second vessel conditions: T = 4°C, pH uncontrolled, V = 200 mL. The first vessel was initially seeded with 2% (wt/vol) amoxicillin trihydrate (RIA, NJ). pH was controlled via the addition of 2 mol/L NaOH and a Mettler Toledo dosing unit connected to iControl software. An external wet milling loop was connected to the first vessel, with the same milling parameters as Runs 15 and 16.

Figure 5.12 Summary of process data for Run 17, the continuous RC of amoxicillin trihydrate with two MSMPRs in series; A) FBRM counts and turbidity vs. time; B) pH value and temperature vs. time; C) HPLC liquid phase concentrations in MSMPR-1; D) HPLC liquid phase concentrations in MSMPR-2. The dotted line marks the concentration of 6-APA in the liquid phase corresponding to 50% conversion; E) Solids productivity as isolated, post filtration and drying; F) Process diagram with two MSMPRs in series. Process conditions: T_{MSMPR-1} = 25°C, T_{MSMPR-2} = 9°C, pH_{setpoint,MSMPR-1} = 6.3, pH in MSMPR-2 uncontrolled, V_{MSMPR-1} = 300 mL, V_{MSMPR-2} = 200 mL. F = 3.3 mL/min.

Figure 5.13 Summary of results from Run 18, a continuous RC of amoxicillin with higher substrate concentrations. (A) MSMPR-1 liquid phase concentrations measured by HPLC; (B) MSMPR-2 liquid phase concentrations; (C) FBRM fines (<10 μm) and mid-size (10-100
μm) chords over time; (D) FBRM large (100-1000 μm) chords; (E) Comparison of productivity over time from Run 18 (gray) and Run 17 (blue); (F) Comparison of synthesis selectivity from Run 18 (gray) and Run 17 (blue). 30% higher feed concentrations for both substrates and 50% higher enzyme concentration compared to Run 17.

Figure 5.14 ATR-FTIR spectroscopy for measurement of sodium nitrate concentration in water. (A) ATR-FTIR spectra for a range of concentrations of sodium nitrate concentration; (B) IR peak area for peak at 1354 cm⁻¹ versus sodium nitrate concentration. Sodium nitrate was dissolved in deionized water. The peak was integrated from 1277 to 1445 cm⁻¹ with a two-point baseline subtraction between the same range of wavenumbers. T = 25°C.

Figure 5.15 Residence time experiments for MSMPR-1 and MSMPR-2 from the pilot plant apparatus. Both vessels were initialized with a known concentration of sodium nitrate dissolved in deionized water. \( V_{\text{MSMPR-1}} = 300 \text{ mL}; \ V_{\text{MSMPR-2}} = 200 \text{ mL} \). (A) Sodium nitrate concentration versus time for MSMPR-1 and MSMPR-2. The solid curves represent fits of each set of data to the equation \( c(t) = c_0 - c_0(1 - e^{-t/\tau_c}) \); (B) Pilot plant apparatus. The wet mill was connected to MSMPR-1 with a flow rate of 12 mL/min. The wet mill outlet and outlet from MSMPR-1 were withdrawn through a sieve of 300 μm using 2 mm I.D. Tygon tubing.

Figure 5.16 (A) Aqueous solubility of 6-APA and D-4-HPGME versus pH value at 25°C, as fit by Equation (5-2). (B) Overall conversion of 6-APA and D-4-HPGME versus recrystallization pH value. Gray dashed circles are shown at values of overall conversion for each substrate at their respective isolation pH values selected for experimental verification. Feed solutions were 240 mmol/L 6-APA and 234 mmol/L D-4-HPGME and the single pass conversion of both was set to 50% \((m_{\text{reacted},i} = 0.5m_{\text{initial},i})\).
Figure 5.17 6-APA recrystallization in deionized water with cosolutes present. (A) Liquid phase concentration of 6-APA over time with different cosolutes present; (B) In situ microscopy of 6-APA recrystallization in presence of cosolutes; (C) In situ microscopy of pure component 6-APA in deionized water. T = 13°C, pH_{final} = 4.0, V = 200 mL, c_{4-HPGME} = 20 mmol/L, c_{Amoxicillin} = 10 mmol/L, 1.2 g of 6-APA seeds were added.

Figure 5.18 PXRD diffraction patterns of 6-APA seeds (RIA, NJ), 6-APA recrystallized in pure deionized water, and 6-APA recrystallized in the presence of cosolutes (amoxicillin & 4-HPGME).

Figure 5.19 Solubility of 6-APA in presence of cosolutes (10 mmol/L amoxicillin, 110 mmol/L 4-HPGME) in comparison to pure component pH curve (from [83]). Green data point corresponds to liquid phase concentration of 6-APA after 90 minutes from data in Figure 5.17. T = 13°C.

Figure 5.20 (A) Effect of seeding density on 6-APA recovery. 10, 20, 30, 40, 50% seeds correspond to 0.4, 0.8, 1.2, 1.6, 2.0 g 6-APA seeds, respectively; (B) Effect of wet milling on 6-APA recovery, with two different seed densities. T = 13°C, pH_{final} = 4.0, V = 200 mL, c_{4-HPGME} = 20 mmol/L, c_{Amoxicillin} = 10 mmol/L. For milling experiments: IKA Magic Lab, MK Module, 4000 RPM, F_{mill} = 12 mL/min.

Figure 5.21 (A) Effect of differing rates of 2 mol/L NaOH addition on crystallization rate of 4-HPGME; (B) In situ microscopy of pH-swing 4-HPGME crystallization with immediate pH shift; (B) In situ microscopy with 6 mL/h addition rate of 2 mol/L NaOH. The size of the microscopy images is 1 mm². T = 13°C, pH_{final} = 8.0, c_{Amoxicillin} = 10 mmol/L, c_{6-APA} = 110 mmol/L.

Figure 5.22 Summary of data from Run 19, a continuous RC of amoxicillin over a 72-hour time period. (A) Turbidity, measured via in situ microscopy; (B) FBRM counts for three chord length ranges (<10 μm, 10-100 μm, 100-1000 μm); (C) Liquid phase component concentrations for MSMPR-1; (D) Liquid phase component concentrations for MSMPR-2; (E) Amoxicillin productivity; (F) Synthesis selectivity, estimated by the ratio of 6-APA consumption to 4-HPG production. T_{MSMPR-1} = 25°C, T_{MSMPR-2} = 7°C, pH_{setpoint,MSMPR-1} = 6.3, pH not controlled in MSMPR-2. Feed concentrations: 240 mmol/L 6-APA, 350 mmol/L 4-HPGME. Residence time of MSMPR-1 was increased from 90 min to 150 minutes at t = 52 hours.
Figure 5.23  (A) In situ microscopy in MSMPR-1 of Run 19. The size of each image is 1 mm². Spherical enzyme support particles are outlined in red; (B) Purolite amino-C6 support particles after Run 19; (C) Support particles after stirred in 300 mL of 50 mmol/L sodium phosphate, pH 7.5 for 2 hours.

Figure 5.24  Process data for the pilot-scale continuous enzymatic RC of amoxicillin trihydrate. A) FBRM counts and turbidity vs. time. B) pH value and temperature vs. time. C) Offline microscopy images of crystals. D) HPLC liquid phase concentrations in MSMPR-1. E) HPLC liquid phase concentrations in MSMPR-2. F) Solids productivity as isolated, post filtration and drying.

Figure 6.1  Disturbance simulations for continuous RC of cephalexin. (A) PGME feed flow rate disturbances – PG concentration over time after step change; (B) 7-ADCA feed flow rate disturbances – 7-ADCA concentration over time after step change; (C) Enzyme deactivation disturbance – PGME and 7-ADCA concentrations over time before and after 40% step change reduction in enzyme concentration. All disturbances were introduced at 720 minutes, after an initial steady state was achieved.

Figure 6.2  Run 20 (amoxicillin continuous RC) HPLC data for solution phase concentrations in MSMPR-1 showing points of disturbances being introduced and removed. Milling was removed at 5 hours and resumed at hour 7. The pH control was removed 11 hours into the run and reestablished at 12 hours into the run.

Figure 6.3  Run 20 (amoxicillin continuous RC) FBRM data tracking crystal chord length distribution (top) and PVM data tracking counts in different size bins. Also shown are the mean (square weighted by chord length) in μm from FBRM and the mean length of crystals as measured by PVM (bottom).

Figure 6.4  pH value over time during Run 20. pH control disabled at t = 10.7 hours and reactivated at t = 12.1 hours. Red dotted line is the fit to Equation (6-1).

Figure 6.5  Amoxicillin solids productivity throughout course of Run 20, at beginning and end of each simulated disturbance.

Figure 6.6  PAT useful for characterizing 2-phase solid/liquid systems along with offline characterization tools. Figure reproduced from McDonald et. al [156].
Figure 6.7  Attenuation of Raman scattering. (A) Raman spectra for water, 100 mmol/L 6-APA in water saturated with amoxicillin (~10 mmol/L), and 4% amoxicillin slurry; (B) Raman peak areas with varying slurry densities of amoxicillin trihydrate. pH = 6.3, T = 25°C, V = 200 mL, exposure time = 5 seconds.

Figure 6.8  Detection of solid 4-HPGME in an amoxicillin slurry. (A) Raman spectra of water, a 2% (wt) amoxicillin slurry, saturated with 4-HPGME, and increments of 1% and 2% (wt) 4-HPGME solids added; (B) Alternative axes of Panel (A) to show peak at 1734 cm⁻¹ corresponding to 4-HPGME solids, outlined in gray on Panel (A); (C) Evolution of 1734 and 852 cm⁻¹ peak areas with increasing 4-HPGME solids in a 2% amoxicillin slurry. pH = 6.3, T = 25°C. After measurement of water, solution was saturated with amoxicillin and 4-HPGME, and then 2% amoxicillin solids were added. V = 200 mL, exposure time = 5 seconds.
LIST OF SYMBOLS AND ABBREVIATIONS

Subscripts

\( i \) \( i^{th} \) moment of the size distribution
\( j \) \( j^{th} \) component in solution (P, B, S, Nu)

P Product (amoxicillin or cephalexin)
B Byproduct (4-HPG or PG)
S Acyl-donor substrate (4-HPGME or PGME)
Nu \( \beta \)-lactam nucleophile (6-APA or 7-ADCA)
0 Initial amount

Abbreviations

4-HPG D-4-hydroxyphenylglycine
4-HPGME D-4-hydroxyphenylglycine methyl ester
6-APA 6-aminopenicillanic acid
7-ADCA 7-desacetoxycephalosporanic acid
AE Atom economy
AEH \( \alpha \)-Amino ester hydrolase
AIC Akaike Information Criterion
AMI Aqueous mass intensity
API Active pharmaceutical ingredient
ATR-FTIR Attenuated total reflectance Fourier-transform infrared spectroscopy
CD Circular dichroism
CLD Chord length distribution
CSD  Crystal size distribution
CM  Continuous manufacturing
CSTR  Continuous stirred tank reactor
FBRM  Focused beam reflectance measurement
ISPR  In situ product removal
HPLC  High performance liquid chromatography
MSMPR  Mixed-suspension mixed-product-removal crystallizer
NiNTA  Nickel nitriloacetic acid
PAT  Process analytical technology
PG  D-phenylglycine
PGA  Penicillin G acylase
PGME  D-phenylglycine methyl ester
PMI  Process mass intensity
PVM  Particle vision and measurement
PXRD  Powder x-ray diffraction
QbD  Quality by Design
RC  Reactive crystallization
RME  Reaction mass efficiency
SNR  Signal to noise ratio
SSE  Sum squared error
SEM  Scanning electron microscopy
WT  Wild-type protein

*Latin characters*

\[ A_j \]  Apelbat constant for component \( j \)
\[ B \] Total nucleation rate
\[ B_0 \] Surface energy constant
\[ B_1 \] Primary nucleation rate
\[ B_2 \] Secondary nucleation rate
\[ B_j \] Apelbat constant for component \( j \)
\[ C_j \] Apelbat constant for component \( j \)
\[ b \] Secondary nucleation exponent
\[ c_j \] Concentration of component \( j \)
\[ c_j^* \] Saturation concentration of component \( j \)
\[ c_{pt,j}^* \] Saturation concentration of component \( j \) at the isoelectric point
\[ [E] \] Free enzyme concentration
\[ F_{\text{max}} \] Maximum fractional yield
\[ G \] Crystal growth rate
\[ g \] Crystal growth rate exponent
\[ l \] Ionic strength
\[ k_{B1} \] Primary nucleation rate constant
\[ k_{B2} \] Secondary nucleation rate constant
\[ k_{\text{cat}} \] Enzyme rate constant
\[ k_G \] Crystal growth rate constant
\[ K_i \] Equilibrium constant for enzyme-substrate complex
\[ k_v \] Crystal volumetric shape factor
\[ L \] Crystal length
\[ M_T \] Slurry density
\[ m \] Slurry density exponent
[\textit{Nu}] Concentration of nucleophile (6-APA or 7-ADCA)

\(n(L, t)\) Crystal size distribution

\(P\) Productivity

\(pK_{a,j}\) Log\(_{10}\) acid dissociation constant of component \(j\)

\(R_j\) Reaction rate of component \(j\)

\(S\) Supersaturation ratio

\(t\) Time

\(T\) Temperature

\(V\) Volume

\(t_{ind}\) Induction time

\(X\) Conversion

\(x_j\) Mole fraction of component \(j\)

\(z\) Charge number of ionic species

\textit{Greek characters}

\(\alpha\) Youshko and Svedas’ alpha parameter

\(\beta\) Youshko and Svedas’ beta parameter

\(\gamma\) Youshko and Svedas’ gamma parameter

\(\mu_i\) i\textsuperscript{th} moment of the CSD

\(\rho\) Density

\(\tau\) Residence time

\(\tau_l\) Residence time of the liquid phase

\(\tau_s\) Residence time of the solid phase

\(\Theta_{obs}\) Observed optical rotation

xxiv
$\phi_j$ Specific optical rotation of component $j$
SUMMARY

The enzymatic manufacture of beta-lactam antibiotics can be improved and intensified by integrating the synthesis and separation of the antibiotic within a single vessel. Reaction and crystallization within the same unit operation, or reactive crystallization (RC), improves substrate conversions and productivity by protecting the antibiotic from enzymatic degradation. Additionally, incorporation of this process on a continuous platform allows for improved control, greater capacity for the same process footprint, reduced environmental impact, and improved process performance. The use of biocatalysts instead of traditional metal catalysts allows for the operation in mild aqueous conditions, reducing organic solvent usage and eliminating the need for cryogenic refrigeration units which are highly energy intensive. Additionally, biocatalysts yield improved reaction efficiency and decreased total waste production. Applying process analytical technology (PAT) to continuous processes allows for the on-line monitoring of important process variables, such as product purity, but also allows for the monitoring of process conditions such that conditions are never reached which promote a decline in product purity. A model for the reactive crystallization of amoxicillin and cephalexin was utilized to determine attainable operating conditions, ensuring acceptable productivity and conversion. Two similar continuous RC processes were developed for the manufacture of amoxicillin and cephalexin, and various challenges with the incorporation of these processes were addressed. The process was operated robustly for short and long-term runs and the sensitivity of the process to disturbance variables was evaluated via modelling and experimentally. This process will enable a more cost efficient and sustainable production
of amoxicillin and cephalexin, and possibly similar antibiotics, with potential for on-demand production of drug substances, which has been shown to be particularly important when considering recent events involving supply chain breakdowns and shortages involving the Covid-19 pandemic, as well as current and potential foreign conflict.
CHAPTER 1. Introduction

1.1 Beta-lactam antibiotics

Beta-lactam antibiotics are the most prevalently used antibiotic class worldwide, with about $15 billion in sales and representing 65% of the total world market for antibiotics in 2003 [1]. Beta-lactam antibiotics have also been produced industrially for over 70 years, yet their manufacture remains a topic of discussion due to the significant increase in demand worldwide, primarily in middle- and low-income countries [2]. The two classes of beta-lactam antibiotics are penicillins and cephalosporins, which have been utilized extensively in the treatment of various bacterial infections for several decades [3]. Amoxicillin and cephalexin are the two most prevalent beta-lactam antibiotics, prescribed on a worldwide scale of approximately 30,000 and 15,000 metric tons/year, respectively. Due to increased demand as well as supply chain shortages, even highly developed countries such as the USA have experienced shortages for these critical front-line drugs. A scalable, lower cost, and on-demand production of these drugs would help alleviate these shortages, improving availability to those who require treatment. Innovation in the production of beta-lactam antibiotics such as amoxicillin and cephalexin may accelerate development and commercialization of new production processes and facilities, improving worldwide production capacity and availability.
1.2 Enzymatic beta-lactam synthesis

In comparison to traditional chemical synthetic pathways, the biocatalytic synthesis of beta-lactam antibiotics addresses many of the environmental concerns of large-scale pharmaceutical manufacturing. Biocatalytic synthesis can be conducted in mild aqueous conditions, reducing the usage of organic solvents (such as dichloromethane and methanol regularly used in chemical beta-lactam antibiotic synthesis), as well as lessening the energy demand due to cryogenic refrigeration units [4]. Additionally, biocatalytic synthesis of beta-lactam antibiotics is generally a single-step stereoselective condensation reaction between an activated acyl-donor and beta-lactam nucleus; whereas, the chemical production requires separate protection, coupling, and deprotection steps, leading to a much lower atom economy (AE), or the percent mass of reactants that is found in the final product [1, 5]. The enzyme Penicillin G acylase (PGA) from *E. coli* has been applied extensively in the production of the beta-lactam nucleus-containing 6-aminopenicillanic acid (6-APA), a feedstock to the production in some semi-synthetic beta-lactam antibiotics (e.g., ampicillin, amoxicillin), via hydrolysis of penicillin G produced from fermentation in yeast. More recently, PGA has been used in the synthesis of semi-synthetic beta-lactam antibiotics, by coupling beta-lactam nucleus-containing moieties such as 6-APA or 7-aminodesacetoxycephalosporanic acid (7-ADCA) to an acyl sidechain, such as D-phenylglycine methyl ester (PGME), releasing the product active pharmaceutical ingredient (API) from the active site. Other enzymes have been shown to catalyze the synthesis of semi-synthetic beta-lactam antibiotics or biotransformation of their primary substrates, such as PGA from other organisms (e.g., *K. citrophila*, *X. citri*, *P. rettgerri*) as well as other classes of enzymes (e.g., Penicillin V acylase, α-amino acid ester hydrolase...
(AEH), cephalosporin acylase, glutaryl acylase) [6]. Other than PGA, AEH has been investigated the most due to its high activity and increased reaction promiscuity [7-11]; however, AEH has not been used industrially due to its low stability. The main disadvantage of using enzymatic beta-lactam antibiotic synthesis is the enzymes themselves catalyze the hydrolysis of the activated acyl-donor (e.g., PGME) as well as hydrolysis of the product [12]. To mitigate these side reactions as well as other aspects of the enzyme, PGA has been engineered for improved synthesis selectivity and substrate specificity [13-20]. Additionally, the PGA catalyzed synthesis of beta-lactam antibiotics has been engineered for improved efficiency, such as in situ product removal [21-24], enzyme immobilization [25-31], and reaction engineering [32-37].

1.3 Continuous manufacturing

Continuous manufacturing (CM) offers many benefits over batch processing in pharmaceutical manufacturing, such as higher efficiency, easier scalability due to smaller required unit operations, improved product quality, and reduction of the environmental impact of pharmaceutical production [38-42]. Due to these advantages, regulatory agencies have urged pharmaceutical manufacturers to investigate and implement technologies required to implement CM in their processes [38, 43]. Recently, the CM of beta-lactam antibiotics is positioned to meet many of the shortcomings of current manufacturing methods. A significant challenge in CM implementation in the pharmaceutical industry is the strict product specifications necessitating the use of process analytical technology (PAT) for online process monitoring [44-46]. In the case of beta-lactam manufacturing, the
capacity is surely the most critical issue to address, which CM is well suited to do as the vessel volume is processed every residence time, greatly improving volumetric productivity as well as reducing time usage for startup/shutdown protocols. Also, the recent Covid-19 pandemic underscores the necessity for achieving the ability to rapidly scale up production in the case of the breakdown of international supply lines for pharmaceuticals and important intermediates/feedstocks. Ideally, a continuous process would incorporate end-to-end processing, which reduces the accumulation of intermediates between steps and requires less human intervention; however, matching the capacity of multiple unit operations in series can be challenging.

1.4 Reactive crystallization of amoxicillin and cephalexin

A commonly applied approach to reduce secondary hydrolysis of beta-lactam antibiotics is in situ product removal, as the product is removed from the same phase as the enzyme, preventing enzymatic hydrolysis. The most used method of in situ product removal for beta-lactam antibiotic synthesis is reactive crystallization (RC), defined as the combination of reaction and crystallization concurrently, often improving process dynamics. RC is also a form of process intensification, reducing the complexity of chemical processes. RC is beneficial when the product is often isolated in its desired form, such as is often the case for pharmaceuticals [47-50] or salts such as calcium carbonate [51-54]. Additionally, RC is especially advantageous cases where the product possesses significantly lower solubility than other components in the system, such as amoxicillin, ampicillin, and cephalexin [55-59]. For both amoxicillin and cephalexin, the products
possess lower solubility in water than their respective enzymatic synthesis substrates [60]. For cephalexin, the byproduct D-phenylglycine (PG) possesses a low solubility and precipitates upon significant accumulation, complicating further downstream processing, which further necessitates RC to reduce cephalexin hydrolysis and PG formation.
CHAPTER 2. Kinetic Characterization of penicillin g acylase from 

_E. coli_ for the synthesis of amoxicillin and cephalixin

2.1 Introduction and Motivation

Chapter 2 is based on the previously published study [61].

β-lactam antibiotics have been great contributors to the fight against bacterial infections for almost 80 years. Since the introduction of β-lactam antibiotics, many other classes of antibiotics (e.g. macrolides, tetracyclines, aminoglycosides) have been developed for the treatment of different infections; nonetheless, β-lactam antibiotics remain the most used class of antibiotics on a dose-consumption basis [62]. Additionally, global demand is increasing, primarily among low- and middle-income countries [2]. β-lactam production using traditional chemical processes requires the use of organic solvents, cryogenic refrigeration units, and protecting groups, leading to an energy- and material-intensive process that produces large amounts of hazardous waste. Due to this situation, extensive research has been conducted to identify alternative synthetic procedures [1]. Biocatalytic pathways have drawn considerable interest as they can generally be run under mild aqueous conditions (e.g., neutral pH, 25-37°C), eliminating the need for organic solvents, strong acids or bases, and energy-intensive heating and refrigeration units. Today, several plants employ the Penicillin G acylase-based technology, such as those of Centrient in Delft/Netherlands or GSK in Singapore.

Penicillin G acylase (PGA) from _E. coli_ was initially used to hydrolyze penicillin G to produce 6-aminopenicillanic acid (6-APA)[63], which is a precursor to several semi-
synthetic β-lactam antibiotics (e.g., ampicillin, amoxicillin). More recently PGA has been repurposed to synthesize semi-synthetic β-lactam antibiotics, specifically penicillins and cephalosporins. The reaction network in which PGA catalyzes cephalexin synthesis as well as two hydrolysis reactions is outlined in Figure 2.1.

Figure 2.1: Reaction network for the synthesis of cephalexin from 7-ADCA and PGME. Synthesis denotes the condensation of PGME and 7-ADCA to form the desired product. Primary hydrolysis denotes hydrolysis of PGME to form a byproduct. Secondary hydrolysis denotes hydrolysis of the desired product cephalexin to form a byproduct PG and 7-ADCA. For the amoxicillin system, 7-ADCA is replaced with 6-APA, PGME is replaced with 4-HPGME, and PG is
replaced with 4-HPG. The crystal structure of PGA from E. coli (PDB code: 1GK9) is shown in the center of the figure.

A major drawback of enzymatically synthesizing β-lactam antibiotics is the tendency of the biocatalyst to catalyze primary and secondary hydrolysis: that is, hydrolysis of the activated acyl-donor (e.g. PGME) and product antibiotic (e.g. cephalexin), respectively. While the yield of the reaction is obviously a major concern, the unwanted byproducts are often sparingly soluble in water; therefore, it is important to minimize byproduct formation to reduce the risk of unwanted byproduct precipitation, complicating downstream isolation and processing of the desired product. As a result, PGA has been the subject of research to enhance its selectivity and stereoselectivity for the synthesis of β-lactam antibiotics [14, 15, 17, 18, 64].

The model developed in [65] was applied for the synthesis of ampicillin using PGA at low substrate concentrations and later by [66] at higher substrate concentrations, albeit without consideration of inhibitory affects. This model uses the initial rates of synthesis and hydrolysis to estimate three kinetic parameters ($\alpha$, $\beta_0$, $\gamma$), where $\alpha$ represents the ratio of the catalytic efficiency ($k_{cat}/K_M$) of secondary hydrolysis of product P to primary hydrolysis of substrate S, $\beta_0$ represents the reactivity of the nucleophilic β-lactam nucleus (termed “nucleophile”) at very low concentrations, and $1/\gamma$ the maximum initial-rate selectivity (the ratio of product P production to byproduct P₂ production) at near-infinite nucleophile concentration. $k_{cat}$ and $K_M$ represent the apparent catalytic rate constant and Michaelis-Menten binding constant for the hydrolysis of a given species, respectively.
Experimental data fit to Equations (2-1) and (2-2) allow for determination of $\alpha$, $\beta_0$, and $\gamma$ for a specific reaction and biocatalyst.

$$
\alpha = \frac{\left(\frac{k_{cat}}{K_m}\right)_p}{\left(\frac{k_{cat}}{K_m}\right)_S} \quad (2-1)
$$

$$
\left(\frac{\nu_{P_S}}{\nu_{P_n}}\right)_{\text{init}} = \frac{1}{\gamma} \left(\frac{[Nu]}{\frac{1}{\beta_0 \gamma} + [Nu]} \right) \quad (2-2)
$$

$v$ represents the velocity of a particular reaction in units of concentration/time. The parameters $\alpha$, $\beta_0$, and $\gamma$ have been used to compare unique enzymes and enzyme variants and ascertain sources of variations in their synthetic properties, whether they be altered binding of product and substrates or modified reactivity of the $\beta$-lactam nucleus. While this model has been used effectively for low substrate concentrations, the use of higher substrate concentrations to synthesize and crystallize (i.e., reactively crystallize) $\beta$-lactam antibiotics provides significant benefits in yield and productivity [55-57, 67, 68]. If the model by Youshko and Śvedas or an enhanced version were accurate and predictive for the synthesis of $\beta$-lactam antibiotics at higher substrate concentrations, it could be used for the model-based design and evaluation of these types of reactive crystallization systems and the engineering of biocatalysts tailored for these systems. For example, the model by Youshko and Śvedas was modified [57] for ampicillin synthesis using PGA from E. coli to account for changes in the reactivity of the acyl-enzyme complex over a range of pH values and to account for substrate inhibition by 6-APA. Here we consider formulation of a model for the synthesis of the most used cephalosporin and second most common beta-lactam antibiotic, cephalexin.
The selectivity of PGA, especially of key variants such as βF24A and commercial variant Assemblase®, has not been thoroughly characterized for the synthesis of cephalexin, the second largest (by volume) produced β-lactam antibiotic worldwide, neither at low concentrations typically employed in academic studies nor at high substrate concentrations. As these compounds are preferentially synthesized at high substrate concentration conditions, a better understanding of the enzymatic performance of PGA and its key variants could lead to better variant selection and reaction engineering. This work serves to achieve two goals: 1) Characterize the selectivity of the wild-type PGA from *E. coli*, its βF24A variant, and of Assemblase® for cephalexin synthesis under high substrate concentrations and 2) Assess the suitability of the commonly used model [65] and Equations (2-1) and (2-2) in the identification and evaluation of the differences between variants for synthesis by noting inconsistencies between reaction data and the model and proposing a model that more accurately describes the phenomena observed in cephalexin synthesis reactions. As the kinetics of β-lactam antibiotic synthesis depends on the target and the specific acyl-donors, and nucleophiles employed in its synthesis, model-based reaction engineering, which is needed to accelerate the development of biocatalytic β-lactam production processes, likely differs for each combination of target and precursors.

2.2 Materials and Methods

Materials

Cephalexin monohydrate was obtained from RIA International LLC (East Hanover, NJ); D-phenylglycine methyl ester hydrochloride (D-PGME·HCl, ≥95%) from Sigma (St.
Louis, MO); 6-aminopenicillanic acid (6-APA, ≥98%), 7-aminodesacetoxycephalosporanic acid (7-ADCA, ≥98%), and amoxicillin trihydrate (≥98%) from TCI America (Portland, Oregon); 2-(4-hydroxyphenyl)-D-glycine methyl ester hydrochloride (4-D-HPGME, ≥95%) from Alfa Aesar (Willard Hill, MA); HPLC grade methanol from Fischer Scientific (Waltham, MA). Assemblase® was kindly donated by DSM-Sinochem and their successor Centrient (Delft, The Netherlands).

**Generation of penicillin G acylase from E. coli clones**

Penicillin G acylase from *E. coli* ATCC 11105 was cloned into pET28 vector via introduction of NcoI and XhoI restriction sites to the N and C termini of the gene, respectively. Prior to restriction, the signal sequence of the gene (first 25 codons) and stop codon at the C-terminus of the gene were removed, to allow for addition of a poly-histidine (6x) tag, by PCR amplification using the following primers with the restriction site **bolded** -

- Forward primer: CGCGCCATGGGCGAACAGAGCAGCAGCGAA;
- Reverse primer: CGCGCGCTCGAGGCGCTGCACATGCAGC.

Following amplification, the gene was double digested using NcoI and XhoI restriction enzymes and ligated into pET28 which was previously double digested by the same enzymes. Clones were then transformed into chemically competent BL21(DE3) *E. coli* cells and selected for using kanamycin-agar (25 mg kanamycin/L) plates and validated by PCR followed by Sanger sequencing. To generate the βF24A variant, site-directed mutagenesis was combined with overlap extension PCR using the previously mentioned primers for amplification of the total gene and the following primers with the mutation site **bolded** –

- Forward primer:
GTGAACGCGCCCGCAGGCAGGGCTGATGCGGC; Reverse primer: CGGCATACGCGCCGCTGGATGCGGCCTGGTATGCGGC. Clones were generated in the same fashion as the wild-type and were confirmed via Sanger sequencing.

*Expression and purification of penicillin G acylase from E. coli*

Prior to expression, a starter culture of BL21(DE3)-PGA cells was grown. 500 mL of expression culture media was prepared prior to expression by adding 10 g of tryptone, 2.5 g of yeast extract, and 2.5 g NaCl to 480 mL of 1X PBS, pH 7.2, followed by autoclaving. Next, 20 mL of an autoinduction media was added followed by 500 μL of 25 g/L kanamycin in water. 10 mL of starter culture was then added to the 500 mL expression culture in a 2 L baffled shaker flask and incubated at 25°C for 24 h while shaking. The resuspended cells were then lysed via sonication on ice and centrifuged to remove cellular debris. The lysate was collected and applied to Ni-NTA agarose beads on ice in a gravity column for 1h. PGA was eluted from the Ni-NTA column using imidazole and desalted using a PD-10 desalting column, storing in 50 mmol/L sodium phosphate pH 7.0. Expression in the cytosol followed by nickel affinity purification resulted in approximately 45 mg of pure protein per liter of culture, much higher than the 2 mg per liter of culture acquired via purification from periplasmic extract obtained via osmotic shock extraction, described by [13, 69]. Purity of PGA was always >95% and assessed via intensity of the bands of pure protein in an SDS-PAGE gel (Figure A.6) in comparison to impurities.
Collection of Reaction Data

Initial-rate reactions were buffered using phosphate buffer at the specified concentration and pH value. To initiate reactions, a specified mass of reactants was dissolved in 10 mL of phosphate buffer to achieve a desired stock solution concentration. The pH was then adjusted to the desired value using 5 mol/L HCl and/or 10 mol/L NaOH. The reactant solution was then incubated in a 1.2 mL microfuge tube in an Eppendorf Thermomixer R heating block set to 25°C, the temperature of all reactions conducted in this study. The reactant solution was then added to an enzyme-buffer solution incubated at 25°C to bring the reactant concentrations and enzyme concentration to the desired value at a total volume of 1 mL per reaction. The concentration of the enzyme stock used to create the enzyme-buffer solution was determined via Bradford protein assay. Time-course reactions were conducted unbuffered in DI water using the Metrohm 902 Titrando system. Time-course reactions were conducted with an initial reaction volume of 20 mL in a 50 mL glass jacketed vessel connected to an external water bath loop and were well-mixed using a pitched impeller. The pH was automatically controlled at the desired value via addition of 1 mol/L NaOH using the Metrohm 800 Dosino unit with a 5 mL borosilicate buret. Upon initiation of the reaction, 50 μL aliquots of reaction solution were withdrawn at various times and diluted in at least 4 volumes of 100 mmol/L sodium acetate buffer pH 4.3 to quench the reaction. The total time of reactions varied from 4 to 120 minutes. All reactions were conducted at pH 7.0 to allow for higher concentrations of 7-ADCA to be dissolved, as 7-ADCA solubility is highly pH dependent.
Analysis of Reaction Samples

Quenched reaction samples were analyzed via reverse phase HPLC using a Hichrom Ultrasphere C18 Column, Diameter 4.6 mm, 5 μm particle size, 250 mm length. A gradient method was employed to achieve baseline separation of all components. Mobile phase A was 10 mmol/L sodium phosphate buffer pH 7.0. Mobile phase B was HPLC grade methanol.

For the analysis of cephalexin reactions, the column was equilibrated in 95% mobile phase A and 5% methanol at a total flow rate of 1 mL/min. At t = 1.5 minutes, a linear gradient shifted from 95% to 85% mobile phase A until t = 4 minutes. Another linear gradient shifted the mobile phase composition from 85% to 50% mobile phase A until t = 7 minutes. The mobile phase composition was then held at 50% mobile phase A until t = 9 minutes and then immediately changed to the initial composition to re-equilibrate the column. The retention times of components were roughly, 3.5, 4.0, 10.0, and 12.0 minutes for phenylglycine, 7-ADCA, cephalexin, and PGME, respectively.

For the analysis of amoxicillin reactions, the same column was equilibrated using the same mobile phases at the same concentration and flow rate as the cephalexin analytical protocol (95% mobile phase A and 5% mobile phase B). From t = 0 minutes to t = 7 minutes, a linear gradient shifted from 95% to 50% mobile phase A with the balance mobile phase B. The mobile phase was then held at 50% mobile phase A until t = 8 minutes and then changed to 95% mobile phase A to re-equilibrate the column. The retention times of
components were roughly 2.5 minutes, 4.0 minutes, 7.0 minutes, and 10.0 minutes for 4-HPG, 6-APA, amoxicillin, and 4-HPGME, respectively.

Computational methods

Data processing and model fitting were achieved using MATLAB R2020b. Fitting of time-course data was conducted using the “patternsearch” function to minimize the sum squared error between the model fit and experimental data. The “gamultiobj” solver function was used to generate pareto optimal fronts for the multiobjective optimization of a cephalexin synthesis CSTR. The system of equations solved, optimization inputs, and objective functions are defined in the Supplemental Information.

2.3 Results

2.3.1 Determination of initial reaction rates for synthesis and hydrolysis of amoxicillin and cephalexin and precursors using Penicillin G Acylase from E. coli

2.3.1.1 Cephalexin Synthesis

The α parameter for each variant was experimentally determined by separately examining the initial rates of primary and secondary hydrolysis (Figure A.1). The results showed that the concentration dependence of secondary and primary hydrolysis followed Michaelis-Menten kinetics. Additionally, initial rates of cephalexin synthesis reactions were determined for the wild-type, βF24A, and Assemblase® over a range of 7-ADCA concentrations to determine the effect of nucleophile concentration on cephalexin synthesis
selectivity, \( \frac{v_{CEX}}{v_{PG}} \). Initial-rate cephalaxin synthesis reactions were conducted for each variant over a range of 7-ADCA concentrations and for the wild-type enzyme at 50 mmol/L, 100 mmol/L, and 150 mmol/L PGME concentrations to determine the impact of varying substrate concentrations on the selectivity of cephalaxin synthesis. The model parameters reported in Table 2.1 were estimated by fitting to the data in Figure 2.2 and in Figure A.1.

Figure 2.2: (A) Initial-rate cephalaxin synthesis selectivity for the wild-type, βF24A, and Assemblase® variants for 7-ADCA concentrations 2.5-50 mmol/L. Reaction conditions: pH = 7.0, CPGME = 100 mmol/L, CPGA = 500 nmol/L. (B) Initial-rate cephalaxin synthesis selectivity for the wild-type for 7-ADCA concentrations 5-60
mmol/L. Each curve represents the same PGME concentration for each reaction.

Reaction conditions: pH = 7.0, C_{PGA} = 500 nmol/L.

Table 2.1: Parameters from fitting to initial-rate data for cephalaxin synthesis, secondary hydrolysis, and primary hydrolysis using Equations (2-1) and (2-2) using data provided in Figure A.1 and Figure 2.2(A).

<table>
<thead>
<tr>
<th>Parameter (units)</th>
<th>Wild-type</th>
<th>BF24A</th>
<th>Assemblase®</th>
</tr>
</thead>
<tbody>
<tr>
<td>K_{M,PGME} (mM)</td>
<td>47 ± 1</td>
<td>32 ± 7</td>
<td>29 ± 2</td>
</tr>
<tr>
<td>k_{cat,PGME} (s^{-1})</td>
<td>33 ± 2</td>
<td>37 ± 6</td>
<td>39 ± 7</td>
</tr>
<tr>
<td>K_{M,cephalexin} (mM)</td>
<td>8.1 ± 0.8</td>
<td>2.5 ± 0.2</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>k_{cat,cephalexin} (s^{-1})</td>
<td>32 ± 3</td>
<td>31 ± 1</td>
<td>33 ± 1</td>
</tr>
<tr>
<td>α_{cephalexin} †</td>
<td>5.7 ± 0.8</td>
<td>11 ± 3</td>
<td>10. ± 1</td>
</tr>
<tr>
<td>β_{0,cephalexin} (M^{-1})</td>
<td>204 ± 5</td>
<td>177 ± 3</td>
<td>160 ± 10</td>
</tr>
<tr>
<td>γ_{cephalexin} †</td>
<td>0.076 ± 0.003</td>
<td>0.03 ± 0.01</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>(1/γ)_{cephalexin} †</td>
<td>13.1 ± 0.5</td>
<td>30 ± 10</td>
<td>17 ± 6</td>
</tr>
</tbody>
</table>

As shown in Figure 2.2(A), the selectivity of all three variants followed an expected trend as outlined by Equation (2-2), which is a monotonic increase of selectivity as well as decreasing slope as 7-ADCA concentration increases. At higher 7-ADCA concentrations, the selectivity of the βF24A variant appeared to be greatest. At lower 7-ADCA concentrations, the selectivity of all three variants appeared to be very similar.

To determine whether 7-ADCA binds to the enzyme separately to the formation of the acyl-enzyme complex, the effect of 7-ADCA on the rate of secondary hydrolysis was
investigated. The intersection of all curves slightly to the left of the y-axis of the Lineweaver-Burk plot in Figure 2.3 suggests that mixed inhibition is present but is dominated largely by competitive inhibition. In the limit of pure competitive inhibition, the intersection would lie on the y-axis.

Figure 2.3: Lineweaver-Burk plot for the inhibition of cephalexin hydrolysis by 7-ADCA for WT-PGA. \( v_{\text{initial}} \) represents the initial activity of cephalexin hydrolysis. Each data series represents different 7-ADCA concentrations and each solid line the best fit to Michaelis-Menten hydrolysis of cephalexin with a competitive inhibitor for each separate inhibitor concentration. Reaction conditions: pH = 7.0, C\(_{\text{PGA}}\) = 300 nmol/L.
A fit to a Michaelis-Menten model with competitive inhibition resulted in a close fit to experimental data and $K_I$ value of $3.2 \pm 0.1$ mmol/L, confirming that competitive inhibition is adequate in describing the data in Figure 3. These results are on the same order of those previously published ($K_I = 7$ mmol/L) indicating 7-ADCA competitively inhibits hydrolysis of D-2-nitro-5-[(phenylglycyl)amino]-benzoic acid (NIPGB) by PGA [70]. If 7-ADCA competitively inhibits hydrolysis of some activated acyl-donating compounds catalyzed by PGA, then perhaps 7-ADCA may bind to the active site, thereby blocking the binding of other species whose binding sites are in the same location or proximity. As the binding sites for 7-ADCA and PGME are directly adjacent to each other and the binding site for PGME is located more deeply towards the core of the enzyme, it is feasible that 7-ADCA may sterically block access of PGME to its binding site. In that case, even though 7-ADCA and PGME would not be competing for the same active site, it would yield apparent competitive inhibition as there would be two possible states of the system: 1) 7-ADCA bound to the active site, blocking PGME binding and 2) PGME bound which may proceed to hydrolysis or synthesis. Uncompetitive inhibition would not be observed in this case as the formation of the acyl-enzyme complex prior to 7-ADCA binding would lead to the nucleophilic attack of 7-ADCA or water and product or byproduct release. If 7-ADCA not only inhibited the hydrolysis of cephalexin but also the binding of PGME and formation of the acyl-enzyme complex as proposed, this would lead to a decrease in synthesis rate with increasing 7-ADCA concentration, a phenomenon recently observed for immobilized PGA with cephalexin synthesis as well as with amoxicillin synthesis with increasing 6-APA concentration. [71].
The maximum fractional yield, $F_{max}$, of cephalexin synthesis for the reaction based on the model in [72] (Scheme 2) may be estimated using Equations (3) and (4) below, which were developed by [73] and applied to ampicillin synthesis by [57].

\[
F_{max} = \frac{\beta n_0}{\alpha} \left( \frac{\alpha}{1 + \beta n_0} \right)^{1 - \frac{\alpha}{1 + \beta n_0}}^{-1} \quad (2-3)
\]

\[
\beta = \left( \frac{1}{\beta_0 + \gamma n_0} \right)^{-1} \quad (2-4)
\]

Using these equations and $\alpha$, $\beta_0$, and $\gamma$ previously determined, the estimated maximum fractional yield $F_{max}$ of cephalexin synthesis under the same conditions as the experiment in Figure A.5 reaction 13 was 40% for the $\beta$F24A variant, whereas the actual value was 52%. This indicates that the use of initial-rate kinetic parameters to extrapolate to time-course cephalexin synthesis may result in inaccurate model predictions. Selective inhibition of cephalexin hydrolysis could explain greater experimental yields than model prediction but would disagree with the results of Figure 2.3. Models incorporating phenomena from either hypothesis are discussed later in this work.

The effect of increasing substrate concentrations on cephalexin synthesis selectivity was investigated while maintaining a constant molar ratio of 2.5:1 (PGME:7-ADCA) with the results plotted in Figure 2.4.
Figure 2.4: Initial selectivity and initial cephalexin synthesis rate versus total magnitude of substrate concentrations for cephalexin synthesis using wild-type PGA at a constant (PGME:7-ADCA) ratio of 2.5:1. Reaction conditions: pH = 7.0, C_{PGA} = 500 \text{ nmol/L}.

As the overall substrate concentrations increase at constant PGME:7-ADCA, the selectivity towards cephalexin synthesis increased monotonically in the range examined, which is expected, as increased 7-ADCA concentrations improve synthesis selectivity and PGME does not play a strong role in initial selectivity. As 7-ADCA concentration increases, the initial cephalexin synthesis rate increased as well.
2.3.1.2 Amoxicillin Synthesis

Initial rate hydrolysis reactions were conducted for amoxicillin as well as 4-HPGME for all three variants with the rate versus substrate concentration plots found in the appendix in Figure A.2. As expected, all curves fit Michaelis-Menten kinetics. Initial rate amoxicillin synthesis experiments were conducted over a range of 6-APA concentrations with the results seen in Figure 2.5 and the parameters obtained from the data found in Figure A.2 and Figure 2.5 to Equations (2) and (3) are found in Table 2.2.

![Graph showing initial rate amoxicillin synthesis selectivity for wild-type, βF24A, and Assemblase® variants for 6-APA concentrations 25-150 mmol/L. Reaction conditions: pH = 6.2, C4-HPGME = 100 mmol/L, CPGA = 1.5 μmol/L.]

Figure 2.5: Initial rate amoxicillin synthesis selectivity for the wild-type, βF24A, and Assemblase® variants for 6-APA concentrations 25-150 mmol/L. Reaction conditions: pH = 6.2, C4-HPGME = 100 mmol/L, CPGA = 1.5 μmol/L.
Table 2.2: Parameters from the model fit for amoxicillin synthesis, secondary hydrolysis, and primary hydrolysis using Equations (1) and (2) using data provided in Supplemental Figure 2 and Figure 6.

<table>
<thead>
<tr>
<th>Parameter (units)</th>
<th>Wild-type</th>
<th>BF24A</th>
<th>Assemblase®</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{MA-HPGME}$ (mM)</td>
<td>12 ± 1</td>
<td>11 ± 1</td>
<td>18.3 ± 0.9</td>
</tr>
<tr>
<td>$k_{cat,4-HPGME}$ (s$^{-1}$)</td>
<td>13 ± 1</td>
<td>21 ± 2</td>
<td>22 ± 1</td>
</tr>
<tr>
<td>$K_{M,amoxicillin}$ (mM)</td>
<td>2.2 ± 0.1</td>
<td>3.4 ± 0.3</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>$k_{cat,amoxicillin}$ (s$^{-1}$)</td>
<td>13.3 ± 0.2</td>
<td>20.6 ±</td>
<td>16.5 ± 0.8</td>
</tr>
<tr>
<td>$\alpha_{amoxicillin}$ †</td>
<td>5.3 ± 0.7</td>
<td>2.9 ± 0.5</td>
<td>5.3 ± 0.6</td>
</tr>
<tr>
<td>$\beta_{0,amoxicillin}$ (M$^{-1}$)</td>
<td>56 ± 7</td>
<td>42 ± 2</td>
<td>47 ± 3</td>
</tr>
<tr>
<td>$\gamma_{amoxicillin}$ †</td>
<td>0.40 ± 0.07</td>
<td>0.33 ± 0.05</td>
<td>0.40 ± 0.07</td>
</tr>
<tr>
<td>$(1/\gamma)_{amoxicillin}$ †</td>
<td>2.5 ± 0.5</td>
<td>3.1 ± 0.5</td>
<td>2.5 ± 0.5</td>
</tr>
</tbody>
</table>

†Parameter is unitless
Based on similar kinetic parameters obtained for amoxicillin synthesis and hydrolysis, it does not appear that any of the variants tested possessed enhanced biocatalytic performance in the case of amoxicillin, as would be supported by higher $\alpha$, $\beta_0$, or $1/\gamma$ values.

2.3.2 Kinetic model development, fitting, and parameter estimation for cephalexin synthesis

Three models which have been previously proposed to describe $\beta$-lactam antibiotic synthesis by PGA are shown in Figure 2.6.
Figure 2.6: Proposed Models 1, 2, and 3. Model 1: Model Proposed by Youshko and Svedas (Y-S); Model 2: Y-S Model with Competitive Inhibition by 7-ADCA; Model 3: Y-S Model with Random Binding of 7-ADCA and PGME. E corresponds to free enzyme, S the acyl-donor, Nu the nucleophilic beta-lactam nucleus, EA the acyl-enzyme complex, P the product antibiotic, P₂ the byproduct. Note: All unshaded model parameters and reactions correspond to Model 1.

$K_{ni}$ corresponds to the competitive inhibition binding constant and $K_{n1}$ and $K_{n2}$ correspond to the binding constants of 7-ADCA to the free enzyme as well and the enzyme-PGME complex, respectively. $\eta$ corresponds to the ratio of rate of acyl-enzyme formation by PGME with and without 7-ADCA bound to the active site. Time-course synthesis reactions
were conducted, which are shown in Appendix Figure A.4, with Model 2 fits shown for each reaction. Models 1-3 shown in Figure 2.5 were fit to the data, with parity plots of each model shown in Figure 2.7.

Figure 2.7: Parity plots for best fits of Models 1-3 for cephalexin reaction data.  
(Bottom right) Comparison of model fits by sums-of-squared errors (SSE) and Akaike Information criterion (AIC).
The model parameters yielded along with a statistical analysis of the fits of Model 2 to time-course cephalexin reaction data are shown in the Supplemental Information in Appendix Tables A.1 and A.2. Figure 2.7 shows that Models 2 and 3 describe the experimental reaction data more accurately than Model 1 throughout all concentration ranges tested. Akaike Information criterion (AIC) were calculated and used to compare between models and penalize models with a higher number of parameters [74, 75]. Additionally, Model 2 yielded a marginally improved fit over Model 3, as indicated by the lower values for the sums-of-squared errors (SSE) and AIC. This suggests that PGA may not be able to facilitate a binding event of 7-ADCA that is separate from PGME binding, to proceed down the synthetic pathway.

2.3.3 Pareto optimization for homogenous continuously stirred reactor for cephalexin synthesis

The use of the model for the design of enzymatic reactors was examined by completing simulations for a single CSTR with continuous 7-ADCA and PGME feeds. Pareto optimal fronts, shown in Figure 2.8, were generated to concurrently optimize either fractional yield (moles of cephalexin produced per mole of PGME consumed) or PGME conversion (moles of PGME consumed per mole in feed) with productivity (mass cephalexin produced per unit volume of reactor solution per unit time).
Figure 2.8: Pareto optimal fronts generated for the operation of a CSTR for the synthesis of cephalexin. (A) Fractional yield, defined as the fraction of PGME consumed which results in cephalexin, versus productivity for various PGME conversions. The bold curves show how the extremes of each pareto front change with respect to productivity and fractional yield. (B) PGME conversion versus productivity for various 7-ADCA conversions. $C_{PGA} = 5 \, \mu M$, $T = 25^\circ C$, $pH = 7.0$

Shown in Panel A of Figure 2.8, a tradeoff exists between productivity and fractional yield. For a given PGME conversion, as productivity is increased, fractional yield must decrease. Additionally, as PGME conversion is decreased, both productivity and fractional yield are increased, likely due to the increased PGME concentration within the reactor yielding greater synthetic rates and performance. Shifting focus to Panel B of Figure 2.8, as 7-ADCA conversion increases, the productivity at a given PGME conversion is greater.
The binding of PGME to PGA improved for the βF24A variant and Assemblase® over the wild-type, as indicated by their lower $K_M$ values for PGME. On the other hand, it appears the βF24A variant and Assemblase® also possessed a higher affinity towards the binding of cephalexin than the wild-type. The $\alpha$ value for the wild-type was roughly half of the value for the βF24A variant as well as Assemblase®, suggesting that the wild-type more preferentially binds and forms an acyl-enzyme complex with the substrate PGME rather than cephalexin in comparison to the two variants. These results differ from those reported in literature [76], where the $K_M$ value of cephalexin hydrolysis found for the βF24A variant was roughly 2x higher $K_M$ and the value of PGME hydrolysis was roughly 30% lower than the wild-type. These findings suggest that the βF24A mutation may not offer the preferential binding of the substrate as previously indicated for cephalexin synthesis.

As indicated by Figure 2.3, the selectivity of cephalexin synthesis increased for all variants as 7-ADCA concentration increased due to the greater likelihood of a nucleophilic attack occurring by 7-ADCA rather than water at higher 7-ADCA concentrations. For the conditions evaluated, it appears that all variants exhibited similar selectivity of cephalexin synthesis at lower 7-ADCA concentrations, as indicated by the similar $\beta_0$ values yielded. The outlier among variants appeared to be βF24A, which exhibited slightly higher selectivity at higher 7-ADCA concentrations than the wild-type and Assemblase®, as indicated by a lower $\gamma$ value. While it appears that the βF24A mutation results in deleterious effects with regards to the binding and reaction of substrate versus product, it is possible that upon binding of PGME, the acyl-enzyme complex is in a position allowing for the more efficient nucleophilic attack of 7-ADCA in comparison to the wild-type. It
has been demonstrated that the removal of the hydrophobic phenylalanine in position βF24 induces a conformational change in the binding mode of phenylacetic acid (PAA), a molecule of similar structural characteristics to PGME [14]. As 7-ADCA is more readily bound to the enzyme at higher concentrations, the effects of this improved conformational change could be more pronounced at higher 7-ADCA concentrations as well. This hypothesis could not be evaluated simply by obtaining the crystal structure of PGME bound PGA for both variants as its binding, reaction, and release occur too quickly to obtain a reliable crystal structure.

While it is understood that increased concentrations of 7-ADCA increases the selectivity of cephalexin synthesis and decreases the rate of cephalexin hydrolysis, it is likely that an increase in both the PGME and 7-ADCA concentrations would lead to two competing effects: 1) An increase in phenylglycine formation due to the increase in activated acyl-donor bound to the enzyme and 2) an increase in synthesis to hydrolysis for those bound activated acyl-donor molecules due to the presence of higher 7-ADCA concentrations. The data in Figure 4 suggests that the impact of increasing 7-ADCA concentration outweighs the impact of increasing PGME concentration on the initial-rate production of phenylglycine, which suggests that for cephalexin synthesis, substrate concentrations should be maintained at higher levels to ensure high selectivity of the enzyme and low byproduct formation. The increase in selectivity with overall increase in substrate concentrations may be exploited to achieve improved synthetic performance in scenarios where high substrate concentrations are required, as indicated in the studies published by [77] and [78] in aqueous solution-precipitate systems for the synthesis of ampicillin. However, the enzymatic synthesis of ampicillin uses 6-APA which is a less
potent nucleophile than 7-ADCA [72], though, 7-ADCA possesses a lower solubility than 6-APA in an aqueous environment [67]. For example, in the reactive crystallization of cephalexin, high substrate concentrations are required to generate enough supersaturation to induce nucleation and growth of cephalexin monohydrate crystals. Additionally, a reactor designed to optimize for selectivity should promote higher substrate concentrations.

Since the selectivity at the lowest PGME concentration tested was higher at greater 7-ADCA concentrations, Figure 2.3(B) suggests that as the PGME concentration approaches the $K_M$ value of PGME, it is possible that a significant reduction in the total fraction of acyl-enzyme complex present allows for the separate binding of 7-ADCA to the active site of the enzyme, leading to a larger fraction of substrate proceeding down the synthetic pathway. It has been suggested in previous works that the nucleophile may bind prior to the formation of the acyl-enzyme complex [79], although the ability of 7-ADCA to bind prior to the formation of the acyl-enzyme complex has yet to be thoroughly investigated. In comparison to 6-APA, it has been shown that 6-APA competitively inhibits ampicillin hydrolysis by PGA with a $K_I$ value of 8 mM [57], a lesser degree than seen for 7-ADCA here.

When comparing maximum yields predicted from initial-rate parameters derived from Model 1 with experimentally measured yields, significant differences were observed. Improved fits to time-course reaction data by Models 2 and 3 indicate that this was possibly due to the assumption of Model 1 that 7-ADCA may not bind to the free enzyme, which was shown in Figure 2.3 to be a limiting assumption. The use of initial-rate instead of time-course reaction data to estimate model parameters of a complex reaction network may have also contributed to inaccuracies in the fit of initial-rate model parameters and their
application in predicting maximum yields. Therefore, the use of initial-rate model parameters to describe the kinetics of cephalexin synthesis by PGA is inaccurate in comparing between variants as the predictions based on these parameters are inaccurate. In fact, a fit to Model 2 using the initial-rate parameters found in Table 2.1 resulted in an SSE an order of magnitude greater than the fit incorporating solely time-course data. Indeed, it is difficult to estimate all the parameters accurately and independently in Model 2 even using time-course data, due to their correlation in the model predictions. These correlations are illustrated in Appendix Table 2.2.

When testing the applicability of Model 2 to the simulation of a CSTR for cephalexin synthesis, it is possible to make a few observations. Greater productivity can be achieved at higher 7-ADCA conversions for a CSTR due to decreased inhibition at higher 7-ADCA conversions, as the reactor is operating at outlet conditions and therefore lower 7-ADCA concentrations. Additionally, at higher 7-ADCA conversions, the attainable region of PGME conversion is decreased, likely due to the constraint that phenylglycine, the byproduct of PGME and cephalexin hydrolysis, may not reach concentrations higher than 50 mmol/L. Since higher 7-ADCA conversions result in lower concentrations experienced within the reactor, the selectivity of cephalexin synthesis within the reactor also suffers, resulting in lower PGME turnover due to excessive byproduct formation. Model 2 may be applied to more complex reactor geometries, as it is likely that at some point 7-ADCA inhibition may play a lesser role in cephalexin synthesis, and a tubular reactor in series downstream from a CSTR may be more beneficial for improved productivity and product yield due to the improved selectivity observed in the presence of higher 7-ADCA concentrations.
2.4 Conclusions

It was determined that a model developed by Youshko and Svedas, which only allowed for 7-ADCA binding following formation of the acyl-enzyme complex, does not fully describe the synthesis of cephalexin under elevated substrate concentrations. Three models for cephalexin synthesis were fit to time-course reaction data, with the best model found to be the model by Youshko and Svedas combined with competitive inhibition due to 7-ADCA. This finding aligned with the observation of 7-ADCA inhibition of both cephalexin hydrolysis and synthesis under initial-rate conditions. Additionally, the βF24A variant and Assemblase® did not exhibit significantly improve synthesis of cephalexin or amoxicillin over than the wild-type enzyme at the concentrations evaluated, indicating the wild-type enzyme may be sufficient for the application of PGA in processes involving higher substrate concentrations. Simulations of a CSTR operation for cephalexin synthesis were performed using the new model to determine optimal regions for PGME conversion, fractional yield, and productivity. A tradeoff between conversion and fractional yield with productivity was observed, likely due to the interplay between 7-ADCA consumption, inhibition, and selectivity. A process with a single well-mixed reactor was also simulated including instantaneous crystallization of cephalexin, which was found to improve the optimal regions attainable due to lower rates of secondary hydrolysis.
CHAPTER 3. Amoxicillin trihydrate solubility and crystallization in presence of cosolutes

3.1 Introduction

The solubility of the desired product and its synthesis precursors/byproducts in a mixture largely dictates the design of an efficient crystallization system. When applied to reactive crystallization (RC), the crystallization must be conducted under the same conditions as the reaction, placing a design constraint on the system [80]. For example, if the reaction only proceeds at higher temperatures or pH values that increase the solubility of the desired product, the use of RC may not be warranted [81]. In the case of amoxicillin trihydrate, the solubility is lowest (7 - 8 mmol/L) at neutral pH values while the solubilities of other components in the system (6-APA, 4-HPGME, 4-HPG) are generally at least two orders of magnitude higher [82-85]. Thus, crystallization of the product could be achieved at neutral to slightly acidic pH values where PGA yields modest activity, while higher pH values yield improved activity, but with a tradeoff in synthesis selectivity due to lower nucleophilicity of the beta-lactam nucleus [57]. Additionally, the Michaelis constant (discussed in Chapter 2) for amoxicillin on PGA is 2-3 mmol/L, while for the acyl-donor 4-HPGME it is 10-20 mmol/L, indicating a specificity of the enzyme to binding and hydrolyzing amoxicillin when both species are present in similar quantities in the liquid phase, thus being a driving force to implement strategies to reduce product hydrolysis. All these characteristics make the system for enzymatically synthesizing amoxicillin trihydrate with PGA a viable candidate for RC; on the other hand, the addition of crystallization during the synthesis of amoxicillin significantly increases the complexity of the overall
There are obvious tradeoffs when selecting conditions such as pH value. To address this increased complexity, models for the solubility and crystallization of amoxicillin trihydrate could guide process design. As other synthesis components are present in high concentrations, investigation of any effect of other components on the solubility and crystallization of amoxicillin is required. Additionally, investigation of the solubility of other synthesis components is also warranted to ensure that a process is not operated at conditions that would promote the crystallization of additional components that would contaminate the product slurry.

In this chapter, we investigated the solubility of amoxicillin trihydrate in an aqueous environment as a function of pH, temperature, cosolute concentration, and ionic strength. Additionally, we studied the effect of pH value on the solubility of 4-HPGME and 4-HPG (6-APA solubility versus pH has been reported numerous times in literature [83, 86, 87]). Next, we investigated the primary and secondary nucleation of amoxicillin trihydrate in water and examined the impact of cosolutes on crystallization. An emphasis was placed on studying secondary nucleation, as it has been shown to dominate in self-seeded systems such as mixed-suspension mixed-product-removal (MSMPR) style crystallizers [88, 89]. The crystallization was modelled using classical nucleation and growth kinetics and solved using the method of moments [90].

### 3.2 Materials and Methods

#### Materials
Amoxicillin trihydrate (≥99%) and 6-aminopenicillanic acid (6-APA, ≥99%) was acquired from RIA (Hanover, NJ); 2-(4-hydroxyphenyl)-D-glycine methyl ester hydrochloride (4-D-HPGME, ≥95%) from Alfa Aesar (Willard Hill, MA); 2-(4-hydroxyphenyl)-D-glycine (≥99%) from TCI America (Portland, OR); HPLC grade methanol from Fischer Scientific (Waltham, MA).

Experiments concerning Raman and ATR-FTIR were conducted at constant temperature (T=25°C) in a 250 mL vessel in a Mettler Toledo (Columbus, OH) Optimax system, stirred at 300 RPM to ensure it was well-mixed. Raman spectra were measured using a Mettler Toledo ReactRaman system with a 785 nm laser at laser power of 300 mW. The exposure time was varied from 5 s to 10 s based on the concentration of components measured and whether or not a solid phase was present. IR spectra were recorded using a Mettler Toledo ReactIR 10 system. A Mettler Toledo FBRM was used to monitor the solid phase. A pH electrode (InLab Semi-Micro-L) from Mettler Toledo was used to monitor the pH value of the solution.

Methods

Solubility measurement

To investigate component solubility, a Metrohm 902 Titrando pH titration system was used with a 100 mL jacketed batch vessel attached to a pH probe, dosing pump, and pitched blade impeller. The vessel was maintained at 25°C (unless otherwise specified) and held 30 mL of solution. The probe, dosing device, and impeller were controlled and monitored using Metrohm Tiamo software. The pH probe was calibrated before each
experiment. The solution in the vessel was well mixed for the duration of the experiments and samples were collected though an opening in the vessel.

To create a pH versus solubility curve for a specific component, a saturated solution was prepared by adding enough of the component to deionized water that undissolved particles were visible. 1 mol/L NaOH was used as the base and 1 mol/L HCl was used as the acid. Some components required a base to maintain the pH value as they dissolved (e.g., 6-APA, amoxicillin, 4-HPG), while some required acid (e.g. 4-HPGME). By adding base or acid in 1-100 µL quantities using a dosing device, the pH value was changed in increments of 0.2 to 0.5 units. If no solid particles were visible (i.e., the solution ‘ran clear’), more of the component was added to the reactor to maintain saturation. The solution was allowed to equilibrate for 15 minutes after each pH change and samples were collected in triplicate and analyzed using HPLC.

To measure the effect of component concentration on amoxicillin solubility, the components were dissolved in a saturated amoxicillin solution. The concentration of the component was increased by 25 to 50 mmol/L each time, and a 15-minute period was allowed between the time the desired pH was met before a sample was taken. Usually, a change in pH was an indication of a component dissolving as they all have ionizable groups and either donate or accept a proton upon dissolution and ionization. Amoxicillin saturation was maintained by ensuring that suspended particles were visible in the solution. At each component concentration, triplicate samples were collected and analyzed using HPLC.

A similar procedure was applied to investigate amoxicillin solubility in the presence of both 6-APA and 4-HPGME. 4-HPGME concentration was varied at levels of 0, 10, 25,
50, 75, and 100 mmol/L while 6-APA was added at 0, 50, 75, 100, and 150 mmol/L quantities at each 4-HPGME concentration. This yielded a total of 30 data points, each with HPLC samples collected in triplicate. The supersaturation of 4-HPGME for the 100 mmol/L data set was achieved by dissolving it at a low pH and subsequently increasing the pH value.

Across all experiments, the collected samples were centrifuged, and 50 µL of the supernatant was added to a known volume of 50 mmol/L sodium phosphate, pH 7.0 buffer solution. To ensure accurate HPLC readings, the collected solution was diluted with a known volume of buffer solution such that no component had a concentration over 5 mmol/L, which is the upper limit of the HPLC calibration curve. The diluted samples were mixed using an agitator and centrifuged. The concentrations of amoxicillin, 4-HPG, 4-HPGME, and 6-APA in the sample were then analyzed using HPLC.

Spectroscopy

For the measurement of spectra, the vessel was charged with 200 mL of deionized water. The temperature of the liquid phase was set to 25°C and was maintained automatically by the Optimax heating/cooling jacket. Solid components were added to the top of the vessel using a funnel. Dissolution of amoxicillin and 6-APA was promoted using 2 mol/L NaOH and dissolution of 4-HPGME was promoted using 2 mol/L HCl. For all experiments, a pH value of 6.50±0.05 was reached before Raman and IR determination and the pH was lowered using 2 mol/L HCl and raised using 2 mol/L NaOH. For homogenous samples, complete dissolution of solute(s) was confirmed with FBRM (<50 total counts).
Amoxicillin crystallization

Primary nucleation is the formation of crystals in a clear supersaturation solution and was investigated using Crystal16 to determine the impact of supersaturation on induction time [91]. Multiple 10 mL samples of amoxicillin dissolved in 100 mmol/L sodium phosphate buffer at different concentrations and were incubated at 25°C for about 15 minutes in a water bath and stirred using a magnetic stir bar at 500 RPM. Immediately before loading into 1 mL vials for Crystal16, the pH of the solution was decreased to a desired value to generate supersaturation for primary nucleation. Primary nucleation was detected by a decrease in transmission from the initial value of 100%, with the time required for the onset of transmission reduction considered as the induction time.

Batch crystallization experiments were conducted in a 500 mL jacketed vessel and monitored using FBRM, PVM, and offline microscopy to monitor the solid phase, pH probe and thermocouple to monitor and control liquid phase conditions, and offline HPLC to measure the liquid phase concentration of amoxicillin. The HPLC analysis protocol was the same described in Chapter 2 for the analysis of amoxicillin reaction solutions. To prepare for a crystallization experiment, a known amount of amoxicillin trihydrate (RIA, NJ) was dissolved in 300 mL deionized water and the pH was increased to increase amoxicillin solubility using 2 mol/L NaOH. After the amoxicillin was dissolved, the solution was filtered using a grade 1 Whatman filter and sampled for HPLC analysis as the initial point. The filtrate was added to the vessel, stirring was initiated at 300 RPM, and the
temperature control was set to 25°C using the thermocouple connected to iControl software. Time was allowed to reach the desired temperature. For unseeded crystallization experiments, the pH value was lowered to a set value to drive supersaturation, via manual 2 mol/L HCl addition. For seeded crystallization experiments, the pH value was lowered to an intermediate value to produce a slightly supersaturated solution (to avoid seed dissolution), a known mass of seeds were added, and then the pH value was immediately lowered to the final value. Liquid phase samples were collected by withdrawing 1 mL of slurry, filtering using a 0.2 μm filter, and diluting the filtrate in 50 mmol/L sodium phosphate buffer pH 7.0. The crystal size distribution (CSD) of the seeds and final slurry was measured via offline microscopy and was approximated by a log-normal distribution.

3.3 Determination of solubility of amoxicillin system components

3.3.1 Effect of pH, temperature, and ionic strength

The effect of temperature and pH on the solubility of amoxicillin and the effect of pH on the solubility of 4-HPGME and 4-HPG were examined (Figure 3.1).
Figure 3.1: (A) Effect of temperature on the solubility of amoxicillin at pH = 6.5; (B) Effect of pH value on the solubility of amoxicillin at 25°C; (C) Effect of pH value on the solubility of 4-HPGME at 25°C; (D) Effect of pH value on the solubility 4-HPG at 25°C. The solid line in Panel (A) is the fit to Equation (3-1). The solid lines on Panels (B-D) are fits to Equation (3-2). Error bars are one standard deviation over three measurements.
The solubility of amoxicillin increased with increasing temperature and the slope of the curve increased with increasing temperature, indicative of behaviour corresponding to the Apelbat equation [92]:

\[
\ln x_i = A_i + \frac{B_i}{T} + C_i \ln T
\]  

(3-1)

where \(x_i\) is the mole fraction of solute \(i\) in a specific solvent and \(T\) is the temperature in Kelvin. The constants \(A_i, B_i, C_i\) are fit to the data for each solute-solvent combination and are dependent on thermodynamic parameters such as the enthalpy, Gibbs free energy, and entropy of solution as well as the activity of the solvent. The mole fraction was approximated by dividing the solubility \(c^*\) of amoxicillin (in mol/L) by the molarity of pure water (55.56 mol/L), which is only justified when \(x_i \ll 1\). Equation (3-1) yielded a good fit to the data in Figure 3.1(A). The inclusion of the \(C_i\) term was critical, with a fit to Equation (3-1) with the \(C_i\) term removed (terms the “Vant Hoff equation”) shown in Appendix A Figure A.8. The model parameters for the Apelbat equation are found in Table 3.1.
Table 3.1: Parameters of Apelbat equation fit to the data in Figure 3.1(A) by least-squares regression for the temperature dependence of amoxicillin solubility in water.

<table>
<thead>
<tr>
<th>Parameter (units)</th>
<th>$A_{\text{Amoxicillin}}$ (–)</th>
<th>$B_{\text{Amoxicillin}}$ (K)</th>
<th>$C_{\text{Amoxicillin}}$ (–)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Value</td>
<td>-4.57x10^2±0.79x10^2</td>
<td>1.85x10^4±0.28x10^4</td>
<td>6.78x10^1±1.1x10^1</td>
</tr>
</tbody>
</table>

While, in the context of amoxicillin RC, it would be preferable to operate a crystallizer at lower temperatures to improve crystallization yield by lowering amoxicillin solubility, the activity of the biocatalyst, PGA, is also sensitive to temperature and must be accounted for.

Figure 3.1(A-C) indicate that amoxicillin and 4-HPG exhibit increasing solubility with increasing pH value, while 4-HPGME exhibits the opposite effect. Additionally, a change in solubility with pH value with similar compounds has been shown to be described well by the Pitzer equation [93]

$$c_j^* = c_{pl,j}^* \left(1 + \frac{10^{-pK_{a,j}}}{10^{-pH}}\right)$$  \hspace{1cm} (3-2)

where $c_j^*$ is the solubility of component $j$, $c_{pl,j}^*$ the solubility at its isoelectric point, and $pK_{a,j}$ the acid dissociation constant of the sensitive functional group in a specific pH range. The data for all components investigated fit Equation (3-2) well, with estimated acid dissociation constant parameters shown in Table 3.2.
Table 3.2: Summary of parameters used to model the solubility of amoxicillin, 4-HPGME, 4-HPG, and 6-APA. Model parameters for amoxicillin, 4-HPGME, and 4-HPG are from data in Figure 3.1(B-D) fit to Equation (3-2). The fraction in Equation (3-2) is inverted in the case of 4-HPGME solubility. \(^a\)From [57]. \(^b\)From [78].

<table>
<thead>
<tr>
<th>Component</th>
<th>(c_{pl}^*) (mmol/L)</th>
<th>(pK_a)</th>
<th>(pK_{a,\text{reported}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>6.5±0.1</td>
<td>7.40±0.10</td>
<td>7.48 [82]</td>
</tr>
<tr>
<td>4-HPGME</td>
<td>14.5±0.3</td>
<td>6.99±0.08</td>
<td>-</td>
</tr>
<tr>
<td>4-HPG</td>
<td>109.5±2.7</td>
<td>8.86±0.23</td>
<td>8.98 [94]</td>
</tr>
<tr>
<td>6-APA</td>
<td>20.0(^a)</td>
<td>4.83(^b)</td>
<td>4.83(^b)</td>
</tr>
</tbody>
</table>

According to Figure 3.1, 4-HPG exhibits the highest minimum solubility, which is preferable in the case of amoxicillin RC as the system pH may be varied significantly without worry of developing significant supersaturation of its byproduct by pH-swing. 6-APA has the lowest \(pK_a\), which results in a much higher solubility than other components at neutral pH values, where an enzymatic RC process would likely be operated. The minimum solubility of 4-HPGME of 14 mmol/L for 4-HPGME and its opposite trend with pH value compared to all other amoxicillin synthesis components indicate that at neutral
pH values, the solubility 4-HPGME could potentially be a limiting factor. Due to this limitation, it is possible that slightly acidic pH values may be preferable operating conditions as these conditions yield acceptable PGA activity but retain modest 4-HPGME solubility.

3.3.2 Effect of cosolutes

As shown in similar systems, cosolutes may impact the solubilities of each other due to solute-solute interactions [95]. Here the individual effect of each cosolute (6-APA, 4-HPGME, 4-HPG) on the solubility of amoxicillin was investigated (Figure 3.2).

![Figure 3.2: (A) 6-APA, 4-HPGME, and 4-HPG concentration versus amoxicillin solubility. Error bars are one standard deviation from three sample measurements. (B) Ionic strength due to ionized 4-HPGME, 6-APA, and NaCl versus amoxicillin solubility.](image-url)
solubility, calculated using the Henderson-Hasselbalch equation and Equation (3-3).

\[ \text{pH} = 6.5, \ T = 25^\circ C. \] Solid lines are to guide the eye.

Out of all three components, the solubility of amoxicillin was most significantly impacted by 6-APA, followed by 4-HPGME, and 4-HPG having the least effect (Figure 3.2(A)). The ratio of ionized to non-ionized species at a pH value of 6.5 for each component are 47, 0.32, and 4.0x10^{-3}, implying that the more ionized cosolutes more significantly impact the solubility of amoxicillin. The ionic strength for the data in Figure 3.2(B) was calculated using the Henderson-Hasselbalch equation

\[ \text{pH} = pK_{a,j} + \log_{10} \frac{A^-}{HA} \]  

where \( HA \) is the acid and \( A^- \) is the conjugate base and ionized species and Equation 3-4 below to account for all ionized species in solution (including sodium and chloride ions due to acid or base added):

\[ I = \frac{1}{2} \sum_{i=1}^{n} c_i z_i^2 \]  

where \( I \) represents the ionic strength in solution, \( c_i \) and \( z_i \) the molar concentration and charge number of ions of species \( i \). Sodium chloride was used as a positive control for ionic strength. The data in Figure 3.2(B) indicate that while there is a correlation between increasing amoxicillin solubility with increasing ionic strength for 6-APA and 4-HPGME,
ionic strength alone does not explain the cause of the elevated solubility due to the minimal impact that high concentrations (up to 0.5 mol/L) of sodium chloride had on amoxicillin solubility. It is possible that the ionized functional groups of the more ionizable species (6-APA, 4-HPGME) interact preferentially with amoxicillin in water, stabilizing amoxicillin in the liquid phase and increasing its observed solubility. Ultimately, the presence of 6-APA and 4-HPGME appear to impact the solubility of amoxicillin due to component specific interactions. As both are present in the case of the RC of amoxicillin, the solubility of amoxicillin in the presence of a range of concentrations of both 6-APA and 4-HPGME were measured (Figure 3.3).

Figure 3.3: Amoxicillin solubility in various mixtures of 4-HPGME and 6-APA.

Each curve corresponds to a single concentration of 6-APA, from left to right higher concentrations.
concentrations of 4-HPGME. pH = 6.5, T = 25°C. The lines between data points are present to guide the eye.

Clearly, the impact of both 6-APA and 4-HPGME on amoxicillin solubility is accumulative, although the impact of 4-HPGME appears to saturate somewhere between 0 and 25 mmol/L, whereas 6-APA continues to increase the solubility of amoxicillin even up to 150 mmol/L 6-APA. The data in Figure 3.3 indicate that higher concentrations of 6-APA, while they have been shown to improve the selectivity of amoxicillin synthesis, would result in higher concentration of amoxicillin present in the liquid phase, and in turn higher rates of secondary hydrolysis. For example, RC conditions discussed later in Chapter 5 (100 mmol/L 6-APA, 80 mmol/L 4-HPGME) result in an effective solubility of amoxicillin of 14 mmol/L, roughly double its pure component solubility. Significantly enhanced solubility of product must be accounted for in the case of RC, particularly when isolating a reaction intermediate, as the rates of product hydrolysis will be enhanced.

3.3.3 Raman spectroscopy to investigate aqueous phase cosolute interactions

Raman spectroscopy provides detailed information about the vibration and motion of intramolecular bonds but has also been shown to sometimes be a viable technique for the detection of intermolecular interactions. Raman spectroscopy has been used to explain interactions between 7-ADCA and PGME which allowed a large metastable zone width for 7-ADCA crystallization [95]. In situ Raman spectroscopy was used to measure FT-Raman data of solutions of amoxicillin, 6-APA, 4-HPGME, as well as mixtures of
amoxicillin and 4-HPGME and mixtures of amoxicillin and 6-APA, in an effort to probe liquid phase interactions that may explain the elevation of amoxicillin’s aqueous solubility. The data in Figure 3.4 exhibit the development of a shoulder on the left side of the 852 cm$^{-1}$ peak, which has been reported to correspond to aromatic ring breathing for amoxicillin’s phenyl ring [96].
Figure 3.4: *In situ* Raman spectra of water, amoxicillin, 4-HPGME, and a mixture of 4-HPGME and amoxicillin, all dissolved in water. (A) Full size spectra; (B)
Spectra focused on peak at 852 cm\(^{-1}\). pH = 6.5, T = 25°C, V = 200 mL. \(c_{\text{Amoxicillin}} = 30\) mmol/L, \(c_{\text{4-HPGME}} = 30\) mmol/L.

The development of a shoulder on the 852 cm\(^{-1}\) peak may imply that π-π aromatic ring stacking occurs between amoxicillin and 4-HPGME, which is plausible due to both compounds containing a phenyl ring in their structure. Aromatic ring-stacking interactions which stabilize amoxicillin in solution could partially explain the elevated solubility of amoxicillin in the presence of 4-HPGME.

3.4 \textbf{Amoxicillin crystallization}

3.4.1 \textit{Primary nucleation}

Crystal16 was used to determine induction time as a function of amoxicillin supersaturation in water (Figure 3.5).
Figure 3.5: Primary nucleation studies for amoxicillin. (A) Working principle for Crystal16, from [97]; (B) Example of output data from Crystal16, laser transmission over time for various starting supersaturation ratios; (C) Induction time versus supersaturation; (D) \( \ln t_{ind} \) versus \( \ln S)^{-2} \). Line is data fit to Equation (3-5). T = 25°C. V = 1 mL.

Figure 3.5(A) demonstrates the working principle for Crystal16. Upon primary nucleation of amoxicillin, normalized transmission of a laser is decreased. For every sample, the time in which the initial onset of transmission reduction was indicated as the induction time,
As expected, as the supersaturation ratio increased, the induction time decreased as the metastable limit was approached [98] (Figure 3.5(C)). At constant temperature, induction time and supersaturation ratio have been shown to be related by the equation

$$\ln t_{ind} = A + B_0 (\ln S)^{-2} \quad (3-5)$$

where $A$ is the pre-exponential factor and $B_0$ is the primary nucleation constant [99, 100] based on classical nucleation theory. The supersaturation was calculated using the equation

$$S = \frac{c_{AMX}}{c_{AMX}^*(T, pH, c_{6-APA}, c_{4-HPGME})} \quad (3-6)$$

where $c_{AMX}$ is the concentration of amoxicillin and $c_{AMX}^*$ is the solubility of amoxicillin, which has been already shown to be a function of temperature, pH, and 6-APA and 4-HPGME concentrations. The fit of the data in Figure 3.5(C) to Equation (3-5) is shown in Figure 3.5(D), with $B_0 = 0.674$ and $A = -0.210$. The value of $B_0$ for ampicillin, which has a very similar structure and crystallization behavior, as amoxicillin, was found to be 1.27 in a separate study [55, 99], which is the same order of magnitude as the value obtained in this study. Additionally, the impact of 6-APA on the induction time of amoxicillin was examined and compared to similar supersaturation in pure water, and no difference was observed when the change in solubility due to the presence of 6-APA was accounted for when calculating supersaturation. Although the goal of this study was to focus on secondary growth kinetics as well as growth kinetics, knowledge of primary nucleation parameters simplified the overall fitting procedure later for all other crystallization parameters.
3.4.2 Modelling amoxicillin crystallization with and without presence of precursors

Batch seeded crystallization experiments were conducted with amoxicillin trihydrate in water, with the goal of developing a model to describe the crystallization of amoxicillin applicable to a RC process (Figure 3.6).
Figure 3.6: Batch crystallization of amoxicillin in water. (A) Concentration of amoxicillin in the liquid phase over time, with and without 100 mmol/L 6-APA present; (B) Examples of CSDs measured at end of crystallization via offline microscopy. Distinct CSDs are differentiated by color. Light blue and royal blue
CSDs were in the presence of 100 mmol/L 6-APA and red and green CSDs were in pure water. $T = 25^\circ\text{C}$. $pH_{\text{final}} = 6.5$. Supersaturation calculated in the presence of 6-APA was estimated using the solubility of amoxicillin measured in presence of 100 mmol/L 6-APA (11.3 mmol/L).

Figure 3.6(A) shows that by including 6-APA, the rate of depletion of amoxicillin from the liquid phase was similar to amoxicillin in water. Additionally, based on the relative amount of total supersaturation generated, the CSDs varied by a modest amount Figure 3.6(B). The rates of primary nucleation $B_1$ and secondary nucleation $B_2$ may be described as

$$B_1 = k_{B_1} \exp\left[\frac{-B_0}{\ln^2(S)}\right]$$

(3-7)

$$B_2 = k_{B_2} M_T^m (S - 1)^b$$

(3-8)

where $k_{B_1}$ is the primary nucleation rate constant, related to the pre-exponential factor $A$ mentioned earlier, $k_{B_2}$ is the secondary nucleation rate constant, $M_T$ is the slurry density, $m$ and $b$ are the slurry density and secondary nucleation exponents, respectively. Crystal growth $G$ based on classical growth kinetics may be described by the equation

$$G = k_G (S - 1)^g$$

(3-9)

where $k_G$ is the crystal growth rate constant, and $g$ is the growth rate exponent. The rates of primary and secondary nucleation are combined to yield the total nucleation (Equation (3-10)).
\[ B = B_1 + B_2 \tag{3-10} \]

The evolution of the CSD for needle-like crystals may be modelled using a one-dimensional population balance model simplified by the method of moments (Equations (3-11) through (3-14)).

\[
\frac{\partial (n(L,t)V(t))}{\partial t} + \frac{\partial (n(L,t)G(t)V(t))}{\partial L} = B(t)V(t) \tag{3-11}
\]

\[
\frac{d\mu_0(t)}{dt} = B(t)V(t) \tag{3-12}
\]

\[
\frac{d\mu_i(t)}{dt} = i\mu_{i-1}(t)G(t) \tag{3-13}
\]

\[
\frac{dc(t)}{dt} = -3\rho k_v G \mu_2 \tag{3-14}
\]

where \( n \) represents the crystal size distribution, \( L \) the crystal length, \( V \) the volume of slurry, \( \mu_i \) the \( i \)th moment of the CSD, \( \rho \) the crystal density, and \( k_v \) the crystal shape factor. Parameter bounds were constrained during parameter fitting to yield physically meaningful values and are shown in Table 3.3.
Table 3.3: Parameter bounds used in constrained parameter fitting for amoxicillin crystallization.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol</th>
<th>Lower bound</th>
<th>Upper bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth rate constant</td>
<td>$k_G$</td>
<td>0</td>
<td>$\infty$</td>
</tr>
<tr>
<td>Growth rate exponent</td>
<td>$g$</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Primary nucleation constant</td>
<td>$k_{B1}$</td>
<td>0</td>
<td>$\infty$</td>
</tr>
<tr>
<td>Secondary nucleation constant</td>
<td>$k_{B2}$</td>
<td>0</td>
<td>$\infty$</td>
</tr>
<tr>
<td>Secondary nucleation exponent</td>
<td>$b$</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Slurry density exponent</td>
<td>$m$</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

The growth rate exponent was constrained between 1 and 2 according to the crystal growth theory of Burton et. al. [101]. An example of data fit to the model described by Equations (3-6) through (3-9) and Equations (3-11) through (3-13) using the parameter constraints outlined in Table 3.3 are shown in Figure 3.7. Data were fit to the model using the MATLAB R2020a function “fmincon” by minimizing the logarithm of the sum squared error of the moments and liquid phase concentration with equal weighting for each.
Figure 3.7: Example of model fit to the training set of liquid phase amoxicillin concentrations during amoxicillin crystallization.

The model yielded a modest fit the data, regardless of the presence of 6-APA or not, as long as the change in solubility of amoxicillin was accounted for using the data in Section 3.3.2. Additionally, a representative fit of the model to an experimental CSD is shown in Appendix Figure A.7. A summary of parameters obtained from the model fitting procedure are shown in Table 3.4.
Table 3.4: Parameter values for amoxicillin crystallization data fit to the crystallization model detailed. Error is 95% confidence intervals.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol</th>
<th>Fit value</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth rate constant</td>
<td>$k_G$</td>
<td>$6.1 \pm 1.8$</td>
<td>$\mu m , min^{-1}$</td>
</tr>
<tr>
<td>Growth rate exponent</td>
<td>$g$</td>
<td>$2$</td>
<td>-</td>
</tr>
<tr>
<td>Primary nucleation constant</td>
<td>$k_{B1}$</td>
<td>$10.5 \pm 3.0$</td>
<td>$min^{-1} , L^{-1}$</td>
</tr>
<tr>
<td>Secondary nucleation constant</td>
<td>$k_{B2}$</td>
<td>$9 \times 10^5 \pm 2 \times 10^5$</td>
<td>$min^{-1} , L^{-1}$</td>
</tr>
<tr>
<td>Secondary nucleation exponent</td>
<td>$b$</td>
<td>$1$</td>
<td>-</td>
</tr>
<tr>
<td>Slurry density exponent</td>
<td>$m$</td>
<td>$0.20 \pm 0.04$</td>
<td>-</td>
</tr>
</tbody>
</table>

The resultant ratio of $k_{B2}$ to $k_{B1}$ indicates that the system is dominated by secondary nucleation, which is preferential in the case of a self-seeding system such as a mixed-suspension mixed-product-removal (MSMPR) crystallizer. Additionally, a value of 0.2 for the slurry density exponent $m$ indicates that secondary nucleation is not strongly driven by crystal-crystal collisions, rather crystal-impeller collisions [102].

3.5 Conclusions
The solubility and crystallization kinetics of amoxicillin in an aqueous environment were reported in this chapter. Additionally, we investigated the effect of temperature, pH value, and cosolutes involved in amoxicillin enzymatic synthesis on its solubility and the effect of 6-APA on its crystallization. It was found that the dependence of amoxicillin solubility on temperature is fit by the Apelblat equation. 6-APA most significantly increased the solubility of amoxicillin at higher cosolute concentrations, but 4-HPGME also caused a small increase in solubility which was also observed in the presence of with 6-APA. Liquid phase hydrogen bonding or aromatic ring stacking between 6-APA and amoxicillin may be the cause for the liquid phase stabilization observed in amoxicillin mixtures. Crystallization kinetics were only examined at 25°C, although processes involving reactive crystallization of amoxicillin in the presence of its precursors might be operated at room temperatures or higher to ensure acceptable biocatalyst activity. Primary nucleation of amoxicillin occurs slowly, until a supersaturation of 1.8 to 2.2 is reached, and was not impacted by the presence of 6-APA or 4-HPGME. The secondary nucleation of amoxicillin generated a much larger quantity of crystals, indicating that a self-seeding (e.g., MSMPR) crystallizer would be preferable to ensure sufficient growth surface area is generated.
CHAPTER 4. Development and characterization of size-based enzyme separation for enzyme retention and product withdrawal in a continuous reactive crystallizer

4.1 Introduction

Chapter 4 is based on the previously published study [71] in which Hossein Salami was a significant contributor. Patrick Harris contributed significantly to experiments involving immobilized enzymatic reaction, as well as the design of experiments involving separator efficiency.

For a long time, the chemical industry has benefited from the advantages of continuous processes, such as higher productivity, the ability to process higher concentration of reactants, relatively smaller reactor size, potentially smaller environmental footprint [40], and higher flexibility in operation and control [103, 104]. The pharmaceutical industry, on the other hand, lags in capitalizing on this technology and is mainly dominated by batch systems. One reason is the existence of an already well-developed knowledge and equipment base, which is “reinforced” by tight regulations and intense time pressure to introduce a new drug to the market. However, recently there has been a push to move towards using continuous processes in this industry [105, 106]. This shift is motivated by the listed advantages and is encouraged by pharmaceutical companies as well as regulatory agencies such as the United States Food and Drug Administration (FDA) [38, 107]. Changing from batch to continuous processes also provides an opportunity for implementation of Quality by Design and for introducing new process
observation and control technologies in the newly designed processes [107]. Crystallization is one of the most common processes in the pharmaceutical industry where it is primarily used for purification of small-molecule drugs and in the preparation of marketed drug forms with specific attributes, such as dissolution rate [108]. Considering that many small-molecule drugs are administered in crystal form, development of robust continuous crystallization processes is critical for exploiting the aforementioned benefits.

In some cases, combining the crystallization with the synthesis step into a Reactive Crystallization (RC) process may be useful. In general, there are two motivating factors to combine these two steps. The first and more obvious is the potential for process intensification. If possible, combining both reaction and crystallization steps into one unit operation may save capital and operating costs. Examples include cases where the synthesis reaction has fast kinetics under a wide range of conditions so that process variables can be set to optimize the crystallization kinetics. Fast reactions are common in the production of inorganic compounds such as Li$_2$CO$_3$ [51], or coupling and neutralization reactions for organic compounds [105, 109]. Many of the RC processes reported in the literature deal with such systems; examples include continuous reactive crystallization of ferrihydrite and sodium cefuroxime [50, 110, 111]. In many processes, however, conditions favorable to both reaction and crystallization might not exist. One can think of temperature, a strong handle on the kinetics of both steps, but usually with opposite effects. There are several reports in the literature where either different process times, or different parts of a single vessel [48], or multiple integrated vessels [112] are used to perform reaction and crystallization steps. Conditions at different stages are set to favor the corresponding step. While usually referred to as RC, we note that in these studies reaction and crystallization
steps do not occur simultaneously, but rather as separated steps integrated into an overall process.

The second motivating factor for combining reaction and crystallization is when one can improve the efficiency of the other. Examples include improving the enantiomeric excess, shifting the reaction equilibrium by simultaneous crystallization of the desired product, or controlling the rate of supersaturation generation [109]. It has been shown that \textit{in situ} crystallization of the antibiotic in enzymatic synthesis of beta-lactam antibiotics can lead to improvement in process yield and conversion [113, 114]. Figure 4.1 illustrates the synthesis of amoxicillin (AMX) using the biocatalyst Pen G Acylase (PGA), where \textit{in situ} crystallization helps protect the desired product from hydrolysis. A similar concept can be applied to produce other beta-lactam antibiotics such as cephalexin (CEX).
Figure 4.1: Enzymatic synthesis of beta-lactam antibiotics amoxicillin and cephalaxin catalyzed by PGA. 6-APA refers to 6-aminopenicillanic acid, 7-ADCA to 7-aminodesacetoxy-cephalosporanic acid, PGME to D-phenylglycine methyl ester, 4-HPGME to 4-hydroxy-D-phenylglycine methyl ester, and HPG to 4-hydroxy-D-phenylglycine. Crystallization can protect the antibiotic intermediate from the secondary hydrolysis reaction.

As mentioned, developing continuous RC processes for cases with fast reaction kinetics (e.g., ionic reactions, neutralization, etc.) is relatively straightforward, with crystallization demanding most of the attention. However, in many cases, particularly in the pharmaceutical industry, this task is complicated by challenges in the synthesis step. Synthesis of some products requires a complex cascade of reactions that might not be compressible into one continuous vessel, or the reaction might need a catalyst to proceed
with an acceptable rate (e.g., enzymatic synthesis of beta-lactam antibiotics shown in Figure 4.1) [115]. There are two possibilities for a continuous enzymatic process. The soluble enzyme can be continuously supplied to the system together with the reaction species to maintain a certain concentration; considering the biocatalyst value, this case is not expected to be economical. A more viable solution is to retain the biocatalyst in the process. Most methods to do this require immobilizing the biocatalyst on some type of organic or inorganic carrier. Use of packed bed reactors is common, where enzyme is immobilized on beads that are then packed to form the biocatalyst bed [116-119]. Alternative approaches include ultrafiltration membranes [120-122], hollow fiber membranes [123], or bi-phasic separation systems [124-126]. Unfortunately, most of these setups are not applicable to RC processes where the presence of a second solid type (i.e., crystals) complicates the process. A major challenge in adapting enzymatic reactive crystallization processes, such as that of Figure 4.1, to continuous manufacturing is establishing a separation strategy that allows for continuous, selective, and isokinetic removal of product crystals (i.e., when solids density and size distribution of the withdrawn sample closely match those of the crystallizer) from the RC vessel while maintaining (or recycling) the biocatalyst.

Solutions for the above separation problem range from size-based methods to those that use an external force field such as gravity. Separation based on gravity is only feasible if the two solids have significantly different densities and settling velocities. Microfluidics-based solid-solid separation devices are also introduced for several applications [127], but the extreme tendency to clogging and low throughput limits their application for large-scale processes.
Size-based methods for separation are the simplest in their working principle but require a difference between the size of crystals and that of the biocatalyst carrier. Note that a key design goal for our separation process is that we desire a near-perfect retention of the biocatalyst in the vessel, but not a full recovery of all crystals and some may be retained in the vessel for further growth. Considering that there are a variety of commercially available organic beads for biocatalyst immobilization, there are two possibilities to separate the two solid types based on their size: (1) choose a carrier size smaller than the crystal size \( L_{\text{carrier}} < L_{\text{crystal}} \), and (2) choose a relatively large carrier such that \( L_{\text{crystal}} < L_{\text{carrier}} \). In the first option, a sieve with appropriate size can be used to separate the large crystals and recycle the beads to the RC vessel. The main issue with this approach is that it is almost certain that a significant number of beads will be trapped in the crystal cake during sieving, which not only leads to loss of biocatalyst but also significantly contaminates the crystal product. In the second approach large beads are maintained on top of the sieve and can be scrubbed off and recycled back to the RC vessel (along with some entrapped crystals). If the right combination of \( L_{\text{carrier}} \) and \( L_{\text{filter}} \) is used this method can provide a high biocatalyst recovery. However, performing the separation step downstream of the RC vessel adds a non-continuous element to the otherwise continuous process that is our goal. Furthermore, biocatalyst carriers might be severely damaged while being scrubbed off the sieve to be recycled.

To avoid the issues in the preceding paragraph, one can move the filter to the RC vessel to perform the separation. Figure 4.2(A) provides a schematic representation of the proposed separation strategy. This separation method offers several advantages, including the possibility of a truly continuous process, no need for scrubbing the beads off a sieve
and possible damages, flexibility in the separator design from choosing the filter geometry to filter size, and a large and three-dimensional filter area that minimizes the potential of clogging. However, the requirement of $L_{\text{crystal}} < L_{\text{filter}} < L_{\text{carrier}}$ leads to two issues. First, the size of the carrier might impact the activity of the immobilized biocatalyst, i.e., effectiveness factor, through introducing mass transport limitations [128, 129]. Second, the morphology of the growing crystals might produce long needle-like particles, with a length greater than the carrier size, opposing the set separation criterion. Indeed, this is the case for amoxicillin trihydrate crystals that typically grow as long, 1D needle-like particles. Figure 4.2(B) shows optical images of an AMX slurry produced in a continuous crystallization process. As can be seen, some particles grow to be as long as 800 μm, making it very challenging to choose an immobilization carrier that satisfies $L_{\text{crystal}} < L_{\text{carrier}}$ without a major blow to the biocatalyst effectiveness.

Figure 4.2: (A) Schematic representation of the carrier-crystal separation method proposed for continuous reactive crystallization using immobilized biocatalyst in a Mixed Suspension Mixed Product Removal Reactor (MSMPR) vessel. Inlet to the
mill is withdrawn from the filter to avoid milling the carrier beads. Listed values correspond to those used in pH-induced continuous crystallization experiments (with no beads present) with intermittent milling described in the Methods section. A photo of an example 400 μm mesh used to make the filter is shown in the bottom right corner. (B) Optical microscopy of amoxicillin trihydrate crystals produced in pH-induced continuous crystallization experiments when no milling (left) or intermittent milling (right) is used. The scale bar in (B) applies to all four microscope images.

In this work, the applicability of the proposed solid-solid separation method to the continuous enzymatic RC of beta-lactam antibiotics with immobilized biocatalyst is assessed. First, the feasible range of the separation criterion \( L_{\text{crystal}} < L_{\text{filter}} < L_{\text{carrier}} \) is examined by determining the impact of carrier size on the biocatalyst activity and studying the applicability of intermittent wet milling of the slurry for reducing the crystal size, which can provide more flexibility in choosing the carrier size while satisfying the criterion. Finally, the effect of the proposed separation strategy on slurry withdrawal is studied to find the conditions \( (L_{\text{filter}} \text{ and pumping speed}) \) for a consistent isokinetic withdrawal where dilution and sieving effects are minimized during the slurry transfer from the MSMPR vessel.

### 4.2 Materials and Methods
Materials

Lifetech ECR8404M and ECR8404F enzyme carriers were donated by Purolite Life Sciences (King of Prussia, PA). 50% glutaraldehyde was purchased from Amresco (Solon, OH). Cephalexin monohydrate and amoxicillin trihydrate were purchased from RIA, Hanover, NJ. 4-hydroxy-D-phenylglycine methyl ester hydrochloride (4-HPGME HCl) >95% was purchased from Thermo Fisher Scientific (Haverhill, MA). D-phenylglycine methyl ester hydrochloride (PGME) >95% was purchased from Sigma (St. Louis, MO). 7-Aminodesacetoxycephalosporanic acid (7-ADCA) >98% and 6-aminopenicillanic acid (6-APA) >98% were purchased from TCI America (Portland, OR).

PGA assays

The βF24A variant of PGA from *E. coli*[130] was expressed using *E. coli* BL21(DE3) and purified via nickel affinity chromatography. Immobilization support sizes ranging from 300 to 800 µm were obtained by processing Lifetech ECR8404M using sieves of sizes 300 µm, 425 µm, 600 µm, and 800 µm. Support sizes 150-300 µm were obtained using raw samples of Lifetech ECR8404F. Carriers were activated for immobilization by first washing resin four times with 50 mmol/L sodium phosphate pH 7.0 with a ratio of 1:2 (support mass-buffer volume). Resins were then gently mixed in a solution of 1% (v/v) glutaraldehyde in 50 mmol/L sodium phosphate pH 7.0 for 1 hour at room temperature. Resin was then washed four times with 50 mmol/L sodium phosphate buffer pH 7.0 with a ratio of 1:4 (support mass-buffer volume). 400 mg of each size range of immobilization resin were then gently mixed with 2.3 mL of 5.5 mg/ml (Bradford assay) PGA-βF24A in 50 mmol/L sodium phosphate at 4°C. After 16 h, the concentration of
remaining enzyme in the supernatant was measured via Bradford assay and was below the
detection limit of the assay (0.05 mg/mL) for all samples. Immobilized enzyme (at
different size ranges) was reacted with concentrations of 6-APA ranging from 50 – 150
mmol/L and 100 mmol/L 4-HPGME for amoxicillin synthesis and concentrations of 7-
ADCA ranging from 50 – 150 mmol/L and 100 mmol/L PGME for cephalaxin synthesis.
Reactions were buffered with 50 mmol/L sodium phosphate pH 6.5 and were limited to
<10% conversion to ensure initial rate reaction conditions. Briefly, 10 mL of reaction
solution incubated in a 25°C water bath for >10 minutes was added to 400 mg of enzyme
supports in 20 mL gravity columns. The reaction was mixed by inverting the sealed gravity
column. 50 µL of reaction solution was withdrawn at time points ranging 1 – 15 minutes
and diluted in methanol for high-performance liquid chromatography (HPLC, Shimadzu)
analysis with a 4.6 x 250 mm HiChrom Ultrasphere ODS column. The amoxicillin and
cephalexin synthesis activity were determined from the slope of the concentration versus
time curve for amoxicillin and cephalaxin. The selectivity of the reaction was determined
by taking the ratio of amoxicillin to 4-HPG accumulation or the ratio of cephalaxin to PG
accumulation in the cases of amoxicillin or cephalaxin synthesis, respectively.

**Crystallization and slurry withdrawal**

Continuous crystallization experiments were conducted to produce an amoxicillin
slurry which was then used to test withdrawal with different filter sizes and pumping
speeds. Considering that amoxicillin solubility in water is a function of pH [84], instead of
using the PGA enzyme for these tests supersaturation was induced by controlling the
crystallizer pH value (in the actual enzymatic RC amoxicillin synthesis generates supersaturation). Accordingly, a 45 mmol solution of amoxicillin (amoxicillin stocks were first purified by batch crystallization) at pH = 8.3 (high solubility, ~45 mmol/L) was prepared and was placed in a 2-L feed tank cooled to 7°C to minimize degradation over the course of the continuous crystallization experiment. Reactor pH was controlled at 5.6 – 5.8 (low solubility, ~ 7 mmol/L) using a Mettler Toledo SP-50 dosing unit by adding 0.5 mol/L HCl. The crystallizer was a jacketed vessel that was maintained at 25.0 °C and was mixed at 250 RPM using a pitched-blade stainless-steel impeller with ~1.5-cm off-bottom clearance. Inlet and outlet flowrates were controlled using Ismatech peristaltic pumps (REGLO-ICC 3 channels). The reactor volume was set to 240 mL and the inlet and outlet flowrates to a net 3 mL/min providing a residence time of 80 minutes (outlet flowrate at 10 mL/min but intermittent). The residence time was chosen so that the reactor solids density was close to that of the design point for the continuous enzymatic RC. An 80-minute residence time corresponds to a solids density of about 14 g/L of slurry at steady state for the case with intermittent milling. Figure 4.2(A) shows the overall process and the parameters used for continuous crystallization experiments. Two sets of experiments were performed with and without wet milling of the slurry. Intermittent wet milling was implemented by recirculating the slurry through an IKA Inc. Magic Lab mill with an MK module (with a constant 12 mL/min flowrate in/out of the mill). Different milling intensities (3000 – 8000 RPM) and frequencies were tested. Eventually, 5000 RPM and a milling rate of 15-minutes-ON 7-minutes-OFF were selected for final tests. Each continuous crystallization was operated for at least four residence times to ensure reaching the steady state, which was confirmed by a relatively constant crystal count, turbidity, and
outlet concentration as measured by a ParticleTrack G400 FBRM probe, an EasyViewer 100 PVM probe, and HPLC, respectively. The slurry at steady state was then used for performing the slurry withdrawal tests with the separator filter. The filter was offline prior to these tests to omit its potential impact on arriving at steady state.

Slurry withdrawal tests were performed on both milled and un-milled slurries using separator filters with sizes ranging from 149 – 800 μm. Filters (with a diameter of approximately 0.5 in. and height of 4 in.) were made using stainless steel mesh screens with a straight weave (Utah Biodiesel). The filter and connected outlet tubing were located close to the vessel wall and at a depth of about 50% of the liquid level [131]. For each filter, a series of withdrawal speeds ranging from 3 to 20 mL/min were tested to study the impact of filter size and withdrawal speed on representative slurry withdrawal. The properties (crystal solids density and crystal mean size) of the samples withdrawn were then compared against those of the reactor slurry to identify the withdrawal method closest to isokinetic. For each sample, solids density was estimated by drying the withdrawn sample at 70°C and then subtracting the solid mass generated due to evaporative crystallization (measured by weighing control samples containing only the liquid phase) from the total mass to find the mass of primary crystals. Measurements were performed in triplicate for each withdrawal condition. Crystal mean size was estimated by optical microscopy and image analysis to estimate the length of the labeled crystals.

4.3 Results

4.3.1 Biocatalyst activity
Biocatalyst performance in this process is typically characterized by its synthesis activity and selectivity. The former refers to the rate of antibiotic generation, and latter to the ratio of antibiotic generation to that of the hydrolysis reaction product. The effect of carrier size on enzymatic activity and selectivity for cephalexin synthesis has been studied both theoretically and experimentally for specific carriers [128, 129]. The general expectation is that biocatalyst kinetics improve when using smaller carriers for immobilization to minimize the mass transfer limitations. The appropriate carrier size meets requirements imposed by both solid-solid separation and enzyme kinetic points of view. Note that loss in activity results in a larger amount of enzyme being required for a specific design and increases operational cost. Loss in selectivity leads to lower product yields as well as higher byproduct formation. In the case of cephalexin, high byproduct formation may lead to its precipitation and contamination of the final product. Therefore, an ideal carrier for this system should have acceptable activity and selectivity while still allowing for the preferential retention of the biocatalyst support and withdrawal of the crystal slurry.

Figure 4.3 shows the impact of the size of beads carrying the PGA enzyme and the concentration of the beta-lactam nucleophile (6-APA or 7-ADCA) on the selectivity and activity of amoxicillin and cephalexin synthesis. Focusing on amoxicillin synthesis, Panel A of Figure 4.3 indicates the catalyst activity decreases with increasing bead size for all concentrations of 6-APA investigated. The effect of size might be slightly more prominent at lower 6-APA concentrations where the enzyme is more active due to less substrate inhibition [128]. On the other hand, the selectivity of amoxicillin synthesis is not a strong function of carrier size, as indicated by no clear trends along the horizontal axis of Panel
C. Shifting focus to cephalexin, the activity of cephalexin synthesis exhibits a strong function of carrier size, decreasing by roughly 50% from the smallest carrier tested to the largest carrier for the 50 mmol/L 7-ADCA reactions. Likewise, the selectivity of cephalexin synthesis decreases with increasing carrier size for all 7-ADCA concentrations, as seen in Figure 4.3(D). The activity of PGA appears to be more sensitive to carrier size for cephalexin synthesis, possibly due to the intrinsically higher reaction rate for cephalexin synthesis using the soluble enzyme.[128] Overall, the experimental results reiterate the fact that enzyme carrier size has a significant impact on its apparent kinetics and performance. Therefore, one cannot freely increase $L_{\text{carrier}}$ to satisfy the separation criterion $L_{\text{crystal}} < L_{\text{filter}} < L_{\text{carrier}}$. Later, it will be shown that intermittent wet milling can help to relax the condition by reducing the $L_{\text{crystal}}$, allowing the use of smaller $L_{\text{carrier}}$ values.
Figure 4.3: Impact of the enzyme carrier size and nucleophile concentration (6-APA or 7-ADCA) on the activity (top) and selectivity (bottom) of the synthesis reaction for amoxicillin (left) and cephalaxin (right) systems.

4.3.2 Crystallization and slurry withdrawal

Figure 4.4 shows an example of the evolution of conditions in the continuous crystallization process from startup to steady state (as mentioned, in these runs pH is used to drive the crystallization). All solid-phase data (Panels A and B, crystal counts and solution turbidity) point to a relatively constant behavior after about 6 hours of operation.
(almost 5 residence times). The fixed slope of the added acid line (Figure 4.4(D)) and HPLC data (not shown) also confirmed the approach to steady-state operation. Panel E shows an in situ image of long crystals grown during the initial batch-mode operation when no milling is yet performed; Panel G shows the slurry at around 360 min when intermittent milling has led to a slurry with significantly smaller crystals. The slurry at steady state was used to perform a series of slurry transfer tests with the separator filters. The steady-state solids density for the case in which intermittent milling was implemented during the continuous crystallization was higher compared to the one with no milling (~14 versus ~11 mg/mL), which points to an increased rate of crystallization due to more available growth surface area.

![Figure 4.4: Example of the process dynamics in pH-induced continuous crystallization experiments with intermittent milling from the initial batch-mode operation to steady state (feed concentration 45 mM, residence time 80 min, intermittent milling at 5000 RPM). (A) solution turbidity measured by the PVM](image)
probe, (B) crystal chord length counts measured by the FBRM probe, (C) MSMPR vessel pH controlled by the addition of 0.5 M HCl, (D) the total volume of the added acid for pH control. (E), (F), (G) in situ images captured by the PVM probe during the batch mode, continuous mode right after the first milling cycle, and at the steady state, respectively. The scale bar in (G) applies to (E) and (F) as well.

One major concern for the proposed solid-solid separation method is the potential interference of the separator filter with slurry withdrawal. For a robust continuous RC process, an isokinetic slurry withdrawal is desired. Note that if crystals with \( L_{\text{crystal}} > L_{\text{filter}} \) are produced and cannot leave the system through the outlet, large crystals might accumulate in the system, eventually halting the operation. The slurry withdrawn from the MSMPR might have a lower solids density than the reactor, which would result from selective removal of smaller crystals or other factors related to the resistances imposed by the filter. To study the impact of the separator filter on the slurry transfer, withdrawal of the steady-state slurry was performed by using different filters and at different withdrawal rates for systems with and without intermittent milling.

Before analyzing the impact of filter size on slurry withdrawal, it is worth mentioning that the likelihood of a crystal passing the filter drops sharply as the particle size (as defined by a characteristic dimension, its length for example) becomes greater than the filter size. However, due to the needle-like shape of amoxicillin crystals, even those larger than the filter size have a non-zero probability of passing through the filter, providing some flexibility in choosing the acceptable filter size (rather than the hard limit of \( L_{\text{crystal}}, \)
max < L_{\text{filter}}). Note that this is not the case for the spherical biocatalyst carrier, and that the \( L_{\text{filter}} < L_{\text{carrier}} \) condition guarantees that the biocatalyst carrier cannot leave the system through the outlet. This will be discussed further in relation to selecting the milling intensity.

**Slurry transfer: continuous crystallization with no milling**

**Dilution effect.** Figure 4.5 compares solids densities of the withdrawn slurry using different filters at different withdrawal rates against that of the MSMPR. For example, Panel A shows that at a slurry withdrawal rate of 5 mL/min, solids density increased with increasing the filter mesh size. Values are normalized with respect to the reactor solids density at steady state. Figure 4.5 clearly shows that there is a significant dilution effect in the slurry transfer due to the presence of the separator filter. Even for the coarsest filter, 800 μm, and at the highest withdrawal rate, the solids density is still about 55% less in the withdrawn slurry. Expectedly, this loss becomes larger as the filter size or withdrawal rate is reduced. Another observation is that even when no filter is placed on the outlet, it is still challenging to withdraw a representative sample, and large-diameter tubing should be used; this is mainly due to occasional clogging initiated by some very long crystals. Tubing diameter five to ten times the size of the largest crystals is desirable to avoid this issue [132]. Intermittent pressure-driven withdrawal at high speeds is usually used for isokinetic withdrawal in continuous crystallization processes [132, 133]. However, as will be shown, analysis of the crystal size confirms that the main reason behind significant dilution during slurry withdrawal is the inability of larger crystals to pass the separator filter. Overall, data
of Figure 4.5 lead to the conclusion that continuously separating the amoxicillin crystals from the biocatalyst carriers with an acceptable size (from the activity and selectivity point of view) is almost impossible with the proposed separation method, mainly due to crystal habit and their strong 1D growth.

Figure 4.5: Efficiency of the slurry withdrawal when filters of 149 – 800 μm are applied to the outlet, at different withdrawal speeds for the AMX slurry made continuously with no milling. All values are normalized with respect to the solids density in the MSMPR vessel at steady state, ~11 mg/mL. (A), (B), and (C) panels correspond to withdrawal speeds of 5, 10, and 20 mL/min, while panel (D) provides a heatmap of all data presented in other panels. Using 5 mL/min and 800 μm filter
results in significant settling of large crystals in the line preventing robust data acquisition (NaN).

**Sieving effect.** The potential of selective removal of crystals due to the separator filter or withdrawal rate was examined by observing the crystal size distribution (CSD) of crystals in the slurry withdrawn using different filter sizes and withdrawal rates. For each sample, CSD was determined using optical microscopy and image analysis to assign a length to each of the detected objects (objects smaller than 25 µm are excluded from the analysis as they are very likely to be unaffected by the filter). Figure 4.6 shows the CSD and the mean size of the samples withdrawn with different filters. For all cases a withdrawal rate of 20 mL/min was used. As can be seen in Figure 4.6(A-D) and in agreement with earlier analysis, there is indeed some probability for particles with $L_{\text{crystal}} > L_{\text{filter}}$ to pass the filter in the case of needle-like crystals. However, a significant correlation can be seen between the size of the filter used and the CSD in the slurry withdrawn. Expectedly, crystals significantly larger than the filter cannot pass the filter and so are missing from the CSD. This can be seen more clearly in the mass-based distribution. As shown in Figure 4.6(E), the number-based mean size is almost similar for all samples since the CSD is dominated by smaller crystals, while mass-based mean size points to a strong correlation with the filter size used for withdrawal. Data in Figure 4.6 also confirm that the dilution effect discussed above is mostly due to selective removal of smaller crystals in the slurry. Note that the presence of very large crystals in the original MSMPR slurry (Panel A) might be partially due to some non-idealities in the withdrawal during the continuous crystallization process; nevertheless, analysis of Figures 4.5 and 4.6 confirms that the filter prevents a robust slurry transfer of AMX slurry made without milling mainly by not
allowing the larger crystals to leave the system.
Figure 4.6: Comparison of the CSD and means size for samples withdrawn from the crystallization process without milling using different filters. Panels (A) to (D) correspond to the crystal size distribution of the original MSMPR slurry at steady state and slurries withdrawn by 800, 300, and 149 µm filters, respectively, obtained by image processing. Panel (E) compares the number- and mass-based mean sizes for all filters. For all tests, withdrawal speed was set to 20 mL/min. NA1 and NA2 correspond to cases when no filter is placed on the outlet and 2.06 and 2.74 mm tubing were used. Particles smaller than 25 µm are excluded from the CSD analysis as they are likely to be unaffected by the filter.
Slurry transfer: continuous crystallization with intermittent milling

The results just described demonstrate the necessity of reducing crystal size to match the size (and activity and selectivity) of a biocatalyst carrier bead; otherwise, the separation criterion cannot be satisfied. Furthermore, without reducing crystal size, the separator filter does not allow isokinetic withdrawal. Considering the analysis of biocatalyst activity and selectivity (Figure 3), a carrier bead with $L_{\text{carrier}} < 425 \, \mu m$ is desired. Therefore, the maximum crystal size must not be larger than this limit to satisfy the separation condition $L_{\text{crystal}} < L_{\text{carrier}}$ (with some flexibility due to the 1D nature of crystals discussed above). Milling provides an appropriate reduction in crystal size and the specific milling parameters used in the present work (see the Materials and Methods section) were chosen to achieve this goal. Further discussion on the potential for using other milling parameters is presented in the next section. Like the previous case, continuous crystallization with intermittent milling was operated for at least four residence times until concentration measurements and PAT signals confirmed steady-state operation.

Dilution effect

Figure 4.7 shows the solids density of the slurry samples withdrawn using different filter sizes. Outlet pump speed varied from 3 to 10 mL/min. Clearly, intermittent milling facilitates withdrawal when compared to the results without milling; several combinations of withdrawal speed and filter size match the solids density produced in the MSMPR. However, as pumping speed and filter size decrease, a dilution effect is introduced into the withdrawal process. This becomes significant for $L_{\text{filter}} < 300 \, \mu m$, and pumping speed
below 5 mL/min. As expected, the worst case corresponds to using the finest filter (149 μm) and the lowest speed (3 mL/min) in which case the outlet withdraws only about 40% of the MSMPR solids content. According to the results of Figure 4.7, using the pump speed of 5 – 10 mL/min and a filter size larger than 300 μm ensures that the withdrawn sample has the same solids density as the crystallization vessel, meaning as long as the diameter of the biocatalyst support is larger than 300 μm, solid-solid separation and robust slurry transfer can be continuously performed using the proposed method. Therefore, 300 μm < \( L_{\text{carrier}} < 425 \) μm corresponds to a workable bead size range for the process.

Figure 4.7: Efficiency of the slurry withdrawal when filters of 149 – 800 μm are applied to the outlet, at different withdrawal speeds for the AMX slurry made with
intermittent milling. All values are normalized with respect to the solids density in the MSMPR vessel at steady state, ~13.8 mg/mL. (A), (B), and (C) panels correspond to withdrawal speeds of 3, 5, and 10 mL/min, while panel (D) provides a heatmap of all data in those panels.

**Sieving effect**

The crystal size distribution of samples withdrawn through different filters was studied to investigate the possibility of selective crystal withdrawal from the crystallizer. Figure 4.8 compares the CSD, along with the number-based and mass-based mean lengths for different filter sizes. For all cases, a pumping speed of 10 mL/min was used. The resulting data imply no significant difference in the size distribution in the crystallizer and the samples withdrawn through different filters (more importantly, there is no clear correlation between mean size and the filter size). This observation is in overall agreement with the results of Figure 4.7(C). Statistical tests such as one-way ANOVA can be used to check whether different groups (filters) have a common mean size and if the difference between mean sizes is significant. For example, comparing the CSD of the sample withdrawn with 234 μm filter against that of the MSMPR results in a p-value of 0.7, confirming that the difference in the mean size is not significant. However, note that even if the filter results in the preferential withdrawal of smaller crystals to a slight degree or if there is a small difference between mean size of different samples, milling prevents the formation and accumulation of enlarged stable particles in the reactor.
As seen in Figure 4.7, using the filter size of 149 μm results in some loss in the solids density in the slurry but does not seem to pose a significant sieving effect (preferential removal of small crystals). This leads us to conclude that another reason for the loss in solids density for the 149 μm filter is the resistance of the filter against the flow, which can strongly affect the flow field and cause weak mixing for the part of the slurry that is inside the filter (while the reactor slurry is still well-mixed). In fact, operating the continuous process for several hours, some settled crystals can be visibly seen at the bottom of this filter, confirming that the weak mixing inside the filter is the main reason for the above loss. While this effect was not significant for coarser filters, solutions such as a more intense mixing, placing a bubbler at the bottom of the filter, or periodic filter exchange can ensure good mixing and avoid any crystal settling inside the filter for long-term continuous operation.

Figure 4.8: Comparison of the crystal size distribution and mean size for samples withdrawn from the crystallization process with intermittent milling using different filters. Panels (A) to (D) correspond to the crystal size distribution of the original MSMPR slurry at steady state, and slurries withdrawn by 800, 300, and 149 μm.
filters, respectively, obtained by image processing. Figure 4.8(E) compares the number- and mass-based mean sizes for all filters. For all tests, withdrawal speed was set to 10 mL/min. NA corresponds to the case when no filter is placed on the outlet. Particles smaller than 25 µm are excluded from the analysis as they are likely to be unaffected by the filter.

Another factor that might impact the withdrawal efficiency when the separator filter is used is the MSMPR solids density at the steady state. To investigate whether the proposed withdrawal strategy is also applicable to cases with a higher solids density, similar tests were performed using a crystal slurry with a significantly higher solid content (21.1 mg/mL compared to 13.8 mg/mL of Figure 4.7). As shown in Figure 4.9, even for the slurry with a significantly higher solid content, representative slurry withdrawal can be achieved using filters with L_{filter} > 300 µm when intermittent milling is applied to keep the size of the crystals in an acceptable range.
Figure 4.9: Efficiency of the slurry withdrawal for a slurry with 21.1 mg/mL solids density when filters of 149 – 800 μm are applied to the outlet, at withdrawal speed of 10 mL/min for the AMX slurry made with intermittent milling. All values are normalized with respect to the solids density in the MSMPR vessel, 21.1 mg/mL.

4.3.3 Impact of milling on crystal shape and filtration

One possible downside of using intermittent milling to reduce the size of the crystals, and thereby facilitate their separation from the biocatalyst carrier, is generation of significant crystal fines. These small particles easily pass through the filter but might complicate downstream processing, for example by increasing the filtration times. Figure 10 compares the filtration rate of the AMX slurries produced with and without intermittent milling. The presence of fines induced by milling appears to result in slightly slower filtration. This side effect is one factor preventing the use of very intense milling to reduce the crystal size for better solid-solid separation. Other factors limiting milling include
formation of local thermal hotspots that might cause degradation of the amoxicillin (or cephalixin) molecules and foam generation. A potential solution to the slower filtration problem is to remove fines by implementing a pH cycle in a secondary vessel in which the pH value of the slurry with fines is increased (to dissolve the fines) and then decreased to the initial value. A similar approach has been used in other systems for fines removal using temperature cycling [134]. As can be seen in Figure 4.10, implementing only one pH cycle (from 5.8 to 7.9 to 5.8) results in a visible improvement in the filtration rate.

Figure 4.10: Impact of milling, slurry density, and pH cycle on the filtration rate of AMX crystals. (A) filtration rate for AMX slurries prepared in continuous crystallization with and without intermittent milling, with a subsequent pH cycle to redissolve and reduce the number of fines, and with a high slurry density (21.1 versus 13.8 mg/mL). (B) and (C) optical microscopy of AMX crystals prepared with
intermittent milling and milling plus pH cycle. The scale bar applies to both (B) and (C).

Another observation during the continuous crystallization experiments with intermittent milling was a slight change in the crystal growth habit of amoxicillin crystals. As shown in Figure 4.10, crystals from the system with milling have a lower aspect ratio and show a slightly greater growth along directions other than their major axis. This might be due to the fact that milling is much more likely to break the crystals along their major axis and while the intrinsic growth rates for different planes remain constant, this results in particles with lower aspect ratios. We reserve a more systematic investigation of the combination of wet milling and pH cycles for improving the size, shape, and filtration rate for a subsequent study.

4.4 Conclusions

A simple size-based method was developed for the solid-solid separation of crystals from biocatalyst carrier particles to enable the continuous operation of enzymatic reactive crystallization processes. The method utilizes a mesh filter through which product slurry is withdrawn. The primary considerations for choosing the appropriate filter characteristics are the size of the biocatalyst carrier beads and the size of the product crystals. Enzymatic assays were performed to evaluate the impact of carrier size on the performance of the immobilized PGA, confirming an increasing loss in both enzyme activity and selectivity.
with larger bead sizes. Intermittent wet milling was shown to be an effective tool in manipulating the size of product crystals so that they could be removed appropriately (without classification and slurry dilution). The selected relationship between bead size and milling allowed the use of relatively smaller carrier beads, and hence more active and selective immobilized biocatalyst, while still satisfying the key criterion for the solid-solid separation $L_{\text{crystal}} < L_{\text{filter}} < L_{\text{carrier}}$. Using the carrier size range of 300 – 425 μm and filter size of 300 μm in combination with intermittent milling at 5000 RPM and a high withdrawal speed was shown to guarantee an almost complete separation while enabling isokinetic product withdrawal.

CHAPTER 5. Development of a continuous enzymatic reactive crystallization process on a pilot scale, operation, and experimental improvement

5.1 Introduction

β-lactam antibiotics have been the first-line treatment for bacterial infections for almost 80 years. Fifteen are on the World Health Organization Essential Medicines (WHOEM) list: i.e., essential drugs for any healthcare system. Their manufacture traditionally consisted of multiple chemical synthetic steps, involving protection, deprotection, and the use of organic solvents, as well as cryogenic steps [135]. More recently, enzymes such as penicillin G acylase (PGA) from *E. coli* have been used under mild, fully aqueous conditions, forming amide bonds between different β-lactam cores and activated side chains. Additionally, the enzymatic pathway more efficiently incorporates
the reactants into the final product, as indicated by an atom economy of 84% in comparison to 36% for the chemical process. However, the enzyme may re-bind the product and hydrolyze it, which has been mitigated to some extent by engineering the enzyme and/or reaction solvent to improve synthetic efficiency [11, 33, 63, 136, 137]. PGA has been used industrially to manufacture amoxicillin, the most consumed antibiotic in terms of volume worldwide (ca. 15,000 metric tons per year (MTPY)). Current processes utilize batch production methods, which are the standard for the pharmaceutical industry, but pose disadvantages in comparison to an equivalent continuous process that has yet to be investigated thoroughly.

Continuous manufacturing often improves the Green Chemistry of processes by reducing the number of intermediate separation steps, reducing the working volume and thus reducing energy consumption, and reducing the startup and shutdown periods [41]. Moreover, drug quality is often superior in continuous processes due to improved control and reduced requirement of human intervention [42]. In addition, particularly for amoxicillin and other pharmaceuticals with large doses (≥ 500 mg) and large production volumes, emphasis also must be placed on waste reduction and the use of more environmentally benign feedstocks, as the process mass intensity (PMI) (or complete E factor, cEF) of pharmaceutical processes is often much higher than in other manufacturing sectors. A key challenge to overcome in developing a continuous enzymatic production process for β-lactam antibiotics has been handling of the enzyme: namely, how to recycle or retain the biocatalyst within the process, due to biocatalyst costs being a large portion of the process operating cost. Previously, a reproducible batch enzymatic process was developed for ampicillin synthesis utilizing large pectin-encapsulated agarose particles
loaded with PGA [56]. These particles were highly susceptible to shear forces, particularly at the tip of the impeller, and required the use of a Taylor-Couette style stirring to ensure resuspension of the slurry. The particles also required recharging with KCl to ensure the gel remained intact. Additionally, particles of such large size (~1 mm) likely exhibited decreased synthetic performance due to increased diffusional limitations [138]. Nevertheless, they were able to demonstrate the first successful application of immobilized PGA used in a RC system by demonstrating the benefits of RC while enabling the recycle of the biocatalyst.

In the present work, we demonstrate the continuous enzymatic RC of amoxicillin and cephalexin, two of the highest volume antibiotics in the world, in a novel process consisting of the combination of a previously developed size-based biocatalyst separator (in Chapter 4) [71] and wet milling to ensure robust process operation (Figure 5.1).
Figure 5.1: (A) Enzymatic reaction and crystallization network for amoxicillin (with components for cephalexin in parenthesis). (B) Pilot plant apparatus (not shown, substrate recovery process). All probes were inserted into MSMPR-1. (C) Process diagram for pilot plant. MSMPR-1 and MSMPR-2 were operated in continuous mode, while downstream unit operations for product recovery, substrate recrystallization, and substrate recovery were operated in batch mode.
In this chapter, the development of this process is detailed for both the cephalaxin and amoxicillin systems. First, we used a model to identify conditions which yield acceptable conversion, productivity, and ensure pure product. We applied these conditions first to the cephalaxin process, and examine issues related to byproduct accumulation and product contamination. The amoxicillin process is also detailed, with improvements related to enhancing amoxicillin desupersaturation and limited substrate yield addressed by leveraging the advantage of the moderate solubilities of our substrates at the operating pH value and their low solubilities at different pH values to selectively recrystallize them from the mother liquor to be recovered and recycled. We analyzed the green chemistry metrics of the process in comparison to prior processes, including the chemical syntheses and batch enzymatic syntheses. We examined the impact of such recovery on the sustainability of the process. Additionally, the conversion and productivity of the amoxicillin process was improved by adjusting feed and enzyme concentrations, with a tradeoff of decreased selectivity and increased byproduct accumulation. Lastly, we examined the long-term stability of the process.

5.2 Materials and methods

Materials

Cephalexin monohydrate, amoxicillin trihydrate, 6-aminopenicillanic acid (6-APA) and 7-aminodesacetoxycephalosporanic acid (7-ADCA) were obtained from RIA International LLC (East Hanover, NJ); D-phenylglycine methyl ester hydrochloride (D-PGME·HCl) and D-4-hydroxyphenylglycine methyl ester (D-4-HPGME) from Wuhan
Preparation of immobilized biocatalyst

The βF24A variant of penicillin G acylase (PGA-βF24A) from *E. coli* ATCC 11105 was cloned into pET28 vector, expressed, and purified by the method outlined in prior work [61]. The soluble purified enzyme was concentrated to about 10 mg/mL using an ultrafiltration membrane centrifuge tube (10K Da, Pall Corp.) and a tabletop centrifuge. Purolite immobilization resins were sieved to 425–500 μm using a stainless mesh sieve. To activate the resins, they were first washed twice with two volumes of 50 mmol/L sodium phosphate buffer, pH 7.0, per volume of resin in a gravity column. The washed resin was then mixed gently by inversion in four volumes per volume of resin in a solution of 1% (v/v) glutaraldehyde in 50 mmol/L sodium phosphate buffer, pH 7.0 for 60 min at room temperature. Afterwards, the resin was washed as previously described and used immediately for immobilization. The mass of resin for immobilization was determined such that a resin to enzyme solution was about 1:4. The soluble enzyme solution and resin were gently mixed by inversion in a gravity column for 18 hours at room temperature, after
which the remaining enzyme in solution was determined by Bradford assay to estimate immobilization yield. The resin was again washed as previously mentioned and stored in fresh wash buffer at 4°C until use.

Collection of liquid phase HPLC samples

Using a pipette, 1 mL of slurry or solution was withdrawn and filtered through a 0.2 μm cellulose membrane filter. The filtrate was then diluted 1:20 (v/v) in 50 mmol/L sodium phosphate buffer, pH 7.0, vortexed, and centrifuged in a microcentrifuge at 14.5k RPM for 5 min prior to loading in HPLC vials.

Analysis of reaction samples

Quenched reaction samples were analyzed via reverse phase HPLC using a Hichrom Ultrasphere C18 Column, having a diameter of 4.6 mm, 5 μm particle size, 250 mm in length. A gradient method was employed to achieve baseline separation of all components. The mobile phase A was 10 mM sodium phosphate buffer pH 7.0. The mobile phase B was HPLC grade methanol.

For the analysis of cephalexin-related mixtures, the column was equilibrated in 95% mobile phase A and 5% methanol at a total flow rate of 1 mL/min. At t = 1.5 min, a linear gradient shifted from 95% to 85% mobile phase A until t = 4 min. Another linear
gradient shifted the mobile phase composition from 85% to 50% mobile phase A until t = 7 min. The mobile phase composition was then held at 50% mobile phase A until t = 9 min and then immediately changed to the initial composition to re-equilibrate the column. The retention times of components were roughly, 3.5, 4.0, 10.0, and 12.0 minutes for phenylglycine, 7-ADCA, cephalexin, and PGME, respectively.

For the analysis of amoxicillin-related mixtures, the column was equilibrated in 95% mobile phase A and 5% methanol at a total flow rate of 1 mL/min. At t = 0 min, a linear gradient shifted from 95% to 50% mobile phase A until t = 8 min. The mobile phase composition was then held at 50% mobile phase A until t = 10 min and then immediately changed to the initial composition to re-equilibrate the column. The retention times of components were about 2.3, 4.4, 5.8, and 6.9 minutes for D-4-HPG, 6-APA, amoxicillin, and D-4-HPGME, respectively.

*Preparation of amoxicillin feed solutions*

The 0.6 mol/L 6-APA feed solution was prepared by mixing 136 g solid 6-APA into 950 mL of DI water while slowly adding 100 mL of 6 mol/L NaOH. The pH of the solution was monitored such that it never rose above a value of 8.5. The 0.5 mol/L 4-HPGME feed solution was prepared by mixing 156 g solid D-4-HPGME in 1610 mL of DI water while slowly adding 140 mL of 6 mol/L HCl. Both solutions were separately
filtered over filter paper following dissolution using a vacuum pump and added to the chilled jacketed feed vessels.

*Preparation of *cephalexin* feed solutions

The 0.36 mol/L 7-ADCA feed solution was prepared by dissolving 81 g in 990 mL DI water while slowly adding about 60 mL of 6 mol/L NaOH until the solution was stable at a pH value of 7.5. The 1.1 mol/L D-PGME feed solution was prepared by dissolving 100 g solid D-PGME•HCl in 450 mL DI water. Both solutions were separately filtered over filter paper following dissolution using a vacuum pump and added to the chilled jacketed feed vessels.

*Amoxicillin pilot plant startup procedure*

To prepare the initial slurry in MSMPR-1, 60 mL of D-4-HPGME feed, 60 mL of 6-APA feed, and 180 mL of DI water were mixed in a beaker and the pH brought to a value of 6.5 using 2 mol/L NaOH or 2 mol/L HCl. The solution was then saturated with amoxicillin by adding 2 g amoxicillin trihydrate, allowing 15 min to equilibrate, and filtered over filter paper with a vacuum pump. The clear solution was then seeded with 6 g of amoxicillin trihydrate, which was pre-ground using a mortar and pestle. To prepare the initial slurry for MSMPR-2, the same steps for MSMPR-1 were followed, except with a 200 mL volume, with all masses and volumes scaled to 2/3 the quantity. The slurries were then poured into their respective vessels and impellers activated at 300 RPM. The
immobilized enzyme was then added to MSMPR-1 to initiate synthesis of amoxicillin. The pumps and wet mill were activated, with pumps running continuously and wet mill activated for 15 min every 20 minutes to reduce crystal size while allowing the mill to cool for 5 minutes every cycle. Additionally, pH control was maintained in MSMPR-1 using the Mettler dosing unit and pH probe by adding 2 mol/L NaOH. Product was withdrawn through the size-based separator, including withdrawal to the wet mill, as described in Ref. [71]. The wet mill was operated using the MK module, course rotor, at 4000 RPM with intermittent cycles of 15 minutes ON and 5 minutes OFF.

*Cephalexin pilot plant startup procedure*

To prepare the initial slurry in MSMPR-1, 27 mL of D-PGME feed, 83 mL of 7-ADCA feed, and 190 mL of DI water were mixed in a beaker and the pH value was brought to a value of 6.5 using 2 mol/L NaOH or 2 mol/L HCl. The solution was then saturated with cephalexin by adding 6 g cephalexin monohydrate, allowing 15 min to equilibrate, and filtering over filter paper with a vacuum pump. The clear solution was then seeded with 6 g of cephalexin monohydrate, which was pre-ground using a mortar and pestle. The slurry was then poured into MSMPR-1 and the impeller activated at 300 RPM. The immobilized enzyme was then added to MSMPR-1 to initiate synthesis of cephalexin. The pumps and wet mill were activated, with pumps running continuously and wet mill activated for 15 min every 20 minutes. Additionally, pH control was maintained in MSMPR-1 using the Mettler dosing unit and pH probe by adding 2 mol/L NaOH using the
iControl software (Mettler) to stabilize the pH value at 6.5. The product withdrawal and wet mill withdrawal were set up in the same fashion as the amoxicillin system.

Product isolation

For amoxicillin, the product slurry was chilled on ice following withdrawal and processed approximately every 170 minutes. The slurry was vacuum filtered over a Whatman 150 mm diameter grade 1 filter paper for approximately 5 minutes. The vacuum was then shut off and the cake was washed with 60 mL of chilled (~4°C) DI water (roughly 3 mL/g dry cake) by gently pouring the water over the cake and immediately turning on the vacuum for approximately 10 minutes. The cake was then removed from the filtration apparatus and allowed to air dry overnight. The mother liquor was then collected and processed to recovery the unreacted substrates 6-APA and D-4-HPGME.

For cephalexin, the product slurry was also chilled on ice following withdrawal and processed approximately every 150 minutes. The slurry was vacuum filtered over a circular, Whatman 82 mm diameter grade 1 filter paper for approximately 5 minutes. The vacuum was then shut off and the cake was washed with 40 mL of chilled (~4°C) 70% (v/v) ethanol in DI water (roughly 5 mL/g dry cake) by gently pouring over the cake and immediately turning on the vacuum for approximately 10 minutes. The cake was then removed from the filtration apparatus and allowed to air dry overnight.
Recovery of amoxicillin substrates

After product collection, the mother liquor was added to a beaker and stirred with an overhead impeller. 1 g of D-4-HPGME seeds were added and immediately 21 mL/h of 2 mol/L NaOH was added using a syringe pump until the pH reached a value of 8 (roughly 60 minutes). The slurry was vacuum filtered for 5 minutes, and the mother liquor was collected. The cake was washed with 30 mL cold IPA, vacuum filtered again, and air dried. Next, to recover 6-APA, the mother liquor was then acidified with 6 mol/L HCl until it reached a pH value of 4.0. 3 g of 6-APA seeds were added to the mother liquor and the pH was maintained at a value of 4.0 using 2 mol/L NaOH. After 90 minutes, the slurry was vacuum filtered over filter paper (150 mm, Whatman, grade 1) for 5 minutes and the remaining mother liquor was disposed of as waste. The recrystallized 6-APA was allowed to air dry. All crystallized components were weighed on a scale after approximately 48 hours of drying.

Calculation of green chemistry metrics

Green chemistry metrics of processes were estimated based on the data collected in this work and available in literature. A summary of the equations used to calculate the green chemistry metrics is shown in Table 5.1.
Table 5.1: Equations used to calculate green chemistry metrics. The complexity of a process corresponds to the number of separate reaction steps.

<table>
<thead>
<tr>
<th>Metric</th>
<th>Equation</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction Mass Efficiency</td>
<td>( \text{RME} = \frac{\text{Mass of product}}{\text{Mass of reactants}} )</td>
<td>kg/kg</td>
</tr>
<tr>
<td>Process Mass Intensity [139, 140]</td>
<td>( \text{PMI} = \frac{\text{Total mass input in system}}{\text{Mass of product}} )</td>
<td>kg/kg</td>
</tr>
<tr>
<td>Aqueous Mass Intensity</td>
<td>( \text{AMI} = \frac{\text{Total mass of water input in system}}{\text{Mass of product}} )</td>
<td>kg/kg</td>
</tr>
<tr>
<td>Relative Process Greenness [141]</td>
<td>( \text{RPG} = \frac{26 \times \text{Complexity}}{\text{PMI}-1} \times 100 )</td>
<td>%</td>
</tr>
</tbody>
</table>

A more detailed accounting of these metrics can be found in the Supplemental Information. For the continuous process, the product was collected over a known period of time after the process reached a steady state. The concentrations of substrates fed into and of waste leaving the system in the liquid phase were measured using HPLC. Product crystal purity was measured using HPLC after redissolving a known quantity in DI water. Mass of product collected over a period of time was measured after drying. Additionally, water content of product was measured using coulometric Karl-Fisher (KF) titration and thermogravimetric analysis (TGA) and confirmed that amoxicillin and cephalexin were isolated in the desired trihydrate and monohydrate forms, respectively.
5.3 Results

5.3.1 Process modelling to identify region of operation

The process model was divided into two separate modules, enzymatic reaction, and crystallization. The development of the enzymatic reaction model is presented in Chapter 2. The modelling of amoxicillin trihydrate solubility and crystallization is discussed in Chapter 3. A model for cephalexin crystallization was adapted from the work of McDonald et al. [90], which was similar to the one developed in Chapter 3 based on classical nucleation and growth kinetics via the method of moments. The effect of molecular diffusion of components within the pores of microporous resins on the kinetics of cephalexin and amoxicillin synthesis are discussed in Chapter 4. The equations for synthesis and consumption ($R_j$) of product ($P$), byproduct ($B$), substrate ($S$) and nucleophile ($N$) are

\begin{align}
R_P &= \frac{c_E}{(k_3K_N + k_4c_N + k_5c_N)} \left( \frac{k_2k_4c_Nc_N}{K_S} - \frac{k_{-4}c_P(k_3K_N + k_5c_N)}{K_P} \right) \\
R_B &= \frac{c_E(k_3K_N + k_5c_N)}{(k_3K_N + k_4c_N + k_5c_N)} \left( \frac{k_2c_S}{K_S} - \frac{k_{-4}c_P}{K_P} \right) \\
R_S &= -(R_P + R_B) \\
R_N &= -R_P
\end{align}
where \( c_j \) corresponds to the liquid phase concentration of each component \( j \) (i.e., \( P, B, S, N \)) these kinetic parameters and binding constants are discussed in Chapter 2. The concentration of the enzyme \( c_E \) is

\[
c_E = \frac{c_{E,0}}{1 + \frac{c_S}{K_S} + \frac{c_P}{K_P} + \frac{c_N}{K_N} + K_N \left( \frac{k_{-4} c_P}{K_P} + \frac{k_{-2} c_P}{K_S} \right) \left( 1 + \frac{c_N}{K_N} + \frac{10^{-pH}}{K_{A1}} + \frac{K_{A2}}{10^{-pH}} \right)}
\]  

(4-5)

where \( c_{E,0} \) corresponds to the initial concentration of free enzyme added, \( K_{A1} \) the 1st proton dissociation constant for the nucleophile, and \( K_{A2} \) the 2nd proton dissociation constant for the nucleophile, both of which impact the nucleophilicity of 6-APA or 7-ADCA at different pH values, which has been shown to impact the selectivity of ampicillin synthesis with Assemblase®[57]. The values for these kinetic and thermodynamic parameters for the cephalexin and amoxicillin systems are shown in Appendix B. The model used to describe amoxicillin solubility and crystallization are discussed in Chapter 3. The same model was applied to cephalexin crystallization, with the model parameters previously reported in [90].

The population balance (Equation (4-6)) and solute balance (Equation (4-7)) for API \( j \) (amoxicillin or cephalexin) used for the modelling of a continuous mixed-suspension mixed-product-removal (MSMPR) crystallizer are

\[
\frac{\partial n_j(L,t)}{\partial t} = -\frac{n_j(L,t)}{\tau_s} - G_j \frac{\partial n_j(L,t)}{\partial L}
\]  

(4-6)
\[
\frac{dc_j}{dt} = \frac{c_{j,0} - c_j}{\tau_s} - R_j - \frac{3k_{V,j} \rho_j G_j \mu_{2,j}}{\tau_\ell}
\]  
(4-7)

where \(\tau_s\) corresponds to the residence time of the solid phase and \(\tau_\ell\) the residence time of the liquid phase, which were assumed to be the same value in simulations. After the process reached steady-state at a given set of conditions, the volumetric productivity and nucleophile conversion were specified as output parameters, as they are often subject to optimization during process development and design. The output of the simulations for the continuous RC of cephalexin is shown in Figure 5.2 below.
Figure 5.2: Simulations of continuous RC of cephalixin. (A) 7-ADCA conversion as a function of residence time and enzyme concentration. (B) Cephalexin volumetric productivity as a function of residence time and enzyme concentration. (C) 7-ADCA conversion as a function of residence time and enzyme concentration with regions of PG and 7-ADCA precipitation removed. (D) Cephalexin volumetric productivity as a function of residence time and enzyme concentration with regions of PG and 7-
ADCA precipitation removed. Simulation conditions: $c_{7-ADCA,feed}=0.26$ mol/L, $c_{PGME,feed}=0.33$ mol/L, $T = 25^\circ$C, $pH = 6.5$. (E-F) 7-ADCA conversion and cephalxin productivity during continuous RC of cephalxin with $c_{7-ADCA,feed}=0.30$ mol/L, $c_{PGME,feed}=0.48$ mol/L and all other conditions the same as Panels (C-D).

The 7-ADCA conversion (Fig.5.2(A)) steadily increases as a function of both residence time and enzyme concentration, until it reaches a plateau and exhibits a diminishing effect due to the accumulation of cephalxin and depletion of 7-ADCA and PGME in the liquid phase, leading to decreased enzyme performance. The volumetric productivity of cephalxin production increases monotonously with enzyme concentration within the region examined, which agrees with observations of previous modelling studies of similar systems [67]. However, there appears to be a maximum productivity at specific residence time for any enzyme concentration (Figure 5.2(B)). An optimum residence time is generally observed at any enzyme concentration (within the ranges of parameters examined), which corresponds to the residence time in which the rate of production of cephalxin equals the rate of hydrolysis of cephalxin. At higher residence times, the rate of cephalxin hydrolysis overtakes the rate of cephalxin synthesis, which has been observed in homogeneous batch experiments for cephalxin synthesis[61, 142, 143]. Additionally, not all points in the space examined in Figure 5.2(A-B) are attainable, due to the precipitation of PG or 7-ADCA. PG may precipitate due to its accumulation due to PGME or cephalxin hydrolysis; however, 7-ADCA may accumulate due to too low enzyme activity, or too high of 7-ADCA feed concentration, due to the continuous nature
of the system and possibility of substrate accumulation. If a constraint was set on PG or 7-ADCA accumulation (40 and 160 mmol/L for each, respectively), the attainable region of operation for the given feed conditions, pH, and temperature is narrowed (Figure 5.2(C-D)). The pure component solubility of 7-ADCA in water at a pH of 6.5 is 44 mmol/L [60]. The relationship between 7-ADCA supersaturation and mean induction time is shown in Appendix B Figure B.1 and indicates that a supersaturation of 3.5 is required (154 mmol/L at pH 6.5) to result in a mean induction time of 6 hours. At higher residence times and enzyme concentrations, the level of PG accumulation was too large and would result in PG precipitation and product contamination. At lower residence times and enzyme concentrations, the level of 7-ADCA would accumulate due to the continuous feed of substrates into the MSMPR, leading to 7-ADCA precipitation and product contamination. Additionally, the impact of increasing substrate feed concentrations was simulated by changing 7-ADCA feed from 260 to 300 mmol/L and PGME from 330 to 480 mmol/L (Figure 5.2(E&F)). The results of simulating higher feed concentrations indicated higher 7-ADCA conversion and cephalaxin productivity could be achieved, although the operating region to avoid 7-ADCA or PG precipitation was narrowed considerably. A narrower attainable region of operation implies greater difficulty maintaining a state of control during operation. For example, small deviations in operating conditions (e.g. pH, temperature), may cause small changes in the concentrations of components in the liquid phase, leading to byproduct or substrate precipitation and likely process shutdown in the case of a narrower operating region, whereas a wider operating region would be more resistant to such disturbances. Due to this, the more conservative feed concentrations with
a wider operating region were selected for initial experimental pilot plant operating conditions.

Due to these observations of the simulation experiments, it is clear that the conditions which the reactor is designed to operate are important in ensuring these two issues of substrate and product precipitation are avoided in order to ensure pure cephalexin is produced. Additionally, as regions of higher productivity are sought by increasing the enzyme concentration for a given residence time, the width of the design space narrows, indicating that process stability may become problematic at these conditions.

5.3.2  *Process operation during a state of control for cephalexin and amoxicillin*

Initially, the process was constructed and fully contained within a fume hood. A series of experiments were conducted to test the operation of various equipment and unit operations such as pumps, dosing unit, and wet mill (Table 5.2).
Table 5.2: Summary of initial pilot runs for system development and optimization.

Runs are numbered chronologically based on the order that they were conducted.

<table>
<thead>
<tr>
<th>Run</th>
<th>Process</th>
<th>Length (h)</th>
<th>Main goal/finding</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2</td>
<td>Testing the pumps and the tubing</td>
<td>2, 4</td>
<td>- Programming and steady operation of the pumps</td>
</tr>
<tr>
<td>3</td>
<td>Continuous Crystallization by pH swing</td>
<td>8</td>
<td>- Potential crystal clogging in the lines.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Programming the pumps in an oscillatory mode (e.g., 5-1-3-1 s)</td>
</tr>
<tr>
<td>4,5</td>
<td>Continuous Crystallization by pH swing</td>
<td>4, 8</td>
<td>- Significant crystal retention in MSMPR for outlet filters &lt; 300 μm</td>
</tr>
<tr>
<td>6</td>
<td>Continuous Enzymatic Reactive Crystallization</td>
<td>8</td>
<td>- Poor mixing in the enzyme holder cage leads to low reaction rates</td>
</tr>
<tr>
<td>7</td>
<td>Continuous Enzymatic Reactive Crystallization</td>
<td>8</td>
<td>- 10 g CEX.H₂O produced continuously</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- ~ 49% 7ADCA conversion</td>
</tr>
<tr>
<td>8</td>
<td>Continuous Enzymatic Reactive Crystallization</td>
<td>15</td>
<td>- Continuous operation for 13 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Robust pH control implemented</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- ~1 g/h CEX.H₂O was produced and isolated during the steady operation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Observation of PG crystals due to poor mixing and training of a CNN image analysis model</td>
</tr>
<tr>
<td>9</td>
<td>Continuous Enzymatic Reactive Crystallization</td>
<td>17</td>
<td>- 35 g CEX.H₂O produced</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- ~2.5 g/h CEX.H₂O was produced and isolated during the steady operation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Continuous operation for 17 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- ~55% 7-ADCA conversion</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- A primary washing protocol was implemented and successfully tested</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Crystals wet milling was tested for modification of crystal shape</td>
</tr>
<tr>
<td>10</td>
<td>Continuous Crystallization by pH swing (AMX)</td>
<td>8</td>
<td>- Studying potential crystal loss in the mill and efficient pumping strategy</td>
</tr>
<tr>
<td>11</td>
<td>Continuous Crystallization by pH swing (AMX)</td>
<td>8</td>
<td>- Studying potential crystal loss in the mill and efficient pumping strategy</td>
</tr>
<tr>
<td>12</td>
<td>Continuous Crystallization by pH swing (AMX)</td>
<td>10</td>
<td>- Evaluation of different milling frequencies for crystal size control</td>
</tr>
<tr>
<td>13</td>
<td>Continuous Enzymatic Reactive Crystallization</td>
<td>16</td>
<td>- A new immobilization matrix was tested (Purolite organic beads)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Operated continuously for 16 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- 13 g CEX.H₂O was produced</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Three washing protocols were tested to identify the best candidate (DIW, MeOH, EtOH)</td>
</tr>
<tr>
<td>14</td>
<td>Continuous Enzymatic Reactive Crystallization</td>
<td>18</td>
<td>- Operated continuously for 18 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Net 34 g CEX.H₂O was produced continuously (~ 2.2 g/h crystal productivity)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Milling from t = 0 helped mitigating severe clogging issues in the lines</td>
</tr>
</tbody>
</table>
During the first runs, the size-based separator was not designed, and it was assumed that either soluble enzyme would be utilized with an ultrafiltration membrane, or an immobilized enzyme would be utilized in combination with a solid-solid separation. Various commercial enzyme supports with covalent chemistry were screened for activity using cephalexin hydrolysis experiments (Table 5.3), with results listed from best to worst activity.

**Table 5.3: Commercial enzyme immobilization supports tested for residual activity compared to soluble enzyme.** Reactions were conducted with PGA-WT-loaded supports in 50 mmol/L cephalexin in 50 mmol/L sodium phosphate buffer at pH 7.0 and 25°C. Residual activity was compared to wild-type in terms of specific activity (U/mg PGA).

<table>
<thead>
<tr>
<th>Carrier</th>
<th>Chemistry</th>
<th>Residual Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purolite amino-C6</td>
<td>glutaraldehyde</td>
<td>40.2</td>
</tr>
<tr>
<td>Purolite amino-C2</td>
<td>glutaraldehyde</td>
<td>35.6</td>
</tr>
<tr>
<td>Purolite epoxy</td>
<td>epoxy</td>
<td>33.4</td>
</tr>
<tr>
<td>Immobead cov-1</td>
<td>epoxy</td>
<td>28.7</td>
</tr>
<tr>
<td>Immobead cov-2</td>
<td>epoxy</td>
<td>34.0</td>
</tr>
<tr>
<td>Immobead cov-3</td>
<td>epoxy</td>
<td>26.4</td>
</tr>
<tr>
<td>Relizyme-EP</td>
<td>epoxy</td>
<td>25.3</td>
</tr>
</tbody>
</table>
All supports screened were of similar size ranges (100-300 μm), although larger supports (300-700 μm) were acquired later for pilot plant experiments as particles larger than the sieve size of 300 μm were required. Additionally, only covalent supports were screened in an effort to reduce the likelihood of biocatalyst leaching into the product stream, which would result in product hydrolysis. Also, the multipoint covalent immobilization of PGA with epoxide and glutaraldehyde chemistry has been shown to improve the resultant enzyme stability [25, 28, 31]. The Purolite amino-C6 glutaraldehyde-functionalized particles achieved the highest residual activity of PGA of 40.2%, possibly due to a larger pore structure improving the diffusion of components within the particles. It is worth noting that all the supports tested were poly-methyl methacrylate (PMMA), which exhibits improved mechanical stability compared to agarose-based particles previously demonstrated for PGA immobilization [56, 79, 144, 145]. Since cephalexin synthesis is a fast reaction, it was used as a “worst case” assay to compare to soluble enzyme, since it would exaggerate the effect of diffusional limitations due to the shorter time scale of reaction [138]. Later tests confirmed that for slower reactions, such as amoxicillin synthesis, Purolite amino-C6 particles achieved 70% soluble enzyme activity. Additionally, the stability of PGA immobilized on Immobead Cov-2 supports over the course of 28 hours of stirring during homogenous cephalexin hydrolysis was tested using polarimetry to detect the product concentration (Figure 5.3).
Figure 5.3: Concentration of cephalexin over the course of 28 hours for cephalexin hydrolysis with PGA-WT-loaded Immobead Cov-2 supports. $c_{\text{Cephalexin,feed}} = 20$ mmol/L in 100 mmol/L sodium phosphate pH 7.0, $T = 25^\circ C$. $V = 20$ mL, Initial feed flowrate = 2 mL/min, manipulated to 1 mL/min at $t = 13$ h and to 2 mL/min at $t = 22$ h using a peristaltic pump. $C_{\text{PGA}} = 1 \mu\text{mol/L}$.

The concentration of cephalexin was tracked over time using polarimetry by measuring the total optical rotation $\Theta_{\text{obs}}$ of the solution and using the equation

$$c_{\text{Cephalexin}} = \frac{\Theta_{\text{obs}} - c_{\text{Cephalexin,feed}}(\vartheta_{\text{PG}} + \vartheta_{7\text{-ADCA}})}{\vartheta_{\text{Cephalexin}} - \vartheta_{\text{PG}} - \vartheta_{7\text{-ADCA}}}$$  \hspace{1cm} (5-1)$$

where $\vartheta_j$ represents the specific optical rotation of component $j$ in $^\circ/(\text{mol/L})$, which were 50, -16, and 32 $^\circ/(\text{mol/L})$ for cephalexin, PG, and 7-ADCA, respectively. The data in Figure
5.3 imply that after an initial transient period, the concentration of cephalexin remained at a constant value, which is indicative of constant enzyme activity. The feed flow rate was decreased to 1 mL/min at $t = 13$ hours (and back to 2 mL/min at $t = 23$ hours), leading to a higher residence time (20 minutes) and higher conversion; nevertheless, the conversion remained consistent over the course of the experiment after initial transient periods due to startup and feed flow rate changes, indicating that no detectable levels of enzyme deactivation were observed over the entire 28 hour period. While deactivation over a 28-hour period was not detected without crystallization, it is possible that crystallization on the surface or within the pores of the enzyme supports could lead to observed deactivation of immobilized PGA due to pore blockage and increased diffusional limitations.

The pumping of slurries and mixing within the MSMPR was tested using a pH swing crystallization of amoxicillin trihydrate (Runs 1-5). The design and optimization of the size-based separator is discussed in Chapter 4, which was utilized in Runs 8 and onward. For enzymatic cephalexin runs, the feed conditions are identical to those specified in the simulations for Figure 5.2, and a residence time of 100 min and enzyme concentration of 5 μmol/L. At first, the size-based separator was utilized without the aid of wet milling, which led to significant clogging of the surface of the mesh sieve which facilitated the separation, as well as clogging and encrustation of tubing (2 mm I.D.) used to pump the slurry (Run 8). Clogging of tubing and the separator was mitigated by utilizing oscillatory flow, programmed at 18 mL/min for oscillations of 5 seconds of forward flow followed by 3 seconds of backward flow. Additionally, during Run 8, significant PG accumulation in the liquid phase and precipitation into the solid phase was observed, leading to the premature shutdown of the process due to a low purity of the product.
Cephalexin. The source of the PG precipitation was determined to be due to diffusional limitations in the commercial macroporous enzyme supports (ChiralVision Cov-2 Immobeads) used, which were of a very large average size (~700 μm), which led to lower selectivity than expected, and further studies on the effect of support size on enzyme performance, discussed in Chapter 4. The size of the supports was decreased by sieving (425-500 μm) but kept above the separator sieve size (300 μm), which improved the enzyme performance.

5.3.2.1 Cephalexin Pilot Runs

A summary of the first successful continuous enzymatic RC experiment, Run 9, is shown in Figure 5.4 below.
Figure 5.4: Summary of results from Run 9. (A) Turbidity and solids production over time; (B) Liquid phase concentrations of all components measured by HPLC; (C) pH value over time, controlled using 2 mol/L NaOH; (D) In situ microscopy of cephalexin crystals on left (size of image is 1 mm²), offline microscopy on right. (E)
Produced samples of >99% purity cephalexin monohydrate. V = 300 mL, F = 3 ml/min. T = 25°C, pH_{setpoint} = 6.5.

Panel (A) indicates that after an initial transient period of approximately 150 minutes, the production of cephalexin stabilized at approximately 1 g/h. HPLC analysis of the filtered liquid phase (Panel (B)) indicates that after approximately 200 minutes the liquid phase concentrations of all components begin to stabilize. The pH was adequately controlled at a set value of 6.5 (Panel (C)), and offline and in situ microscopy (PVM) were used to confirm the needle-like morphology expected for cephalexin monohydrate crystals. The purity of cephalexin product was confirmed using HPLC (Figure 5.5(A,C)) for washed and unwashed crystals, chiral purity via optical rotation (Figure 5.5(B)) and the water content using thermogravimetric analysis (TGA) (Figure 5.5(D)).
Figure 5.5: Product purity analysis for Run 9. (A) Unwashed and washed filter cake and HPLC mass purity analysis; (B) Chiral purity analysis via optical rotation, comparison between collected product and standard (RIA, NJ); (C) HPLC chromatogram of collected cephalexin product (washed); (D) TGA of multiple samples of collected cephalexin. Dotted line corresponds to expected mass after loss of the water of hydration. Yellow point corresponds to mass loss after 24 hours oven drying at 50°C.

A slight yellow color was observed in the filter cake without a deionized water wash (Figure 5.5(A)). The optical purity of the collected sample was very similar to the standard (Figure 5.5(B)). The mass loss during TGA corresponded to water loss, which was likely
the loss of the crystal water. As cephalexin exists in a monohydrate form, the mass of water is approximately 5%, which corresponded to the mass loss of both samples tested with TGA, as well as the sample dried at 50°C for 24 hours in a drying oven. While washing yielded pure product (>99% purity by mass), approximately 21% of product was lost in the water wash, largely due to the solubility of cephalexin in water (~50 mmol/L at pH 7.0). During the next pilot plant experiment, Run 13, which was designed the same process conditions (e.g. feed concentrations, residence time, enzyme concentrations) as Run 9, ethanol and methanol were screened as possible washing solvents, with mass losses of cephalexin, with cephalexin losses of 2% and 15%, respectively. Figure 5.6(A) shows the relative molar concentrations of each component present in the wash filtrate.
Figure 5.6: Relative molar concentrations of components in wash filtrate for cephalexin system during Run 13. (A) Water, 100% ethanol, and 100% methanol as washing solvent. (B) Mother liquor component concentrations versus wash filtrate using 25-100% (v/v) ethanol in deionized water as washing solvent. For all experiments, 5 mL washing solvent/g cephalexin was applied. All washing solvents were chilled to 4°C in an ice bath before use.
Using water and methanol as the washing solvent, the relative concentration of cephalexin in the wash filtrate was the major fraction (~58%) (Figure 5.6(A)). The lack of cephalexin in the ethanol wash filtrate (~16% relative fraction) was possibly due to a lower solubility of cephalexin compared to other components in ethanol. The solubility of cephalexin, PG, and 7-ADCA in mixtures of ethanol and water were measured, with all components exhibiting lower solubility with higher ethanol concentrations. Rather than using pure ethanol, which would be more costly than deionized water and difficult to recycle via distillation, mixtures of ethanol and water were tested as the washing solvent (Figure 5.6(B)). At higher concentrations of water, the fraction of cephalexin in the wash filtrate increased. The relative fraction of cephalexin in comparison to the other components decreased with increasing ethanol concentration. The 75% ethanol mixture exhibited similar distributions of components in the wash filtrate as 100% ethanol, so 75% ethanol in water was selected as the wash solvent in future cephalexin pilot runs.

For Run 9, the commercial enzyme carrier utilized was Immobeads Cov-2. In later runs, the carrier was switched to Purolite amino-C6 after it was acquired and tested for activity. The stability of the PGA-loaded carrier was tested after Run 9 (Figure 5.7).
Figure 5.7: Residual activity of PGA-loaded Immobeads Cov-2 after 8 and 24 hours of process time in Run 9. Activity was assayed using 50 mmol/L cephalaxin hydrolysis, T = 25°C, pH = 7.0. Residual activity was calculated as a percentage of the initial activity of the PGA-loaded support prior to its use in Run 9.

After 8 hours of process time, the observed activity of the PGA-loaded Immobeads Cov-2 decreased 17%, and 22% total after 24 hours of process time. This observed deactivation was possibly due to crystallization on or within the pores of the supports, decreasing the diffusion of components in and out of the support. Elemental analysis of the supports via SEM mediated energy dispersive spectroscopy (EDS) was attempted, with the results inconclusive due the limit of detection being too high.

The last observation to note from Run 9 was regarding the inclusion of wet milling in the cephalaxin runs. The shear imparted within the mill itself generated foam that collected at the top of the vessel, resulting in some level of mixing concerns. The generation
of foam in dense slurries of cephalexin has been reported previously [90], although the majority of the foam generated during continuous pilot runs was observed being discharged from the wet mill outlet. Foam generation was reduced via the inclusion of a food grade antifoam agent (Sigma Antifoam 204).

Next, Run 14 was conducted, applying the lessons and methods learned previously as a final test of the continuous reactive crystallization system for cephalexin production. A summary of the results from Run 14 is shown in Figure 5.8.
Figure 5.8: Summary of results from Run 14, a continuous RC of cephalexin monohydrate. (A) FBRM fines (<10μm) and mid-sized (10-100 μm) chords, pH, and cephalexin production over the course of the pilot run; (B) Liquid phase concentrations of all components measured by HPLC; (C) Pilot plant apparatus for Run 14, complete with external wet milling loop; (D) Offline microscopy of crystals
over time. Residence time from \( t = 0 \) to \( t = 400 \) minutes was 100 minutes. Residence time from \( t = 400 \) minutes to \( t = 1000 \) minutes was 150 minutes.

The solid phase reached a state of control at approximately 600 minutes based on steady FBRM and solids productivity data (Figure 5.8(A)), much later than the liquid phase, which is expected due to the higher order dynamics of crystal nucleation and growth [49, 146]. A decision was made to increase the residence time of the MSMPR at \( t = 400 \) minutes after observation of substrate accumulation, an indication of too low enzyme activity. The enzyme used in Run 14 was reused from Run 13 in an effort to demonstrate reusability of the PGA-loaded supports. On the other hand, as indicated in Figure 5.7, there likely was some degree of deactivation between these two experiments. The residence time was increased from 100 minutes to 150 minutes by decreasing the inlet and outlet flow rates to from 3 to 2 mL/min and manually maintaining the same reactor volume of 300 mL. The concentration of substrates PGME and 7-ADCA were depleted in the liquid phase following the increase in residence time at \( t = 400 \) minutes (Figure 5.8(B)), due to a longer period of time allowed for the enzyme to turn over substrates. After the change in residence time, the conversion of 7-ADCA increased from 42% to 50% and the conversion of PGME increased from 40% to 48%. It should be noted that increasing conversion of PGME may be risky due to the potential for PG accumulation and precipitation. Interestingly, even with the flow rates in the process decreased by 33%, the total production rate of cephalexin increased about 50% from 1.50 to 2.25 g/h (corresponding to an increase in volumetric productivity from 120 to 180 g/L/d). This increase in production rate with a decrease in
flow rate was indicative of a 2.25 times higher slurry density in the reactor, a significant improvement over the initial process conditions. The improvement in process dynamics was likely due to the reduction in inhibition due to 7-ADCA by depleting it in the liquid phase, a phenomenon discussed in depth in Chapters 2 and 4 in this work.

5.3.2.2 Amoxicillin Pilot Runs

After the process was largely developed, the focus shifted to the continuous RC of amoxicillin trihydrate. The amoxicillin system poses many benefits over the cephalexin system, namely a greatly reduced solubility of the API (~7 mmol/L and ~35 mmol/L at pH 6.5 for amoxicillin and cephalexin, respectively). The lower solubility of amoxicillin in comparison to cephalexin results to greater levels of product isolation and lower waste production, a topic discussed in depth in Section 5.3.8. Additionally, the byproduct of amoxicillin synthesis, 4-HPG, possesses a much higher solubility than PG (110 mmol/L versus 30 mmol/L for 4-HPG and PG, respectively) [78, 84], resulting in a lower potential for byproduct precipitation and product contamination. The solubility of 6-APA, the nucleophile for amoxicillin synthesis, also possesses a higher solubility than 7-ADCA at neutral pH values [60, 94]. On the other hand, the acyl-donating species for amoxicillin, 4-HPGME, possesses limited solubility (~100 mmol/L at pH 6.5), resulting in the potential issue of substrate precipitation in the event of 4-HPGME accumulation, possibly due to high substrate feed rates or enzyme deactivation. The process model was applied as discussed in Section 5.3.1 to identify process operation conditions for the first amoxicillin
pilot experiment (Figure 5.9), which utilized a single MSMPR with an external wet milling loop (Figure 5.9(A)).
Figure 5.9: Summary of results from Run 15, the first continuous RC of amoxicillin trihydrate. (A) Process diagram of single MSMPR with external wet milling loop. Both product outlet and wet mill inlet travel through the size-based separator; (B) Liquid phase component concentrations over time measured by HPLC; (C) Offline micrograph of product crystals after 300 minutes process time; (D) FBRM counts, PVM turbidity, and productivity over time. T = 25°C, pH setpoint = 6.5, V = 300 mL.

After approximately 200 minutes, the liquid phase appeared to reach a state of control, while the solid phase required approximately 300 minutes of process operation until the productivity approached a steady value of ~550 g/L/d, a much higher value than the highest observed for the continuous RC of cephalexin (180 g/L/d) in Run 14, mostly due to the lower solubility of amoxicillin. Turbidity oscillations (Figure 5.9(D)), caused by intermittent wet milling cycles (15 minutes on, 5 minutes off), correlate strongly with FBRM fines counts (<10 μm), implying that wet milling contributes to fines generation to some degree. Additionally, most crystals observed in offline microscopy (Figure 5.9(C)) are smaller than 200 μm, and none observed were larger than 300 μm, indicating that the solid phase should be able to pass freely through the mesh sieve (300 μm). Even with crystals larger than the filter size, it is still possible for them to pass through the filter, given their needle-like morphology. The conversion of 6-APA and 4-HPGME were 55% and 75%, respectively, which were lower than process modelling predicted. The amount of amoxicillin present in the liquid phase (28 mmol/L) was much higher than expected solubility in deionized water (7 mmol/L) a pH value of 6.5 (Figure 5.8(B)). A higher
concentration of amoxicillin in the liquid phase is detrimental to process outcomes (e.g. productivity, selectivity, yield) in two ways: 1) Higher amoxicillin concentration in the liquid phase results in a higher rate of secondary hydrolysis catalyzed by PGA and 2) A larger quantity of amoxicillin present in the mother liquor after product filtration leads to reduced product yield. The accumulation of amoxicillin in the solid phase was determined to be initially caused by the impact of 6-APA and 4-HPGME on amoxicillin solubility, as discussed in Chapter 3. With the effect of substrates, the solubility of amoxicillin trihydrate at reactor conditions (100 mmol/L 6-APA, 80 mmol/L 4-HPGME, pH 6.5, 25°C) was 14 mmol/L, meaning that the effective supersaturation of amoxicillin was a value of 2 when the liquid phase concentrations stabilized. A supersaturated solution of amoxicillin during operation indicated that the process was crystallization limited, so a change in operating conditions or process configuration/design may be warranted to reduce the accumulation of amoxicillin in the liquid phase. Two methods to address amoxicillin accumulation in the liquid phase were proposed and tested: 1) An increase in MSMPR residence time to allow for amoxicillin crystallization and desupersaturation and 2) The addition of a second MSMPR downstream in series without enzyme to act as a crystallizer for amoxicillin desupersaturation. The depletion of amoxicillin in the liquid phase in the first MSMPR was desired, as it would lead to lower rates of secondary hydrolysis; however, if desupersaturation of amoxicillin could not be achieved in the first MSMPR by changing process conditions (e.g., feed concentrations, residence time, pH), the crystallization could be decoupled and optimized in the second MSMPR to enhance the yield of amoxicillin and reduce amoxicillin present in the product filtrate. The effect of increasing the residence time of the MSMPR was tested in Run 16 (Figure 5.10).
Figure 5.10: Summary results of Run 16, an experiment to test the effect of increasing the MSMPR residence time. (A) FBRM counts, PVM turbidity, and volumetric productivity over time; (B) Liquid phase concentrations of all components versus time, measured by HPLC of slurry filtrate; (C) 6-APA conversion over time (Red background data are after change in residence time). At t
= 400 minutes, the residence time of the MSMPR was increased from 90 to 120 minutes. T = 25°C, pH\text{setpoint} = 6.3, V = 300 mL.

Additionally, the operating pH of the system was decreased from 6.5 to 6.3 to slightly reduce the solubility of amoxicillin. At t = 400 minutes, the residence time of the MSMPR was changed from 90 minutes (the same as Run 15) to 120 minutes by reducing the inlet and outlet flow rates from 3.3 to 2.5 mL/min to allow greater time for amoxicillin crystallization. The conversion of 6-APA was initially lower due to lower activity of PGA at the lower pH value, but after the increase in residence time, the conversion of 6-APA reached nearly 50% (Figure 5.10(C)), at the cost of higher 4-HPG accumulation (while still below its solubility of 110 mmol/L). Most notably, the concentration of amoxicillin in the liquid phase was lower (~18 mmol/L) than Run 15 which was most likely due to the lower activity of PGA at lower pH values as well as decreased amoxicillin solubility. Changing the MSMPR residence time did not appear to significantly impact the accumulation of amoxicillin in the liquid phase, as indicated by the same liquid phase concentration of amoxicillin before and after the shift in residence time at t = 400 minutes (Figure 5.10(B)).

The effect of adding a second MSMPR in series to act as an amoxicillin crystallizer was tested via a continuous pH-swing amoxicillin crystallization (Figure 5.11).
Figure 5.11: Continuous pH-swing crystallization of amoxicillin trihydrate. Data represent liquid phase concentration of amoxicillin measured by HPLC. Feed conditions: 45 mmol/L amoxicillin, pH = 8.0, T = 4°C. First vessel conditions: T = 25°C, pH_{setpoint} = 6.3, V = 300 mL. Second vessel conditions: T = 4°C, pH uncontrolled, V = 200 mL. The first vessel was initially seeded with 2% (wt/vol) amoxicillin trihydrate (RIA, NJ). pH was controlled via the addition of 2 mol/L NaOH and a Mettler Toledo dosing unit connected to iControl software. An external wet milling loop was connected to the first vessel, with the same milling parameters as Runs 15 and 16.

The liquid phase concentration of amoxicillin was tracked using HPLC and a decrease in comparison to the feed of 45 mmol/L indicated depletion of amoxicillin from the liquid phase and crystallization into the solid phase. The data in Figure 5.11 indicate that even when the first vessel does not approach the solubility of amoxicillin at a pH value of 6.3 (7
mmol/L), indicating that the first MSMPR is crystallization limited, the addition of another hour of residence time within a second MSMPR in series allows for greater amoxicillin crystallization into the solid phase. The data in Figure 5.11 imply that the inclusion of a second MSMPR downstream in series with the main reactive crystallizer could allow for the concentration of amoxicillin in the liquid phase to deplete and approach its solubility. The inclusion of a PFR style crystallizer could also achieve the same effect, although the greater likelihood of encrustation and clogging within slurry transfer tubing could potentially reduce the process’s operational stability.

5.3.3 Enhancement of the amoxicillin RC process via process synthesis

After Run 16, a few issues remained with the operation of the continuous RC of amoxicillin trihydrate, the first being the remaining supersaturation of amoxicillin in the liquid phase even after implementing a higher MSMPR residence time (Figure 5.10(B)). In Run 17, we examined the effect of adding a second MSMPR in series downstream without enzyme to act as an amoxicillin crystallizer. A benefit of a two MSMPR system, with first a reactive crystallizer followed by a crystallizer, is the option for the partial decoupling of the reaction and crystallization. In other words, the second MSMPR may be operated at different conditions (e.g. temperature, pH, solvent composition) to improve the crystallization of amoxicillin. A summary of process data from Run 17 is shown in Figure 5.12.
Figure 5.12: Summary of process data for Run 17, the continuous RC of amoxicillin trihydrate with two MSMPRs in series; A) FBRM counts and turbidity vs. time; B) pH value and temperature vs. time; C) HPLC liquid phase concentrations in MSMPR-1; D) HPLC liquid phase concentrations in MSMPR-2. The dotted line marks the concentration of 6-APA in the liquid phase corresponding to 50% conversion; E) Solids productivity as isolated, post filtration and drying; F) Process
diagram with two MSMPRs in series. Process conditions: $T_{\text{MSMPR-1}} = 25^\circ\text{C}$, $T_{\text{MSMPR-2}} = 9^\circ\text{C}$, $pH_{\text{setpoint,MSMPR-1}} = 6.3$, $pH$ in MSMPR-2 uncontrolled, $V_{\text{MSMPR-1}} = 300\text{ mL}$, $V_{\text{MSMPR-2}} = 200\text{ mL}$. $F = 3.3\text{ mL/min}$.

The process operated for 12 hours with no major disruptions or disturbances. Temperature and pH control effectively kept the values of each at the desired set point in MSMPR-1, which was notable as both conditions impact the synthesis kinetics and stability of PGA [147]. Solid phase PAT indicated that the solid phase was at relatively steady operation after 450 minutes. The liquid phase concentrations in both MSMPRs reached steady values after approximately 300 minutes of process time (Figure 5.12(C-D)), and 6-APA and 4-HPGME conversions leveled off at values of approximately 50% and 60%, respectively. The productivity in MSMPR-1 reached a steady value of 550 g/L/d after 550 minutes, which was very close to the productivity achieved in Run 16. The amoxicillin concentration in the liquid phase of MSMPR-2 was 10 mmol/L, about 41% lower than its concentration in MSMPR-1. The decrease in amoxicillin in the liquid phase corresponded to a 5-7% increase in yield throughout the course of the pilot run. Overall, the inclusion of a second MSMPR was proven to facilitate further crystallization of amoxicillin and improved process yield. While the yield improvement was modest, the potential capacity of these processes if implemented industrially would be so large that even modest improvements in process outcomes (i.e., yield, selectivity) would result in a large decrease in amoxicillin/substrate waste in the product filtrate.
The second major issue to be addressed was the limited conversion of substrates, namely 6-APA as it is the more costly feedstock. The process model in Section 5.3.1 indicated that high substrate feed concentrations and higher enzyme concentration would lead to increased substrate conversions as well as higher productivity. The model also predicted greater accumulation of 4-HPG compared to lower feed concentrations, which could potentially precipitate and contaminate the product slurry, so care needed to be taken to avoid pushing process conditions too far. Higher substrate feed and enzyme concentrations (30% higher substrate, 50% higher enzyme) were tested in Run 18 (Figure 5.13).
Run 18 reached a state of control after a similar period of time as Run 17, as indicated by liquid phase concentrations stabilizing after roughly 360 minutes; however, the degree of accumulation of 4-HPG was significantly higher in Run 18 than Run 17 with 160-170 mmol/L versus 80-90 mmol/L, respectively. As a higher concentration of 4-HPGME is fed into MSMPR-1, it is expected that a higher concentration of 4-HPG will be found due to overall higher reaction rates, although the selectivity (ratio of amoxicillin synthesis to 4-HPG formation rates) was lower for Run 18 (Figure 5.13(F)). Lower selectivity implies that the increase in substrate concentration led to a higher rate of primary hydrolysis, possibly due to increased reaction rate increasing the observed diffusional limitations within the pores of the immobilization support. The rate of secondary hydrolysis was likely not increased significantly, due to a similar concentration of amoxicillin present in the liquid phase in comparison to Run 17. On a positive note, the overall rate of synthesis was increased in Run 18 compared to Run 17 (Figure 5.13(E)), due to the increase in 4-
HPGME feed, which resulted in a higher productivity of 760 g/L/d, 38% higher than Run 17. Higher productivity was expected due to a higher biocatalyst loading and increased 4-HPGME feed concentration, which has been shown in prior modelling [60]. Due to this, a higher slurry density of 4.5% (wt/vol) resulted, compared to 3.3%, which also resulted in a handful of minor clogging events within slurry transfer tubing (2 mm I.D.). Clogging events were corroborated by FBRM with sudden changes in counts (Figure 5.13(C&D)). Additionally, the conversion of both substrates was increased from 50% to 60% for 6-APA and 60% to 85% for 4-HPGME, although a large fraction of the increased conversion of 4-HPGME resulted in 4-HPG formation. The run, as is true for previous runs, generated high purity amoxicillin (>99% (wt)); however, the concentration of 4-HPG in the liquid phase (160-170 mmol/L) was higher than the pure component solubility at pH 6.3 and 25°C. No precipitation of 4-HPG was observed throughout the duration of the experiment, which was possibly due to multicomponent interactions increasing the effective solubility of 4-HPG, or its supersaturation was still in the metastable region and had yet to reach the critical limit for primary nucleation. Obviously, a tradeoff exists between higher productivity and selectivity, and selectivity may play a role in the purity of product. Process conditions such as in Run 18 may be suboptimal for operation due to poorer usage of 4-HPGME, which could make operation economically unviable.

5.3.4 Residence time studies
The residence time of the system was shown to significantly impact process outcomes such as substrate conversion and productivity, with large deviations in residence time potentially leading to byproduct or substrate accumulation and precipitation. The residence time of each MSMPR was determined in separate experiments. Generally, residence time studies are conducted by introducing a small amount of trace compound into the system, and a sensor to measure the effusion of the tracer either into or out of the vessel. In our case, we used attenuated total reflectance Fourier-transform infrared (ATR-FTIR) spectroscopy as the sensor, and sodium nitrate as the trace compound. Sodium nitrate was selected as a trace compound as it yields a linear correlation between its dissolved liquid phase concentration and IR peak area for its peak at roughly 1354 cm\(^{-1}\) up to 200 g/L (higher concentrations saturated the detector) and was easily detectable down to 5 g/L (Figure 5.14), allowing for reliable detection of the tracer for multiple residence times. Additionally, similar tracer experiments have been demonstrated, such as acetonitrile dissolved in water.
Figure 5.14: ATR-FTIR spectroscopy for measurement of sodium nitrate concentration in water. (A) ATR-FTIR spectra for a range of concentrations of sodium nitrate concentration; (B) IR peak area for peak at 1354 cm$^{-1}$ versus sodium nitrate concentration. Sodium nitrate was dissolved in deionized water. The peak
was integrated from 1277 to 1445 cm$^{-1}$ with a two-point baseline subtraction between the same range of wavenumbers. $T = 25^\circ C$.

Linear regression of the data in Figure 5.14(B) yielded the equation $c_{NaNO_3}(g/L) = 3.71 \times A - 1.09$, where $A$, the area of the peak in units of A.U. cm$^{-1}$, was used in to correlate changes in the size of the nitrate peak with the concentration of nitrate throughout the course of residence time experiments. For residence time studies, either a pulse or step change can be utilized. For pulse experiments, a tracer with a very low limit of detection is required; however, for step change experiments, a gradual change in concentration may be observed, allowing for a larger amount of data collection during an experiment, and higher confidence in the fit of average residence time [89]. The data in Figure 5.15 indicates that sodium nitrate is being flushed out of each MSMPR in each separate experiment.
A) Sodium Nitrate Concentration (g/L) vs. Time (hrs)

- MSMPR-1
- MSMPR-2

\[ c_{0,\text{feed}} = \begin{cases} 
  c_0 & t < 0 \\
  0 & t \geq 0 
\end{cases} \]

B) Image of experimental setup with labeled equipment:
- Feed tanks
- Pumps
- pH control
- Collection vessel
- MSMPR-1
- MSMPR-2
- Wet mill (connected to MSMPR-3)
Figure 5.15: Residence time experiments for MSMPR-1 and MSMPR-2 from the pilot plant apparatus. Both vessels were initialized with a known concentration of sodium nitrate dissolved in deionized water. $V_{\text{MSMPR-1}}=300$ mL; $V_{\text{MSMPR-2}}=200$ mL. (A) Sodium nitrate concentration versus time for MSMPR-1 and MSMPR-2. The solid curves represent fits of each set of data to the equation $c(t) = c_0 - c_0\left(1 - e^{-t/\tau_a}\right)$; (B) Pilot plant apparatus. The wet mill was connected to MSMPR-1 with a flow rate of 12 mL/min. The wet mill outlet and outlet from MSMPR-1 were withdrawn through a sieve of 300 μm using 2 mm I.D. Tygon tubing.

Each set of data for MSMPR-1 and MSMPR-2 yielded a close fit to the equation $c(t) = c_0 - c_0\left(1 - e^{-t/\tau_a}\right)$, indicating first-order well mixed dynamics for each vessel. The average residence times $\tau_a$ was 91 and 77 minutes for MSMPR-1 and MSMPR-2, respectively. The expected residence time for each was 90 and 67 minutes for MSMPR-1 and MSMPR-2, respectively. The deviation between the expected and measured residence time for MSMPR-2 was possibly due to error in pump calibration due to worn peristaltic tubing. Between either vessel, it is much more important that the residence time in MSMPR-1 is accurate due to the complex interplay between the enzyme kinetics and API crystallization; alternatively, MSMPR-2 only exists as a crystallizer, so deviations in the residence time of MSMPR-2 compared to intended residence time are unlikely to impact product quality, only product yield. While the data in Figure 5.15(A) indicate that the liquid phase is well mixed in both MSMPRs and exhibits first order dynamics with respect to
feed and withdrawal, the solid phase may still exhibit non-ideal mixing and withdrawal behavior, such as non-isokinetic withdrawal.

5.3.5 Substrate recovery and recycle

Throughout several pilot experiments for the RC of amoxicillin, limited conversion of approximately 50% and 60% for 6-APA and 4-HPGME, respectively, was achieved by PGA based on the difference in feed concentrations and remaining substrates in the product filtrate. Approximately 110 and 120 mmol/L of 6-APA and 4-HPGME, respectively, remain in the liquid phase and wasted in the mother liquor after product isolation. Based on Run 18, conversion can only be pushed higher with a tradeoff of lower selectivity and higher waste production, leading to less efficient use of 4-HPGME. An interesting observation discussed in Chapter 3 is the opposite sensitivities of 6-APA and 4-HPGME solubilities to the pH value in water, with each possessing minimum solubilities of 15 mmol/L at their respective isoelectric points. Due to these characteristics, it was hypothesized that two separate crystallizations may be conducted with the remaining mother liquor by increasing the pH value to a more basic environment near the pI of 4-HPGME, crystallizing 4-HPGME and isolating it, and then lowering the pH value to a more acidic environment near the pI of 6-APA, crystallizing 6-APA and isolating it. The remaining mother liquor should contain amoxicillin, 6-APA, and 4-HPGME near their minimum solubilities, as well as the accumulated 4-HPG. Interestingly, the concentration of 4-HPG observed in the liquid phase during Run 17 (80 mmol/L) was lower than its
minimum solubility (110 mmol/L, discussed in Chapter 3) in water (regardless of pH value), so the risk of 4-HPG precipitation during the substrate recovery process was low.

The concept of substrate recovery was first modeled to test its theoretical impact on conversion. The effect of pH on the solubility of 4-HPGME and 6-APA was modeled using the equation [93]

$$c^*_j = c^*_{pl,j} \left(1 + \frac{10^{-pK_{a,j}}}{10^{-pH}} \right)$$

(5-2)

where $pK_{a,j}$ represents the acid dissociation constant for component $j$ and $c^*_{pl,j}$ represents the solute solubility at the isoelectric point. The $pK_a$ values of 6-APA[78] and 4-HPGME are 4.83 and 6.99 and the $c^*_{pl}$ values are 20 mmol/L and 14 mmol/L, respectively, and were discussed in Chapter 3. It should be noted that the exponential fraction in Equation 1 must be inverted for 4-HPGME as its solubility exhibits the opposite sensitivity to pH compared to 6-APA. The overall conversion was calculated using the equation

$$Overall\ conversion = \frac{m_{reacted,j}}{m_{initial,j} - m_{recovered,j}}$$

(5-3)

where $m_{reacted,j}$ corresponds to the mass of component $j$ reactant converted and was calculated by taking the difference between the feed solution and mother liquor concentrations following product isolation, with each concentration multiplied by the respective volume, $m_{initial,j}$ the mass initially fed into the system and $m_{recovered,j}$ the mass recovered after recrystallization, which was calculated by subtracting the initial mass of substrate in the mother liquor and mass at its solubility at a specified pH value. These
equations were applied over the pH range of 3–10 for both amoxicillin substrates (Fig. 5.16).

Figure 5.16: (A) Aqueous solubility of 6-APA and D-4-HPGME versus pH value at 25°C, as fit by Equation (5-2). (B) Overall conversion of 6-APA and D-4-HPGME versus recrystallization pH value. Gray dashed circles are shown at values of overall conversion for each substrate at their respective isolation pH values selected for experimental verification. Feed solutions were 240 mmol/L 6-APA and 234 mmol/L D-4-HPGME and the single pass conversion of both was set to 50% ($m_{reacted,j} = 0.5m_{initial,j}$).

At the operating pH value for the process of 6.3, both components exhibit modest solubilities (100 mmol/L for D-4-HPGME, >200 mmol/L for 6-APA). As each substrate’s
solubility exhibits opposite sensitivities to pH, supersaturation can be driven independently in two separate crystallizations to produce pure recrystallized substrates. The recrystallization of D-4-HPGME was chosen first as 6-APA exhibits its optimum stability in solution at a pH of 8 [148], roughly where the solubility of D-4-HPGME levels off. Otherwise, if the recrystallization of 6-APA was conducted first, the natural hydrolysis of D-4-HPGME may proceed in solution, reducing the recovery yield. Next, the recrystallization of 6-APA could proceed in the D-4-HPGME depleted solution at a pH value of approximately 4.0, where its solubility reaches its minimum. After the last recovery step, all components present in the liquid phase should be near their minimum solubility, other than the byproduct D-4-HPG, thereby sending the minimum possible concentration of substrates and product out of the system in the waste stream. Figure 5.16(B) indicates that theoretically both components can achieve overall conversions greater than 90% with the use of substrate recovery, even with only 50% single pass conversions for each (observed in Run 17); however, this analysis does not take into account the possibility of cosolute interactions impacting the solubility and crystallization of D-4-HPGME and 6-APA.

The recovery of 6-APA was first tested in an aqueous solution simulating the filtrate following product isolation of the continuous process (Figure 5.17).
Figure 5.17: 6-APA recrystallization in deionized water with cosolutes present. (A) Liquid phase concentration of 6-APA over time with different cosolutes present; (B) *In situ* microscopy of 6-APA recrystallization in presence of cosolutes; (C) *In situ* microscopy of pure component 6-APA in deionized water. T = 13°C, pH_{final} = 4.0, V = 200 mL, c_{4-HPGME} = 20 mmol/L, c_{Amoxicillin} = 10 mmol/L, 1.2 g of 6-APA seeds were added.

For all experiments, the rate of crystallization was fast at the beginning, indicated by the sharp initial decrease in liquid phase 6-APA concentration, and most of the crystal growth occurred within 20 minutes (Figure 5.17(A)). With pure 6-APA, the concentration of 6-
APA in the liquid phase nearly reached its solubility at a pH value of 4.0 (15 mmol/L). With both cosolutes present (4-HPGME and amoxicillin), the rate of crystallization was decreased significantly, with the concentration of 6-APA remaining in the liquid phase at 36 mmol/L, implying some level of inhibition of 6-APA nucleation and/or growth was present. By eliminating each cosolute and repeating, it was determined that amoxicillin was responsible for the inhibition of 6-APA crystallization, as no inhibition was observed when only 6-APA and 4-HPGME were present in the liquid phase. The nature of inhibition was unclear, although a shift in morphology of the crystals was observed, with many small needle-like crystals forming along with the larger, cubic seeds (Figure 5.17(C)). It is possible that due to 6-APA and amoxicillin possessing similar functional groups, amoxicillin interacts and inhibits growth on the surface of 6-APA crystals. These smaller needle-like crystals were not observed during 6-APA crystallization absent of amoxicillin (Figure 5.16(B)). It should be noted that all solid 6-APA crystals isolated were pure (>98.5% (wt)), so the likelihood of cocrystal formation with amoxicillin was low. To investigate the possible formation of a separate polymorph of 6-APA, possibly exhibiting higher aqueous solubility, powder X-ray diffraction (PXRD) was conducted on solid samples isolated after air drying isolated 6-APA from the experiments in Figure 5.17 (Figure 5.18).
Figure 5.18: PXRD diffraction patterns of 6-APA seeds (RIA, NJ), 6-APA recrystallized in pure deionized water, and 6-APA recrystallized in the presence of cosolutes (amoxicillin & 4-HPGME).

All samples exhibited diffraction patterns with well-defined peaks, indicating that the crystals were highly crystalline, eliminating the possibility of oiling out or amorphous precipitate formation. The three samples examined also exhibited similar diffraction patterns, implying that all are comprised of the same polymorph and the presence of cosolutes did not cause the formation of a separate polymorph of 6-APA. The varying of peak intensity across samples is typically due to small differences in sample loading onto the sample tray.
It is possible that the crystallization of 6-APA during its recovery is inhibited by a change in its solubility in the presence of amoxicillin. The solubility of 6-APA was measured in the presence of amoxicillin and 4-HPGME using the same methods discussed in Chapter 3 (Figure 5.19).

**Figure 5.19:** Solubility of 6-APA in presence of cosolutes (10 mmol/L amoxicillin, 110 mmol/L 4-HPGME) in comparison to pure component pH curve (from [83]). Green data point corresponds to liquid phase concentration of 6-APA after 90 minutes from data in Figure 5.17. T = 13°C.

The data in Figure 5.19 suggest that the solubility of 6-APA may be changed slightly in the presence of cosolutes, but not to the extent observed in the recovery experiments in Figure 5.17(A). Since it appears that the concentration of 6-APA is still decreasing at t =
90 minutes in Figure 5.17(A), the phenomenon of 6-APA crystallization inhibition is likely not a thermodynamic limitation, such as in the case of elevated solubility. While it might be possible to wait a long period of time for the concentration of 6-APA to deplete, the stability of 6-APA at acidic pH values is not high, so it was desired to improve the crystallization such that desirable yields are achieved in 1-2 hours. Another possible explanation for the limited crystallization of 6-APA is the inhibition of crystal nucleation and/or growth. To examine this theory, two approaches were taken. First, the number of seeds were varied from 10-50% of the equivalent mass of supersaturated 6-APA. Second, wet milling during the crystallization was investigated. Both strategies were employed to examine the impact of increased growth facets for crystal growth on 6-APA crystallization (Figure 5.20).
Figure 5.20: (A) Effect of seeding density on 6-APA recovery. 10, 20, 30, 40, 50% seeds correspond to 0.4, 0.8, 1.2, 1.6, 2.0 g 6-APA seeds, respectively; (B) Effect of wet milling on 6-APA recovery, with two different seed densities. $T = 13^\circ C$, $pH_{final} =$
\[ V = 200 \text{ mL}, \ c_{4\text{-HPGME}} = 20 \text{ mmol/L}, \ c_{\text{Amoxicillin}} = 10 \text{ mmol/L}. \] For milling experiments: IKA Magic Lab, MK Module, 4000 RPM, \( F_{\text{mill}} = 12 \text{ mL/min}. \]

The data in Figure 5.20(A) indicate that increased seeding density increased the yield of 6-APA crystallization in the presence of cosolutes. After 30% seeding was reached, any increased seeding (up to 50%) yielded roughly the same final concentration of 6-APA in the liquid phase of roughly 36 mmol/L. The improvement in crystallization yield was possibly due to an increase in growth facets, promoting crystal growth. It is possible that multiple growth facets are available for growth when 6-APA is crystallizing in pure deionized water, but in the presence of amoxicillin, one or more of the growth facets are inhibited. The liquid phase interactions between amoxicillin and 6-APA is discussed more in depth in Chapter 3, and it was shown that the nucleation/growth of amoxicillin crystals is not inhibited by 6-APA, but it is possible that amoxicillin interacts in some way with the surface of 6-APA crystals, blocking the advancement of growth facets. The data in Figure 5.20(B) indicate that with wet milling present, higher crystallization yields may be achieved even with lower seed densities. Logically, the effect of increasing surface area for crystal growth by crystal breakage and attrition achieves a slightly better result as adding more seeds (33 mmol/L final 6-APA concentration). With the final optimized 6-APA crystallization with 10% seeding and wet milling, the overall conversion of 6-APA was improved to 87%, much improved over the 50% single-pass conversion.

Next, the recovery of 4-HPGME was investigated. As 6-APA possesses higher stability at a pH value of 8.0, the proposed pH value for 4-HPGME recovery, it was
proposed that 4-HPGME recovery should take place first in the recovery process. Initial 4-HPGME recovery experiments, where the pH was changed quickly to a value of 8.0 using 6 mol/L NaOH, yielded highly aggregated crystals which filtered poorly and yielded cracked filter cakes, possibly due to poor crystal packing within the cake. Cracked filter cakes reduced the washing efficiency of the cakes, leading to the formation of a significant 4-HPG impurity (4% (wt)). If the 4-HPGME was left unwashed, the impurity content of 4-HPG exceeded 10%, which was deemed unacceptable for re-dissolution and recycle back into the process as fresh feed. Literature evidence suggests that filter cake consolidation improves the formation of the cake, although it requires a pressurized filtration system[149]. By decreasing the rate of supersaturation generation of the pH-swing crystallization via decreased NaOH addition, the degree of aggregation was lowered (Figure 5.21).
A) Liquid phase 4-HPGME Conc. (mM) vs Time (min)

- 3 mL/h
- 6 mL/h
- Immediate

Solubility

B) 4% 4-HPG
96% 4-HPGME

C) <1% 4-HPG
>99% 4-HPGME
As expected, slower rates of NaOH addition resulted in slower rates of 4-HPGME crystallization, as indicated by the slower decrease in its liquid phase concentration (Figure 5.21(A)); however, the decreased rate of supersaturation generation led to a lower degree of aggregation, which in turn improved the filterability of the product and eliminated cake channelling (Figure 5.21(C)). Additionally, the final crystallization yield was not impacted by the rate of base addition since all experiments were nearly at the solubility of 4-HPGME. Moreover, unlike in the case of 6-APA, the presence of cosolutes did not appear to result in a supersaturated solution at the end of the crystallization. Along with the improvement in recovered 4-HPGME purity by washing, a further improvement from 96% to >99% (wt) was observed by eliminating channelling. Additionally, by including the recovery process, the single-pass conversion of 4-HPGME was improved from 60% to an overall conversion of 92%. The main disadvantage of this improved recovery process was the increased time requirement, and in turn a possible increase in the total amount of natural hydrolysis of 4-HPGME observed due to extending the time that 4-HPGME is dissolved in water.

For 6-APA and 4-HPGME, the impact of their recovery and recycle on the environmental sustainability and efficiency of the continuous amoxicillin RC process, as
well as comparison to the batch enzymatic process and traditional chemical process is discussed more in depth in Section 5.3.7, but overall, the conversion of each was increased from 50 to 87% and 60 to 92% for 6-APA and 4-HPGME, respectively. This improvement in conversion of each substrate greatly improves the efficiency of substrate use for the amoxicillin crystallization process; however, it does increase the process complexity as well by adding two more crystallizations and two more isolation steps.

5.3.6 Long-term process stability

Until Run 19, all continuous pilot experiments conducted were a maximum of 18 hours long (12 residence times) for the amoxicillin system. A significant amount of insight into the operation, improvement, and performance of the continuous RC process for both amoxicillin and cephalexin was gained during these experiments; however, knowledge of the stability of the process long-term would provide useful information regarding the industrial viability of such a process, as well as what challenges may need to be addressed for its viability. For example, two examples significant considerations of longer-term operation are biocatalyst stability and clogging/encrustation. In Run 19, the amoxicillin continuous RC process was operated for 72 hours continuously (Figure 5.22) under the same conditions described in Run 17.
Figure 5.22: Summary of data from Run 19, a continuous RC of amoxicillin over a 72-hour time period. (A) Turbidity, measured via in situ microscopy; (B) FBRM counts for three chord length ranges (<10 μm, 10-100 μm, 100-1000 μm); (C) Liquid phase component concentrations for MSMPR-1; (D) Liquid phase component concentrations for MSMPR-2; (E) Amoxicillin productivity; (F) Synthesis selectivity, estimated by the ratio of 6-APA consumption to 4-HPG production.
$T_{\text{MSMPR-1}} = 25^\circ\text{C}, T_{\text{MSMPR-2}} = 7^\circ\text{C}$, $pH_{\text{setpoint,MSMPR-1}} = 6.3$, pH not controlled in MSMPR-2. Feed concentrations: 240 mmol/L 6-APA, 350 mmol/L 4-HPGME.

Residence time of MSMPR-1 was increased from 90 min to 150 minutes at $t = 52$ hours.

Overall, the process was operated for 72 hours without any major process upsets (e.g., product contamination) requiring process shutdown; however, a handful of clogs developed in the Tygon tubing used to transfer slurry, usually in the tubes in the external wet mill loop connected to MSMPR-1, approximately once per 24-hour period. Similar to previous runs, the oscillations in turbidity (Figure 5.22(A)) were correlated with FBRM fine counts (Figure 5.22(B)). Additionally, the large chord length bin (100-1000 μm) appears inversely correlated with the mid-size chord length bin (10-100 μm), possibly due to crystal growth from the smaller to larger bin or due to the “snowstorm” effect in FBRM, where there is a bias in detecting smaller chords due to the propensity of smaller crystals to be situated closer to the probe window. After approximately 24 hours, the concentration of substrates accumulated until $t = 52$ hours (Figure 5.22(C&D)), when the residence time of MSMPR-1 was changed to 150 minutes by adjusting the volumetric flow rate from 3 mL/min to 2 mL/min. The accumulation of 6-APA and 4-HPGME in the liquid phase of both MSMPRs was evidence of enzyme deactivation, or reduction in activity, over time. As the rate of PGA thermal deactivation at $25^\circ\text{C}$ is nearly negligible over a period of 72 hours, the observed reduced activity of PGA was likely due to some other phenomenon. The data in Figure 5.22(E&F) indicate that as the productivity declined over the course of
the experiment after the first 24 hours, the selectivity of amoxicillin synthesis declined as well. While PGA deactivation would explain a reduction in productivity, a reduction in selectivity implies that in some way the behaviour of the enzyme in the supports had changed. For example, it is possible that blockage of the pores would decrease the effective diffusivity of the components in the enzyme support, reducing the efficiency (i.e., activity, selectivity) of PGA. In situ microscopy images shown in Figure 5.23 support the hypothesis that blockage of pores slows the rate of diffusion within pores due to the formation of crystals on the surface of the spherical support particles.
Figure 5.23: (A) In situ microscopy in MSMPR-1 of Run 19. The size of each image is 1 mm². Spherical enzyme support particles are outlined in red; (B) Purolite amino-C6 support particles after Run 19; (C) Support particles after stirred in 300 mL of 50 mmol/L sodium phosphate, pH 7.5 for 2 hours.

Although some images were collected which showed particles not significantly coated, a large fraction was similar to those displayed in Figure 5.23 with crystals appearing to coat the surface of the support particles. It is also possible that PGA deactivation was observed
due to attrition of the support particles by shear generated by the impeller, although this would not explain a reduction in selectivity over time. After Run 19 was completed, the support particles were collected and examined. Many of the particles were visually white in color, the same color as amoxicillin crystals, while fresh support particles were brownish orange. After washing in sodium phosphate buffer at a pH value of 7.5, particles visually appeared the same color as when they were initially loaded. Throughout washing, the pH value decreased due to dissolution of amoxicillin, which was confirmed via HPLC analysis of the liquid phase. Based on the remaining productivity in Figure 5.22(E) and assuming a linear relationship between enzyme activity and productivity, 54% of enzyme activity was lost from $t = 24$ hours to $t = 72$ hours, with 46% activity remaining compared to fresh PGA-loaded supports; however, upon washing, the beads returned to 78% of their initial activity. An increase in activity following washing indicates the deactivation was partially reversible by dissolution of the crystals that were blocking the pores.

Overall, the process operated for 72 hours without complete deactivation, although the concentration of 4-HPGME accumulated to almost 190 mmol/L, approximately 80 mmol/L higher than its pure component solubility at the conditions of MSMPR-1. Despite operating the process in a better state of control to avoid such significant substrate accumulation and reduction in productivity, it is likely that some quantity of enzyme would need to be added throughout the course of process operation. On the other hand, this would not fully make up for the reduction in selectivity of the enzyme already present in the MSMPR-1, and a cycle would need to be devised to properly withdraw the enzyme support from the MSMPR and reload fresh or reactivated enzyme. As the supports settle in water much faster than the crystals, it is possible that a gravity-based separation could be devised,
where the enzyme support is withdrawn from the bottom of the reactor and the remaining
slurry retained within the reactor. Additionally, a significant degree of encrustation was
observed on the inner surface of the slurry transfer tubing (2 mm I.D. Tygon) as well as
the inlet to the wet mill. It is likely that tubing would need to be switched periodically
throughout the course of a run to avoid significant clogging issues. As the wet mill is
essential for maintaining a proper separation in size between crystals and enzyme support,
it is critical to ensure that encrustation does not accumulate such that the operation of the
wet mill is hampered.

5.3.7 Green chemistry analysis of processes in comparison to traditional chemical
processes

The data in Figure 5.24 were collected during a 12-h run of the continuous
enzymatic RC pilot plant and were used to evaluate the greenness of the process, and are
the same data collected for Run 17.
Figure 5.24: Process data for the pilot-scale continuous enzymatic RC of amoxicillin trihydrate. A) FBRM counts and turbidity vs. time. B) pH value and temperature vs. time. C) Offline microscopy images of crystals. D) HPLC liquid phase concentrations in MSMPR-1. E) HPLC liquid phase concentrations in MSMPR-2. F) Solids productivity as isolated, post filtration and drying.

The FBRM counts in Figure 5.24(A) indicated the solid phase reached a state of control after roughly 550 min (approximately 6 residence times in MSMPR-1), which is corroborated by the solids productivity (Fig. 5.24(F)). Tight control of pH and temperature
is achieved (illustrated in Fig. 5.24(B)), which is required for enzyme performance and stability. HPLC data indicated that the liquid phase of the system reached a steady state after approximately 400 min (~2.7 residence times of the entire system) (Fig. 5.24(D-E)). Additionally, the immobilized enzyme was tested prior to and after the pilot run and retained about 78% of its initial activity. Reuse of the same batch of immobilized enzyme in future runs did not further reduce the activity, within measurement accuracy.

The Green chemistry metrics of the amoxicillin process with and without raw material (RM) recovery, with comparisons to relevant similar processes are shown in Table 5.4. More details regarding these calculations may be found in the Appendix B. The complexity of the chemical process is a value of three, due to the addition of protection and deprotection steps in addition to the coupling step, while the complexity of the biocatalytic process is a value of one.
Table 5.4: Green chemistry metrics for amoxicillin synthesis via various routes.

Batch-PGA-RC and Cont-PGA-RC with and without raw material (RM) recovery data were collected in this work.

<table>
<thead>
<tr>
<th>Process</th>
<th>Chemical</th>
<th>Batch-PGA-RC</th>
<th>Cont-PGA-RC Without RM recovery</th>
<th>Cont-PGA-RC with RM recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atom Economy (%)</td>
<td>35</td>
<td>85</td>
<td>85</td>
<td>85</td>
</tr>
<tr>
<td>Reaction Mass Efficiency (%)</td>
<td>--</td>
<td>0.368</td>
<td>0.343</td>
<td>0.572</td>
</tr>
<tr>
<td>Process Mass Intensity</td>
<td>50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.0</td>
<td>20.1</td>
<td>20.7</td>
</tr>
<tr>
<td>Aqueous Mass Intensity</td>
<td>&lt;5</td>
<td>22.3</td>
<td>17.2</td>
<td>17.8</td>
</tr>
<tr>
<td>Relative Process Greenness&lt;sup&gt;b&lt;/sup&gt; (%)</td>
<td>156</td>
<td>113</td>
<td>136</td>
<td>132</td>
</tr>
<tr>
<td>Scorecard&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Average&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Avg/Good&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Avg/Good&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Avg/Good&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> From [150], amoxicillin production in 1975

<sup>b</sup> Adapted from [141].

<sup>c</sup> Based on commercial-stage development
The results in Table 5.4 indicate that the reaction mass efficiency of the continuous process is not competitive with the batch process unless RM recovery is incorporated. This limitation is due to the operation of the MSMPRs at outlet conditions, in which amoxicillin accumulation leads to elevated rates of amoxicillin hydrolysis, termed secondary hydrolysis, so that the synthesis/hydrolysis (S/H-) ratio (i.e., selectivity) at the operating point is lower than the average selectivity over the course of a batch RCn. A batch reactor maintains a lower product-to-substrate ratio in the liquid phase at the beginning of the batch, leading to higher selectivity of the enzyme which tapers off towards the end of the batch as product accumulates and substrates deplete. Additionally, the waste production of the continuous process is lower, as is indicated by a lower PMI, due to higher substrate concentration in the feed. For a batch process, both substrates must be dissolved for synthesis to proceed, with solubilities at pH 6.5 of approximately 95 and 60 mmol/L for 6-APA and 4-HPGME, respectively. For the continuous process, however, because the vessel operates at outlet conditions, we were able to feed an equivalent 220 and 300 mmol/L of 6-APA and D-4-HPGME, respectively, in two separate streams at conditions that promote higher solubility of each component (pH 8.0 for 6-APA and pH 4.0 for D-4-HPGME), greatly reducing water usage. The batch process resulted in a similar volumetric productivity of 497 g/L/d compared to 530 g/L/d for the continuous process; however, this analysis does not consider time between batches to empty, sterilize, clean, and refill the vessel and dissolve fresh substrates, so the effective productivity of the continuous process is likely much higher. Although a higher reaction rate is expected for a batch process
compared to a CSTR-type continuous reactor operating at outlet conditions, PGA experiences inhibition due to 6-APA, a phenomenon observed by McDonald et al. during ampicillin synthesis [60], leading to lower reaction rates and consequently lower productivities until 6-APA has been partially depleted in the liquid phase.

Both batch and continuous processes have the potential to incorporate substrate recovery following product isolation, although the continuous process is more suited due to the higher concentrations of 6-APA and D-4-HPGME remaining in the mother liquor (50 mmol/L and 110 mmol/L for the batch and continuous processes, respectively), which is why we decided to demonstrate the idea for only the continuous flow experiment. By including substrate recovery, the RME of the process improves significantly (67% higher) due to more efficient usage of substrates; however, the PMI and AMI are slightly higher due to the addition of water and salt in the form of aqueous sodium hydroxide and hydrochloric acid required to change the pH value of the solution and drive supersaturation for the recrystallizations. Additionally, IPA was required to wash the D-4-HPGME filter cake to liberate the cake of water and reduce the natural hydrolysis of the methyl ester.

Lastly, the relative process greenness (RPG), a measure of waste production relative to the complexity of a process, indicates that RPG for the current continuous enzymatic process (136%) is on par with the established chemical process (156%). However, the chemical process is evaluated at a much later stage of development compared to the RC process demonstrated here, which results in a lesser attribute scorecard association (‘Average/Good’ vs. ‘Average’) [141]. Also, the chemical process incorporates three reactions steps (protection, coupling, deprotection), which inflates the RPG in comparison to the more desirable one-step process, such as in the case of the enzymatically-catalyzed
system. It should also be noted that most of the waste produced by the chemical process is in the form of organic solvents, primarily dichloromethane (DCM), and methanol, with the former posing a significant environmental hazard. However, for the continuous enzymatic process, we generate a smaller amount of waste per unit of product, mostly in the form of water (as indicated by comparing the aqueous mass intensity to the PMI), which has been shown to be readily treated for beta-lactam antibiotics using reverse osmosis (RO) [151]. The main tradeoff of including a water recycle process is the required input of energy to operate separation units, although RO processes require relatively low energy demand. On the other hand, waste may be generated in the form of a much more concentrated purge stream, recycling back pure DI water for further use.

For the continuous cephalexin process (shown in Figure B.1), the process operation data can be seen in Figure B.2. A summary of the green chemistry metrics is shown in Table 5.5.
Table 5.5: Green chemistry metrics for the continuous RC of cephalexin.

<table>
<thead>
<tr>
<th>Reaction mass efficiency</th>
<th>Process mass intensity</th>
<th>Aqueous mass intensity</th>
<th>Relative process greenness</th>
<th>Scorecarda</th>
</tr>
</thead>
<tbody>
<tr>
<td>Continuous cephalexin RC</td>
<td>0.193</td>
<td>60.6</td>
<td>53.7</td>
<td>44</td>
</tr>
</tbody>
</table>

a Adapted from [141].

b Based on late stage-development

Unfortunately, no metrics for the chemical production of cephalexin have been reported in literature, so a comparison of results to the traditional production pathways is not possible. Regardless, the production of cephalexin appears to be much less efficient than amoxicillin, as indicated by a low RME. Additionally, a greater amount of total waste is produced due to the large amount of cephalexin still present in the mother liquor post product isolation (~70 mmol/L, 24 g/L) due to its higher solubility in water in comparison to amoxicillin. We observed that the presence of higher concentrations of PGME increased the apparent solubility of cephalexin, as well as an effect of higher ionic strength, tested by measuring solubility in the presence of aqueous NaCl mixtures (Fig. B.3). The higher solubility of cephalexin led to a decrease in isolation efficiency and overall increase in the PMI and AMI due to the decrease in mass of product isolated. While the synthesis of cephalexin using PGA is more selective [72], the greater amount of cephalexin present in the liquid
phase in comparison to amoxicillin led to higher rates of secondary hydrolysis observed in the reactor. Also, the solubility of the byproduct in the cephalexin system, D-phenylglycine, is sparingly soluble in water (~30 mmol/L), leading to limitations in feed concentrations, enzyme concentration, and residence time. The byproduct of amoxicillin hydrolysis, D-4-hydroxyphenylglycine (4-HPG), has a much higher solubility (~100 mmol/L) and will not precipitate until its concentration reaches nearly 150 mmol/L. The higher solubility of 4-HPG allows for greater flexibility of process conditions yielding better green chemistry metrics. Overall, the relative greenness of the continuous cephalexin process is poor, as indicated by an RPG of 44%. The possible use of an antisolvent, such as ethanol or methanol, to decrease the apparent solubility of cephalexin in the MSMPR may be beneficial to improve the separation efficiency as well as the enzyme performance.

5.4 Conclusions

The development of a continuous enzymatic reactive crystallization of amoxicillin trihydrate and cephalexin monohydrate on a pilot scale was described. PG accumulation during continuous cephalexin pilot experiments were addressed by reducing biocatalyst support sizes, improving synthesis selectivity. The volumetric productivity of the cephalexin process was increased by raising the residence time from 90 minutes to 150 minutes due to reduced inhibition by 7-ADCA at higher substrate conversion. The amoxicillin process generated three times higher productivity than the cephalexin process, but amoxicillin was supersaturated due to the process being crystallization limited. Amoxicillin crystallization was partially decoupled from reaction by including a second
MSMPR in series, allowing for amoxicillin supersaturation to deplete completely. Substrate recovery was investigated to improve the overall conversion of 6-APA and 4-HPGME while maintaining higher productivity. Additionally, modelling predicted that higher concentrations of substrate and enzyme would result in higher amoxicillin productivity and substrate conversions, albeit with increased byproduct accumulation. The long-term process stability was evaluated during a 72-hour continuous amoxicillin RC experiment, with a continual decline in both productivity and selectivity observed from 24 to 72 hours, likely due to pore blockage of the enzyme support by amoxicillin crystals, which was partially reversible by washing the blocked support. The continuous enzymatic process yielded greater reaction mass efficiencies than the batch enzymatic process only when reactant recovery was incorporated. The waste generated by the continuous enzymatic process was much less than the batch enzymatic process, most likely due to the lower feed concentrations for substrates in the latter case. Additionally, the composition of the waste for the continuous enzymatic case was much more environmentally favorable than that produced by the chemical route; possibly, the aqueous waste from the enzymatic processes could be recycled for further use. In comparison to the amoxicillin system, the cephalexin system yielded lower green chemistry metrics, mostly due to the high solubility of the product leading to greater waste discharge in the mother liquor and lesser product isolated in the solid phase. The energy consumption of both processes was low and likely would not contribute significantly to operating costs. The described process may be suitable for development and use on a large scale, as it poses benefits to the sustainability and capacity of production; however, the scalability of certain aspects of the process must also be considered, such as the size-based separator, mixing in the MSMPRs, and the flow
of slurries in tubing or pipes. The implementation of a PGA-catalyzed reactive crystallization process with reactant recovery and recycle could potentially improve the environmental footprint and cost of manufacture for amoxicillin, the largest antibiotic produced worldwide, and in turn improve access to this vital front-line medicine to those affected by shortages.
CHAPTER 6. Impact of disturbances on process operational stability, with insight into the use of process analytical technology

6.1 Introduction

The importance of maintaining a stable process at conditions that ensure high quality product cannot be understated. Numerous studies have been conducted involving the control of crystallization processes, which are often difficult systems to control due to inherent nonlinearities and higher order processes [152-154]. With CM, if a process is operated in a state of control, the likelihood of off-specification product being produced is lowered significantly [42, 43, 154]. During CM, a continuous stream of product is produced, and if at some point the process begins generating off-specification product, the product stream must either be diverted or, in a worst-case scenario, all product downstream sequestered. Two strategies can be used to avoid off-specification product contaminating on-specification product downstream: 1) Process monitoring (i.e., PAT) may be used to determine when conditions arise that promote the formation of off-specification product, and the product stream may be immediately diverted [44] and 2) Process design and control may be applied to ensure the process never reaches a state such that off-specification product is produced [155]. In Chapter 5, the operation of the continuous RC of amoxicillin and cephalexin was demonstrated for short and longer time scales during idealized scenarios which were absent of significant disturbances in input variables or controlled variables. As discussed in Section 5.3.1, the accumulation of substrates or byproducts to levels above their respective solubilities could lead to their precipitation and contamination of the product slurry. Additionally, in Chapter 4, the importance of wet milling was
discussed in the context of the efficiency of the size-based separator utilized for enzyme retention and product withdrawal. Process parameters affecting enzyme performance, and in turn the accumulation or depletion of substrates and byproducts, are likely sources of disturbances that may impact process performance and/or product quality. Additionally, disturbances impacting separator performance (e.g., loss of wet milling) could also be theorized to jeopardize process stability. In this chapter, the impact of disturbances on the operational stability of the continuous RC of amoxicillin is investigated via modelling and experimentally. Additionally, as the ability to monitor the process may be used to detect and correct for disturbances, investigation of the use of various PAT for process monitoring is conducted.

6.2 Materials and Methods

Materials

Amoxicillin trihydrate and 6-aminopenicillanic acid (6-APA) were obtained from RIA International LLC (East Hanover, NJ); D-4-hydroxyphenylglycine methyl ester (4-HPGME) from Wuhan Golden Wing Industry & Trade Co. (Wuhan, China); D-4-hydroxyphenylglycine (4-HPG) from TCI America (Portland, OR).

Experiments concerning Raman and ATR-FTIR were conducted at constant temperature (T=25°C) in a 250 mL vessel in a Mettler Toledo (Columbus, OH) Optimax system, stirred at 300 RPM to ensure it was well-mixed. Raman spectra were measured using a Mettler Toledo ReactRaman system with a 785 nm laser at laser power of 300 mW.
The exposure time was varied from 5 s to 10 s based on the concentration of components measured and whether or not a solid phase was present. IR spectra were recorded using a Mettler Toledo ReactIR 10 system. A Mettler Toledo FBRM was used to monitor the solid phase. A pH electrode (InLab Semi-Micro-L) from Mettler Toledo was used to monitor the pH value of the solution.

Methods

For the measurement of spectra, the vessel was charged with 200 mL of deionized water. The temperature of the liquid phase was set to 25°C and was maintained automatically by the Optimax heating/cooling jacket. Solid components were added to the top of the vessel using a funnel. Dissolution of amoxicillin and 6-APA was promoted using 2 mol/L NaOH and dissolution of 4-HPGME was promoted using 2 mol/L HCl. For all experiments, a pH value of 6.50±0.05 was reached before Raman and IR determination and the pH was lowered using 2 mol/L HCl and raised using 2 mol/L NaOH. For homogenous samples, complete dissolution of solute(s) was confirmed with FBRM (<50 total counts).

6.3 Results

6.3.1 Disturbance testing

The continuous RC model described in Chapter 5 was used to simulate pump disturbances, or changes in pump flow rates, on the concentration of various components
in MSMPR-1. Additionally, as enzyme activity is difficult to monitor instantaneously throughout the course of the process experimentally, the model was useful in determining the impact of enzyme deactivation. As the cephalexin system was susceptible to precipitation of 7-ADCA and PG and the amoxicillin system was much less susceptible to byproduct or substrate precipitation, the cephalexin system was the focus of this study. The data in Figure 6.1 were simulated using the continuous RC model by individually changing PGME feed rate, 7-ADCA feed rate, as well as enzyme deactivation.
Figure 6.1: Disturbance simulations for continuous RC of cephalexin. (A) PGME feed flow rate disturbances – PG concentration over time after step change; (B) 7-ADCA feed flow rate disturbances – 7-ADCA concentration over time after step
change; (C) Enzyme deactivation disturbance – PGME and 7-ADCA concentrations over time before and after 40% step change reduction in enzyme concentration. All disturbances were introduced at 720 minutes, after an initial steady state was achieved.

For a disturbance in PGME feed flow rate (Figure 6.1(A)), a 20% error was required before PG accumulated to a degree where it approached its solubility. For a disturbance in 7-ADCA feed flow rate (Figure 6.1(B)), small errors resulted in small accumulation of 7-ADCA. A 20% error in 7-ADCA feed flow rate was required before the concentration of 7-ADCA reached 160 mmol/L (S~3.5), after which the likelihood of 7-ADCA precipitation increases significantly (as discussed in Chapter 5). Additionally, a 40% reduction in enzyme concentration, or 40% deactivation, resulted in minor increases in concentration of both substrates (Figure 6.1(C)). The concentration of PG decreased in the case of enzyme deactivation as the amount of primary and secondary hydrolysis was reduced, so the risk of PG precipitation is reduced in the event of enzyme deactivation. These simulations reveal that significant disturbances, up to 20% changes, are required in either feed rates to result in process conditions that could promote 7-ADCA or PG precipitation. In the case of the enzyme, greater than 40% deactivation would be required before 7-ADCA would accumulate to a degree where it may precipitate. These results indicate that feed rates and enzyme deactivation only become an issue after significant deviation and that are not highly sensitive parameters for process stability.
The wet mill and pH control were also considered critical to process operation. As mentioned previously, the wet mill ensures crystals remain small enough to pass through the enzyme-crystal separator. If the wet mill loses power, or the pump feeding the mill fails, the crystals could possibly grow so large that they clog the outlet filter, and large crystals will be retained within the reactor. The pH value in the system impacts not only activity and selectivity of PGA [57], but also the solubility of all components, as discussed in Chapter 3. In the case of the amoxicillin RC system, 2 mol/L NaOH is continuously dosed into MSMPR-1 using a dosing unit to maintain the pH at a value of 6.3. The reaction itself causes the pH value of MSMPR-1 to drop, but also the combination of 6-APA and 4-HPGME feeds (pH 8.0 and 4.0, respectively) are slightly acidic due to the higher flow rate of 4-HPGME feed. Without pH control, it was hypothesized that the pH value of MSMPR-1, and consequently MSMPR-2 would be lowered, and in turn the activity of the enzyme would be lowered (PGA has its optimum activity at a pH value of ~7.9), allowing substrate to accumulate. Additionally, the solubility of 6-APA decreases with decreasing pH value to a minimum of 15 mmol/L at a pH value of 4.0.

The goals of an experiment examining the effect of process disturbances are 1) To determine how quickly the disturbance impacts the system and 2) How quickly does the system (or does the system ever) experience a significant disturbance requiring shutdown (i.e., byproduct/substrate precipitation). Both questions were considered in Run 20, a continuous RC of amoxicillin (Figure 6.2), where both loss of pH control and loss of wet milling were examined.
Figure 6.2: Run 20 (amoxicillin continuous RC) HPLC data for solution phase concentrations in MSMPR-1 showing points of disturbances being introduced and removed. Milling was removed at 5 hours and resumed at hour 7. The pH control was removed 11 hours into the run and reestablished at 12 hours into the run.

First, the process was allowed to reach a state of control until $t = 5$ hours and at that point wet milling was paused. Crystal counts and mean size were monitored by FBRM and PVM (Figure 6.3).
Figure 6.3: Run 20 (amoxicillin continuous RC) FBRM data tracking crystal chord length distribution (top) and PVM data tracking counts in different size bins. Also
shown are the mean (square weighted by chord length) in μm from FBRM and the mean length of crystals as measured by PVM (bottom).

From 5 to 7.25 hours, the turbidity measured by PVM decreased in MSMPR-1, which was likely caused by crystal growth (smaller crystals tend to yield higher turbidity). In FBRM, the fines (<10 μm) chord counts decreased consistently, and the mean length of crystals as measured by PVM as well as mean (square weighted by chord length) by FBRM both increased consistently through the same period. At t = 7.25 hours, wet milling was turned on as several clogs, evident by the sharp decrease in turbidity, had developed throughout multiple points in the slurry transfer tubing. Once the milling was reinstated, the system was able to again reach a state of control without any other disturbances, such as clogging, which implies that crystals did not grow in the absence of milling to a point where they could not pass through the separator. These results indicate that with growing crystals, slurry transfer becomes an issue before the efficiency of the size-based separator declines, as the size of the crystals were still small enough to pass through the mesh filter for separation. Clogs can be detected with sudden shifts in FBRM counts and PVM turbidity (Figure 6.3), although detection of larger crystal formation that could promote clogging would be ideal. Ultimately, these clogs do not require a full process shutdown, but typically require replacement or “flushing out” of slurry transfer lines. If crystals had grown to a point where they would not pass through the separator, this would likely require a shutdown where immobilized enzyme is removed, the pH is increased to dissolve amoxicillin crystals, and then the enzyme and amoxicillin seeds are added again. After the process had reached a state of control again, the FBRM mean size (square weighted by chord length) was higher than the previous state of operation, suggesting that a slightly different state of
operation was achieved. All other metrics, including crystal counts and liquid phase component concentrations (Figure 6.2) returned to their previous values.

After re-establishing wet milling and allowing for the system to become steady, the dosing unit providing 2 mol/L NaOH which maintained the pH at a value of 6.3 was disabled. As mentioned previously, pH dictates many of the thermodynamic and kinetic phenomena in the system such as component solubility and enzyme kinetics. Figure 6.4 shows the trend of the pH value after loss of pH control and after pH control was reactivated at t = 12.1 hours.

![Figure 6.4: pH value over time during Run 20. pH control disabled at t = 10.7 hours and reactivated at t = 12.1 hours. Red dotted line is the fit to Equation (6-1).](image-url)
The decrease in pH appears to exhibit first order exponential decay behaviour, which when fit to Equation (6-1)

\[ pH = pH_0 + \Delta pH \left( 1 - e^{-\frac{(t-t_0)}{\tau}} \right) \]  

(6-1)

where \( pH_0 \) and \( t_0 \) are the pH value and time at the time of the initial disturbance, \( \Delta pH \) is the maximum change in pH at \( t = \infty \), and \( \tau = 1.5 \) hours, yielded a tight fit to data and \( \Delta pH = -0.72 \), indicating the final pH value would have been 5.58 if the pH control remained disabled further. As the pH must drop below a value of 5.0 to observe significant PGA deactivation [147], irreversible PGA deactivation is unlikely if pH control is lost. The decision to reactivate pH control at \( t = 12.1 \) hours was due to significant substrate accumulation, which possibly would have resulted in 6-APA precipitation and process shutdown. Ultimately, a pH decrease of 0.63 units did not result in a significant process disturbance. The pH value was increased back to 6.3 in about 15 minutes using 2 mL/min of 2 mol/L NaOH via the dosing unit. The optimum strategy for correcting a pH disturbance may not have been immediate increase in pH, due to the possibility of 4-HPGME precipitation as its solubility decreases at higher pH combined with accumulation of 4-HPGME during operation at lower pH values. The turbidity of solution continually decreased after the pH value was lowered and increased upon re-establishment of pH control. The data in Figure 6.5 imply that the reduced turbidity and accumulation of substrate correlate well with decreased productivity initially; however, upon re-activation of pH control, the productivity continued to decrease even while substrates were depleted.
(Figure 6.2), likely due to the solid phase lagging behind changes in the liquid phase due to its higher order dynamics.

Figure 6.5: Amoxicillin solids productivity throughout course of Run 20, at beginning and end of each simulated disturbance.

Additionally, although productivity appeared higher at the end of the loss of milling, this was possibly the solid phase lagging behind the liquid phase as the liquid phase concentrations were steady throughout the course of the wet milling disturbance (Figure
6.2). While decreased productivity is an adverse effect of losing pH control, the process appeared resistant to the observed change in pH in terms of substrate precipitation during the time period allowed for the disturbance to propagate. As the pH value is easily measured via pH probe, it is unlikely that a disturbance in pH control would continue uncorrected for the length of time observed in Run 20. Additionally, the accumulation of substrate detected by HPLC analysis of the liquid phase is a sign of reduced enzyme activity (Figure 6.2), implying a possible pH disturbance.

6.3.2 Use of PAT

RC process evaluation would not be possible without in-line monitoring using PAT and sensors to obtain qualitative data on the evolution of the solution and crystal phases[46]. Table 6.1 and Figure 6.6 outline PAT common to reactive crystallization processes.
Table 6.1: Summary of PAT evaluated for use in the continuous RC of cephalexin and amoxicillin. Bolded PAT are currently used in the process.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Method</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid phase</td>
<td>ATR-FTIR</td>
<td>Attenuated total reflectance Fourier-transform infrared spectroscopy. An effervescent wave probes the IR spectrum only about one micrometer into the solution; hydrodynamics should prevent crystals from entering the probed region. Spectra can be processed to estimate solution composition.</td>
</tr>
<tr>
<td>Polarimetry</td>
<td></td>
<td>The high specific optical rotation of beta-lactam antibiotics and related synthesis components allows determination of solution composition as total rotation is the sum of each component specific rotation (known) weighted by its concentration. The technique requires a solution path length free of crystals.</td>
</tr>
<tr>
<td>HPLC (offline)</td>
<td></td>
<td>High performance liquid chromatography. HPLC is used to separate and quantify components in solution or solids upon dissolution in a solvent. While ideally an online setup to give real time feedback on the solution phase, the reactor is sampled periodically to give solution-phase information within 15 minutes of sampling.</td>
</tr>
<tr>
<td>Solid phase</td>
<td>FBRM</td>
<td>Focused beam reflectance measurement. Backscattered laser light is used to measure the chord length distribution of crystal population, giving insight into the number, size, and shape of crystals. However, these properties of the population need to be deconvoluted, possibly by data-driven empirical models.</td>
</tr>
<tr>
<td>PVM</td>
<td></td>
<td>Particle vision microscopy. An in situ microscope captures images of individual crystals. Image analysis can be used to estimate/quantify crystal size and shape and identify spurious particles.</td>
</tr>
<tr>
<td>Liquid and solid phase</td>
<td>Raman</td>
<td>Sensitive to both solution and crystal phases. Preliminary work showed that amoxicillin and cephalexin were not strongly Raman active and deconvolution of weak signals from solution and solid phases would not be straightforward.</td>
</tr>
</tbody>
</table>
Figure 6.6: PAT useful for characterizing 2-phase solid/liquid systems along with offline characterization tools. Figure reproduced from McDonald et. al [156].
Attenuated total reflectance Fourier-transform infrared spectroscopy (ATR-FTIR) is often coupled with multivariate models to predict liquid-phase compositions in RC processes[157]. Although ATR-FTIR is useful to ensure solely liquid phase determination, it was not applicable to our system due to low IR signal from all components leading to very low signal-to-noise ratio. In situ Raman spectroscopy is especially useful in systems with co-crystallization or multiple polymorphs[158-162]. PVM is mainly used as an in situ microscope but can corroborate information about particle size alongside FBRM measurements. Additionally, in a separate project, PVM has been used to detect PG precipitation in cephalaxin slurries using deep learning algorithms [163]. Overall, HPLC was the primary method of liquid phase determination and solid purity analysis. While it was an offline determination technique, the time scale for HPLC analysis (~20 minutes) is still a fraction of the residence time of MSMPR-1 (90 minutes for amoxicillin), allowing for relatively fast detection of changes in liquid phase composition.

Raman and ATR-FTIR were both evaluated for use as liquid phase PAT for the amoxicillin system. For 100 mmol/L 6-APA dissolved in water, the signal to noise ratio (SNR), calculated using the method of first standard deviation (FSD), of the most active peak for Raman (852 cm\(^{-1}\)) was 152, while the most active peak for ATR-FTIR had a SNR of 0.1. All other amoxicillin synthesis components were measured using ATR-FTIR and Raman, and all exhibit acceptable Raman scattering but low IR signal. McDonald et. al. used ATR-FTIR to track changes in cephalaxin concentration during its crystallization, albeit with low IR signal [90]. For the purposes of this study, Raman spectroscopy was evaluated as a method of liquid and solid phase PAT. First, the ability of Raman to detect the liquid phase in the presence of solids was examined (Figure 6.7).
Figure 6.7: Attenuation of Raman scattering. (A) Raman spectra for water, 100 mmol/L 6-APA in water saturated with amoxicillin (~10 mmol/L), and 4% amoxicillin slurry; (B) Raman peak areas with varying slurry densities of amoxicillin trihydrate. pH = 6.3, T = 25°C, V = 200 mL, exposure time = 5 seconds.
The homogenous solution of 6-APA (100 mmol/L) in water saturated with amoxicillin had many distinctive peaks in Raman spectra; however, the addition of 4% (wt/vol) amoxicillin solids significantly decreased the intensity of many of the peaks, including the water peak at ~1650 cm\(^{-1}\) (Figure 6.7(A)). On the other hand, the background fluorescence increased in the presence of solids. The data in Figure 6.7(B) imply that an initial significant attenuation of peak intensity is observed between a slurry density of 0.25-0.50%, although some peaks observe a slight increase in intensity at higher slurry densities, such as the peak at 852 cm\(^{-1}\). The reported FT-Raman spectra for amoxicillin in literature suggests the peak at 852 cm\(^{-1}\) corresponds to the aromatic ring breathing of the phenyl ring, which may contribute to the increase in baseline fluorescence due to the intrinsic fluorescence of aromatic amino acids. The duplex peak at 665 cm\(^{-1}\) as well as some other peaks present in the homogenous 6-APA solution completely disappeared at higher slurry densities, indicating a challenge in liquid phase determination in the presence of dense slurries. Slurry densities observed in continuous RC of amoxicillin (Chapter 5) range between 3.0-4.5%, suggesting that Raman may not be useful in liquid phase determination of amoxicillin RC processes, unless a separate stream is diverted and clarified via filtration or centrifugation and used for Raman determination. Additionally, all components for the amoxicillin system (amoxicillin, 4-HPGME, 4-HPG, 6-APA) yielded very similar Raman spectra, suggesting that a more complex algorithm for spectral deconvolution (e.g. partial least-squares regression) would be required to predict all liquid phase component concentrations. However, as Raman was sensitive to the presence of solids, it was inferred that it may possibly be used to assess solid phase purity and the possible presence of the
precipitation of product or byproduct. The data in Figure 6.8 are the results from the result of detecting 4-HPGME impurities in an amoxicillin slurry.

Figure 6.8: Detection of solid 4-HPGME in an amoxicillin slurry. (A) Raman spectra of water, a 2% (wt) amoxicillin slurry, saturated with 4-HGPME, and increments of 1% and 2% (wt) 4-HPGME solids added; (B) Alternative axes of Panel (A) to show peak at 1734 cm\(^{-1}\) corresponding to 4-HPGME solids, outlined in gray on Panel (A);
(C) Evolution of 1734 and 852 cm\(^{-1}\) peak areas with increasing 4-HPGME solids in a 2% amoxicillin slurry. pH = 6.3, T = 25°C. After measurement of water, solution was saturated with amoxicillin and 4-HPGME, and then 2% amoxicillin solids were added. V = 200 mL, exposure time = 5 seconds.

As expected, many of the peaks observed in homogenous solutions of amoxicillin and its cosolutes were attenuated or disappeared in the presence of the initial slurry of 2% amoxicillin saturated in 4-HPGME. A peak at 1734 cm\(^{-1}\) appeared upon addition of 4-HPGME solids, which did not exist in the 2% amoxicillin slurry saturated with 4-HPGME, implying that the peak corresponds to 4-HPGME solids. A separate experiment with a homogenous 4-HPGME solution did not yield the same peak in the Raman spectra, supporting the previous hypothesis. As the 1743 cm\(^{-1}\) peak appeared to be specific to 4-HPGME solids and detectible in amoxicillin slurries, the limit of detection (LOD) measured in subsequent experiments was between 0.10-0.25% 4-HPGME in a 2% amoxicillin slurry. Additionally, higher slurry densities of amoxicillin resulted poorer detection of the 4-HPGME solids. A 0.10-0.25% LOD in a 2% amoxicillin slurry corresponds to 5.0-12.5% impurity content, which is much higher than the drug substance quality standards established by the United States Pharmacopeia of \(\geq 99\%\) purity (wt.).
CHAPTER 7. Conclusions and Future Directions

In this work, we demonstrated a strategy of using a combined experimental and model-based approach to develop and engineer a novel continuous process which utilizes two main phenomena, reaction and crystallization, in the same unit operation to leverage improved productivity, yield, and environmental sustainability of the process. In Chapter 2, we developed a model for the enzymatic synthesis of cephalaxin, incorporating substrate inhibition by 7-ADCA which was shown to significantly impact enzymatic activity and subsequently productivity at higher substrate concentrations. Additionally, wild-type PGA was compared to two variants, a well-studied single variant and an engineered commercial variant. For reaction conditions which could promote RC, the wild-type was found to perform very similarly to both variants, implying that the enzyme was likely engineered for enhanced performance at lower substrate concentrations, where other studies have been performed. While several site-saturation mutagenesis studies have been conducted on the active site of PGA, many studies have avoided looking at the impact of mutating elsewhere due to limiting screening speed. Directed evolution (DE) has overtaken the field of biocatalysis for engineering enzymes for new or enhanced reaction kinetics [164, 165], and it is possible that a DE campaign targeted at PGA with proper assay conditions could yield improved variants designed for enhanced performance under higher substrate concentrations which promote RC. To achieve an improved PGA, a high throughput screen would need to be developed, which is particularly difficult in the case of PGA as the screen would be targeting improved antibiotic production, which is bactericidal. Another strategy would be to take advantage of numerous machine-learning (ML) based approaches which
are currently being developed to engineer proteins for specific reactions[166, 167]. ML could narrow down sites for further mutagenesis studies, and structural based studies ligand binding studies could possibly be used to engineer PGA for the synthesis of other β-lactam antibiotics (e.g., methicillin) [168].

In Chapter 3, we examined the impact of pH, temperature, and cosolute concentrations on the solubility and crystallization of amoxicillin trihydrate. We probed these liquid phase interactions using in situ spectroscopy to better understand their mechanism. Additionally, we fit experimental data to a model for the crystallization of amoxicillin trihydrate, which could be coupled with a reaction model to describe the reactive crystallization of amoxicillin. Further studies may be warranted regarding liquid phase interactions between amoxicillin and synthesis components. Fluorescence emission spectroscopy has been shown to probe aromatic ring-stacking interactions between compounds, which could further support the hypothesis of these interactions between amoxicillin and 4-HPGME[95]. Better understanding of these interactions may allow for the design of complexing agents which may reduce the apparent solubility of amoxicillin and be used as a form of in situ product removal in future processes. Additionally, no model for the breakage of amoxicillin via wet milling has been reported in literature. A model would be useful for process scale up, as well as identifying operating conditions which may result in a non-preferable product CSD.

In Chapter 4, we describe the development of a novel size-based separator for the retention of immobilized biocatalyst within a well-mixed vessel and demonstrated its use for separating immobilized PGA from amoxicillin crystals. We examined the effect of immobilization support size on the performance of the enzyme, which was found to be
significant particularly for cephalexin synthesis due to issues with byproduct accumulation and limited solubility. We also optimized the withdrawal rate and separator sieve size to yield isokinetic slurry withdrawal. Additionally, wet milling was found to be critical in ensuring crystal size remained small enough to ensure the separator was highly efficient. While the size-based separator was robust and efficient for crystal withdrawal, it required the use of relatively large enzyme support particles (425-500 μm) which had a deleterious effect on the enzyme performance. Other strategies for solid/solid separators may be devised, such as a gravity or density-based separator such as a settling tank. Enzyme may also be immobilized on low density polymer particles which could be separated at the liquid/air interface. Additionally, the use of poly-methyl methacrylate (PMMA) particles could lead to issues in process scale up, as shear rates are intensified. While PMMA possesses modest mechanical stability, it is possible that porous glass particles could be a solution[169].

In Chapter 5, we describe the process of operating the continuous RC process for both cephalexin and amoxicillin, and the respective challenges associated with each. For the cephalexin process, byproduct accumulation was significant for large immobilization support sizes, and higher product solubility led to limited productivity. The use of smaller immobilization support sizes combined with reduced enzyme loading led to improved selectivity. Additionally, PGME was found to increase the solubility of cephalexin, and inferred that future processes may benefit from running lean in PGME or incorporating the use of an antisolvent or complexing agent to reduce the observed solubility of cephalexin. For the amoxicillin system, significant accumulation of amoxicillin was observed in the liquid phase of the first MSMPR, which was addressed adding a second MSMPR in series,
operated at a lower temperature to enhance crystallization. Even with product accumulation, the productivity of the amoxicillin system was much higher than the cephalexin system, due to amoxicillin’s low solubility compared to cephalexin; however, the substrate conversion of amoxicillin was limited due to modest enzyme selectivity in the presence of accumulated product. A novel strategy of substrate recovery and recycle for 6-APA and 4-HPGME via a two-step pH swing crystallization was devised, tested, and optimized, which yielded significantly improved reaction mass efficiency and overall conversion of both substrates 50 and 60% to nearly 90% for each. Lastly, we examined the long-term process stability of the amoxicillin process in a 72-hour pilot run and observed significant enzyme deactivation starting at 24 hours. The deactivation was hypothesized to be due to support pore blockage and was partially reversible via dissolution of precipitated amoxicillin on and within the support. Future studies may involve the challenges involved in scaling up of the process or similar processes. The focal points which pose a challenge to scale up and incorporating industrially would be the separator, mixing, and enzyme deactivation. For the separator, it is likely that the flux of the crystals through a certain area of mesh would need to be scaled, which would possibly not scale preferentially with the volume of the reactor as the surface area scales with length squared and volume scales with length cubed. Mixing, as mentioned previously, could be challenging with scale up due to increased shear, which could lead to increased breakage of crystals and support particles leading to premature enzyme deactivation, but also with the addition of acid or base within the process, leading to pockets of very high or very low pH value. It is likely that computational fluid dynamics studies would be useful in designing reactor geometries or configurations that would address the issue of mixing.
Concerning enzyme deactivation, it was shown that roughly 50% of the enzyme deactivated over the course of 72-hours, which resulted in a significant change in slurry density and productivity over time. Strategies considering adding enzyme throughout the course of process operation, or preferential enzyme withdrawal and recharging should be considered. As mentioned previously, a density-based separation may be utilized to allow the support particles to settle at the bottom of the vessel, where they are quickly withdrawn and replaced with fresh enzyme. Magnetized particles may also be used which can easily be separated from the slurry using a magnetic field and have been demonstrated with PGA [31, 170]. As partial reactivation of PGA-loaded supports has been demonstrated, it is possible that a reactivation protocol could be incorporated to utilize enzyme more efficiently, which would likely be a significant portion of the operating cost for similar processes industrially.

In Chapter 6, we investigated the impact of disturbances on the operational stability of the process. Substrate feed rate and enzyme deactivation disturbances were modelled using our process model and were determined to not be significant unless large disturbances were. More complex disturbances such as pH control and wet milling, due to their higher order impact on the rates dictating processes within the system, were examined experimentally in a pilot run. A disturbance in pH control was shown to significantly impact enzyme activity, but not result in enzyme deactivation. We found that the accumulation of substrates during a loss in pH control could ultimately lead to substrate precipitation and product contamination, but only after the disturbance has persisted for more than a residence time. A loss in wet milling was found, as expected, to lead to increased crystal length, which in turn resulted in poor flowability within slurry transfer tubing. Milling was
found to not only impact the separator efficiency, as discussed in Chapter 4, but reduce settling of crystals within slurry transfer tubing and observed clogging events. For both of these disturbances tested, the process was able to return back to a state of control within 2 residence times and without any major process upset (i.e., product contamination), implying the system is somewhat robust in operation. Lastly, we evaluated the use of PAT in detecting disturbances in the continuous RC of amoxicillin and cephalexin. FBRM and PVM were shown to be useful in detecting nucleation events separate from the desired product, such as 7-ADCA and PG in cephalexin slurries. ATR-FTIR was incapable of liquid phase determination due to low signal to noise ratio, but Raman yielded modest signals for all components within the amoxicillin system. Significant liquid phase attenuation was observed in the presence of amoxicillin, limiting the use of Raman for liquid phase determination in the RC of amoxicillin; however, Raman was capable of detecting a separate solid precipitate of 4-HPGME in an amoxicillin slurry, suggesting it shows promise as a solid purity sensor for amoxicillin RC. As the liquid phase dictates all of the rates of the process, for future studies, a liquid phase sensor for component concentrations is critical for an efficient on-line control algorithm to be incorporated. As mentioned previously, Raman and ATR-FTIR are not useful for amoxicillin slurries. All amoxicillin components possess good absorbance in the UV-Vis range, so it is possible such a detector could be utilized in situ, likely with a spectroscopic deconvolutional algorithm such as partial least squares regression[171], to extract component concentrations from UV-Vis spectra. Alternatively, a detector utilizing circular dichroism (CD) spectra for determination of liquid phase components may be viable, as all components in both the amoxicillin and cephalexin system are chiral, meaning they would
exhibit some fingerprint in CD spectral analysis. With effective sensors for the continuous RC system, feedback control algorithms could be used to maintain constant liquid phase composition, enzyme activity, productivity, and ensure pure product. Additionally, with the use of a model, feedforward control such as model predictive control could be applied to predict future behavior and avoid process conditions which may promote impurity formation.

A great deal of the work involved in implementing the aforementioned process on an industrial scale is the resistance of the pharmaceutical industry to process changes (due to regulatory challenges). Additionally, for such a process to be developed in western countries, it is likely that their production would need to be subsidized by the government to be economically feasible (due to low potential for profitability of these drug substances). On the other hand, incorporation of new production facilities worldwide would shore up supply in the case of potential supply line disruptions, as observed in the case of Covid-19. The advances developed in the production of beta-lactam antibiotics (e.g., improved sustainability, continuous manufacturing) would also improve the overall impact that production of these drugs has on the environment, reduce their cost to consumers, and make them more available to those needing treatment.
APPENDIX A. Additional data for enzymatic synthesis of amoxicillin and cephalexin

Figure A.1: Michaelis-Menten plots for hydrolysis kinetics - (left) Specific activity of PGME hydrolysis versus PGME concentration and (right) specific activity of cephalexin hydrolysis versus cephalexin concentration. Black solid curves are fits to
the standard Michaelis-Menten equation. Reaction conditions: pH = 7.0, C_{PGA} = 300-400 nM.

Figure A.2: Michaelis-Menten plots for amoxicillin hydrolysis kinetics - (left) secondary hydrolysis and (right) primary hydrolysis. Black solid curves are fits to the standard Michaelis-Menten equation. Reaction conditions: pH = 7.0, C_{PGA} = 300-400 nM.
Figure A.3: Time course cephalexin synthesis reactions. Initial substrate concentrations: \(C_{\text{PGME},0} = 50 \text{ mM}, C_{\text{7-ADCA},0} = 50 \text{ mM}\). Reactions were buffered with 100 mM sodium phosphate pH 7.0. \(C_{\text{PGA}} = 1 \mu\text{M}\).

**Michaelis-Menten Equation with Competitive Inhibition:**

The following is the Michaelis-Menten equation of a single step reaction consuming a substrate \(A\) in the presence of a competitive inhibitor \(I\).

\[
\nu_0 = \frac{v_{\text{max}}[A]}{K_M \left(1 + \frac{[I]}{K_I}\right) + [A]}
\]
\( v_{\text{max}} \) represents the maximum velocity of the reaction at infinite substrate concentration. 

\( K_M \) represents the Michaelis binding constant for the substrate and \( K_I \) represents the inhibitor binding constant.

Figure A.4: Lineweaver-Burk plot for the inhibition of cephalexin hydrolysis by 7-ADCA for WT-PGA. \( v_{\text{initial}} \) represents the initial activity of cephalexin hydrolysis. Each data series represents different 7-ADCA concentrations and each solid line the best fit to Michaelis-Menten hydrolysis of cephalexin with a competitive inhibitor for each separate inhibitor concentration. (A) Linear fit of each data series (B) Fit of Michaelis-Menten equation with a competitive inhibition by 7-ADCA. Reaction conditions: pH = 7.0, \( C_{\text{PGA}} = 300 \text{ nM} \).

Kinetic Model Equations:
Model 1:

\[
\frac{d[CEX]}{dt} = [e] \left( \frac{k_2k_4[7-ADCA][PGME]}{K_S(k_3K_N + k_4[7-ADCA] + k_5[7-ADCA])} - \frac{k_{-4}[CEX](k_3K_N + k_5[7-ADCA])}{K_P(k_3K_N + k_4[7-ADCA] + k_5[7-ADCA])} \right)
\]

\[
\frac{d[PG]}{dt} = [e](k_3K_N + k_5[7-ADCA]) \left( \frac{k_2[PGME]}{K_S(k_3K_N + k_4[7-ADCA] + k_5[7-ADCA])} \right)
\]

\[
\frac{d[PGME]}{dt} = - \left( \frac{d[CEX]}{dt} + \frac{d[PG]}{dt} \right)
\]

\[
\frac{d[7-ADCA]}{dt} = - \left( \frac{d[CEX]}{dt} \right)
\]

\[
[e] = [e]_0 \left( 1 + \frac{[PGME]}{K_S} + \frac{[CEX]}{K_P} + \frac{K_N}{k_3K_N + k_4[7-ADCA] + k_5[7-ADCA]} \left( \frac{k_{-4}[CEX]}{K_P} + \frac{k_2[PGME]}{K_S} \right) \left( 1 + \frac{[7-ADCA]}{K_N} \right) \right)
\]

Model 2:

\[
\frac{d[CEX]}{dt} = [e] \left( \frac{k_2k_4[7-ADCA][PGME]}{K_S(k_3K_N + k_4[7-ADCA] + k_5[7-ADCA])} - \frac{k_{-4}[CEX](k_3K_N + k_5[7-ADCA])}{K_P(k_3K_N + k_4[7-ADCA] + k_5[7-ADCA])} \right)
\]

\[
\frac{d[PG]}{dt} = [e](k_3K_N + k_5[7-ADCA]) \left( \frac{k_2[PGME]}{K_S(k_3K_N + k_4[7-ADCA] + k_5[7-ADCA])} \right)
\]

\[
\frac{d[PGME]}{dt} = - \left( \frac{d[CEX]}{dt} + \frac{d[PG]}{dt} \right)
\]

\[
\frac{d[7-ADCA]}{dt} = - \left( \frac{d[CEX]}{dt} \right)
\]

211
\[ [e] = \frac{[e_0]}{1 + \frac{[\text{PGME}]}{K_S} + \frac{[\text{CEX}]}{K_P} + \frac{[7-\text{ADCA}]}{K_N} + \frac{K_N}{k_3K_N + k_4[7-\text{ADCA}] + k_5[7-\text{ADCA}]} \left( \frac{k_4[\text{CEX}]}{K_P} + \frac{k_5[\text{PGME}]}{K_S} \right) \left( 1 + \frac{[7-\text{ADCA}]}{K_N} \right)} {\left( 1 + \frac{[\text{PGME}]}{K_S} + \frac{[\text{CEX}]}{K_P} + \frac{[7-\text{ADCA}]}{K_N} \right)} \]

Model 3:

\[
\frac{d[\text{CEX}]}{dt} = [e] \left( \frac{k_2k_4[7-\text{ADCA}][\text{PGME}] + \eta k_2k_4[7-\text{ADCA}][\text{PGME}]^2/K_n}{K_p(k_3K_N + k_4[7-\text{ADCA}] + k_5[7-\text{ADCA}])} \right) \\
- \frac{k_4[\text{CEX}](k_3K_N + k_4[7-\text{ADCA}])}{K_p(k_3K_N + k_4[7-\text{ADCA}] + k_5[7-\text{ADCA}])} \\
\frac{d[\text{PG}]}{dt} = [e](k_3K_N + k_4[7-\text{ADCA}]) \left( \frac{k_2[\text{PGME}] + \eta k_2[7-\text{ADCA}][\text{PGME}]}{K_p(k_3K_N + k_4[7-\text{ADCA}] + k_5[7-\text{ADCA}])} \right) \\
- \frac{k_4[\text{CEX}]}{K_p(k_3K_N + k_4[7-\text{ADCA}] + k_5[7-\text{ADCA}])} \\
\frac{d[\text{PGME}]}{dt} = - \left( \frac{d[\text{CEX}]}{dt} + \frac{d[\text{PG}]}{dt} \right) \\
\frac{d[7-\text{ADCA}]}{dt} = - \left( \frac{d[\text{CEX}]}{dt} \right) \\
\]

\[ [e] = \frac{[e_0]}{1 + \frac{[\text{PGME}]}{K_S} + \frac{[\text{CEX}]}{K_P} + \frac{[7-\text{ADCA}]}{K_N} + \frac{K_N}{k_3K_N + k_4[7-\text{ADCA}] + k_5[7-\text{ADCA}]} \left( \frac{k_4[\text{CEX}]}{K_P} + \frac{k_5[\text{PGME}]}{K_S} \right) \left( 1 + \frac{[7-\text{ADCA}]}{K_N} \right)} {\left( 1 + \frac{[\text{PGME}]}{K_S} + \frac{[\text{CEX}]}{K_P} + \frac{[7-\text{ADCA}]}{K_N} \right)} \]

Model 2 fit values and confidence intervals:
Table A.1: Model 2 parameter fits and confidence intervals.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fit value</th>
<th>Upper 95% CI</th>
<th>Lower 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_2$ (s$^{-1}$)</td>
<td>95.94</td>
<td>102.06</td>
<td>89.82</td>
</tr>
<tr>
<td>$k_3$ (s$^{-1}$)</td>
<td>50.21</td>
<td>52.47</td>
<td>47.94</td>
</tr>
<tr>
<td>$k_4$ (s$^{-1}$)</td>
<td>995.58</td>
<td>1040.48</td>
<td>950.68</td>
</tr>
<tr>
<td>$k_{-4}$ (s$^{-1}$)</td>
<td>95.94</td>
<td>100.42</td>
<td>91.46</td>
</tr>
<tr>
<td>$k_5$ (s$^{-1}$)</td>
<td>25.59</td>
<td>27.10</td>
<td>24.08</td>
</tr>
<tr>
<td>$K_n$ (mM)</td>
<td>63.20</td>
<td>66.05</td>
<td>60.35</td>
</tr>
<tr>
<td>$K_{ni}$ (mM)</td>
<td>50.20</td>
<td>48.63</td>
<td>51.80</td>
</tr>
<tr>
<td>$K_s$ (mM)</td>
<td>65.12</td>
<td>70.05</td>
<td>60.20</td>
</tr>
<tr>
<td>$K_p$ (mM)</td>
<td>12.26</td>
<td>13.14</td>
<td>11.39</td>
</tr>
</tbody>
</table>
Table A.2: Correlation Coefficient Matrix.

<table>
<thead>
<tr>
<th></th>
<th>$k_3$</th>
<th>$k_4$</th>
<th>$\beta_0$</th>
<th>$1/\gamma$</th>
<th>$k_{\text{cat,PGME}}$</th>
<th>$k_{\text{cat,CEX}}$</th>
<th>$K_M,\text{PGME}$</th>
<th>$K_M,\text{CEX}$</th>
<th>$K_{\text{ni}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_3$</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_4$</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\beta_0$</td>
<td>-0.13422</td>
<td>-0.13421</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$1/\gamma$</td>
<td>-0.06607</td>
<td>-0.06606</td>
<td>0.421113</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{\text{cat,PGME}}$</td>
<td>-0.02629</td>
<td>-0.0263</td>
<td>-0.00948</td>
<td>0.135397</td>
<td>1</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>$k_{\text{cat,CEX}}$</td>
<td>-0.03623</td>
<td>-0.03625</td>
<td>-0.02037</td>
<td>0.064151</td>
<td>0.810239</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_M,\text{PGME}$</td>
<td>-0.04805</td>
<td>-0.04807</td>
<td>-0.01305</td>
<td>0.236074</td>
<td>0.914926</td>
<td>0.771356</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_M,\text{CEX}$</td>
<td>0.073737</td>
<td>0.073719</td>
<td>-0.78465</td>
<td>-0.41989</td>
<td>0.506615</td>
<td>0.582056</td>
<td>0.526869</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>$K_{\text{ni}}$</td>
<td>0.043326</td>
<td>0.043308</td>
<td>-0.29434</td>
<td>-0.01793</td>
<td>0.633652</td>
<td>0.74197</td>
<td>0.77685</td>
<td>0.721754</td>
<td>1</td>
</tr>
</tbody>
</table>

To fit experimental data to Model 2, the parameters outlined above were varied, and all model parameters were calculated based on the fitting parameters using the equations available in [65]. $k_3$ and $k_4$ exhibit a high level of correlation. Additionally, many of the Michaelis-Menten kinetic constants for PGME hydrolysis and cephalexin hydrolysis exhibit high levels of correlation with each other, likely due to limited reaction data at very low substrate concentrations leading to inefficient fitting to a supposed Michaelis curve.

Time Course Reactions:
Rxn 1

Rxn 2

Rxn 3

Rxn 4

Rxn 5
Figure A.5: Cephalexin time course reaction data with model 2 fit. Reactions were prepared with initial concentrations of components corresponding to $t=0$ in each subplot. The legend for reaction 1 may be applied to all other reactions.
Figure A.6: SDS-PAGE gel of NiNTA purified PGA with removed signal sequence.
Figure A.7: (Top) Fit of amoxicillin crystallization model to final experimental CSD with the corresponding fit to HPLC data (Middle). (Bottom) Representative
microscope image of amoxicillin crystals. The data were collected for amoxicillin crystallization in pure water.

Figure A.8: Fit of amoxicillin solubility versus temperature to the Vant Hoff equation. $A = -3.86$, $B = -1.47 \times 10^3$. 
APPENDIX B. Additional data for continuous enzymatic synthesis of amoxicillin and cephalexin

Tables B.1 and B.2 outline the measured quantities used to calculate the green chemistry metrics. The water mass constitutes the water used to dissolve the substrates and present in the waste stream, but also water used to wash the amoxicillin product cake. The mass of other solvents and waste includes the mass of all components present in the waste stream, including sodium chloride present due to acid/base neutralization, and methanol present due to the hydrolysis of D-4-HPGME. The aqueous mass intensity of the continuous process with SR is higher than without SR due the required usage of aqueous sodium hydroxide and hydrochloric acid to drive the pH swing recrystallizations. This can also be seen in the slightly higher E-factor due to a greater resultant salt load in the waste stream.
Table B.1: Green chemistry metrics and other process metrics for continuous enzymatic RC process with and without substrate recovery (SR) and batch process.

<table>
<thead>
<tr>
<th>Process</th>
<th>Product recovered (g)</th>
<th>Batch time (min)</th>
<th>Yield</th>
<th>RME</th>
<th>Water mass (g)</th>
<th>Mass other solvents and waste (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cont. w/o SR</td>
<td>18.8</td>
<td>170</td>
<td>0.345</td>
<td>0.343</td>
<td>305</td>
<td>19.6</td>
</tr>
<tr>
<td>Cont. w/ SR</td>
<td>18.8</td>
<td>170</td>
<td>0.575</td>
<td>0.572</td>
<td>315</td>
<td>43.2</td>
</tr>
<tr>
<td>Batch w/o SR</td>
<td>15.5</td>
<td>150</td>
<td>0.375</td>
<td>0.368</td>
<td>346</td>
<td>2.9</td>
</tr>
</tbody>
</table>
Table B.2: Continued green chemistry metrics and other process metrics.

<table>
<thead>
<tr>
<th>Process</th>
<th>E-Factor</th>
<th>Complete E-Factor</th>
<th>AMI</th>
<th>PMI</th>
<th>RPG (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cont. w/o SR</td>
<td>2.84</td>
<td>19.1</td>
<td>17.2</td>
<td>20.1</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cont. w/ SR</td>
<td>2.94</td>
<td>19.7</td>
<td>17.8</td>
<td>20.7</td>
<td>132</td>
</tr>
<tr>
<td>Batch w/o SR</td>
<td>0.88</td>
<td>23.2</td>
<td>22.3</td>
<td>24.2</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The cephalexin continuous enzymatic process was run using a single MSMPR (Fig. B.1).
The process was initially loaded with 125 mg of PGA-βF24A on 15 g of immobilization resin. The initial residence time of the process was set at 100 minutes but was later adjusted at approximately $t = 600$ minutes to 150 minutes to allow more time for substrate turnover. The increase in residence time led to an increase in productivity due to decreased substrate inhibition by depleting 7-ADCA in solution, a phenomenon previously observed for 7-ADCA[61]. All green chemistry metrics calculated for the cephalexin process were calculated from process outputs (solids crystals, waste stream) after the new steady state was reached ($\sim t = 800$ minutes). The operational data for the cephalexin process is shown in Figure B.2 and indicates that FBRM counts, solids productivity, pH, and component liquid phase concentrations reach a steady operational point at approximately 800 minutes.
Figure B.2: Operational data from cephalexin continuous enzymatic process. (A) FBRM fines (<10μm) and midsize counts versus time; (B) pH value versus time; (C) Solids productivity in g/h versus time; (D) HPLC liquid phase concentrations in mmol/L versus time. Feed concentrations are 260 mmol/L and 330 mmol/L for 7-ADCA and D-PGME, respectively. At approximately $t = 600$ minutes, the residence time was changed from 100 minutes to 150 minutes to allow more time for enzyme to turnover substrates.

An observed increase in cephalexin solubility compared to pure component data present in literature was investigated by analyzing the effect of other components on the apparent solubility of cephalexin (Fig. B.3).
Figure B.3: Effect of (A) PGME, (B) 7-ADCA, and (C) NaCl concentration on the apparent solubility of cephalexin in DI water. T = 25°C, pH = 7.0.
Figure B.4: Solubility of cephalexin, PG, and 7-ADCA in different mixtures of ethanol in water. $T = 4^\circ C$.

Increasing PGME concentration appears to linearly correlate with an increase in apparent solubility of cephalexin within the range explored. 7-ADCA does not seem to effect apparent cephalexin solubility, while sodium chloride increases cephalexin solubility, indicating that ionic strength is likely at least partially responsible for the observed increase in the continuous system, which operates with roughly 500 mmol/L sodium chloride present in solution. These results indicate that large concentrations of PGME in the liquid phase or high ionic strength should be avoided as they increase the cephalexin available to the enzyme for hydrolysis.

Table B.3: Green chemistry metrics for cephalexin continuous enzymatic process

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Cephalexin continuous enzymatic process</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass product isolated (g)</td>
<td>5.92</td>
</tr>
<tr>
<td>Batch time (min)</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>Yield</td>
<td>0.208</td>
</tr>
<tr>
<td>RME</td>
<td>0.193</td>
</tr>
<tr>
<td>Water mass (g)</td>
<td>317.8</td>
</tr>
<tr>
<td>Mass other solvents and waste (g)</td>
<td>41.4</td>
</tr>
<tr>
<td>E-factor</td>
<td>7.0</td>
</tr>
<tr>
<td>Complete E-factor</td>
<td>59.6</td>
</tr>
<tr>
<td>AMI</td>
<td>53.7</td>
</tr>
<tr>
<td>PMI</td>
<td>60.6</td>
</tr>
<tr>
<td>RPG (%)</td>
<td>43.6</td>
</tr>
</tbody>
</table>
REFERENCES


61. Harris, P.R., et al., *Selectivity and kinetic modeling of penicillin G acylase variants for the synthesis of cephalaxin under a broad range of substrate concentrations*. Biotechnology and Bioengineering, 2022.


238


