Simultaneous clarification and purification of recombinant penicillin G acylase using tangential flow filtration anion-exchange membrane chromatography

by

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Author’s Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Downstream purification often represents the most cost-intensive step in the manufacturing of recombinant proteins. Conventional purification processes are lengthy, technically complicated, product specific and time-consuming. To address this issue, herein we develop a one step purification system that due to the nature of the non-selective secretion system and the versatility of ion-exchange membrane chromatography can be widely applied to the production of many recombinant proteins. This was achieved through the integration of the intrinsically coupled upstream, midstream and downstream processes, a connection that is rarely exploited.

A bioprocess for effective production and purification of penicillin G acylase (PAC) was developed. PAC was overexpressed in a genetically engineered *Escherichia coli* strain, secreted into the cultivation medium, harvested, and purified in a single step by anion-exchange chromatography. The cultivation medium developed had a sufficiently low conductivity to allow direct application of the extracellular fraction to the anion-exchange chromatography medium while providing all of the required nutrients for sustaining cell growth and PAC overexpression. It was contrived with the purposes of (i) providing sufficient osmolarity and buffering capacity, (ii) minimizing ionic species to facilitate the binding of extracellular proteins to anion-exchange medium, and (iii) enhancing PAC expression level and secretion efficiency. Employing this medium recipe the specific PAC activity reached a high level of 487 $U/L/OD_{600}$, with more than 90% was localized in the extracellular medium. Both, the osmotic pressure and induction conditions were found to be critical for optimal culture performance. Furthermore, formation of inclusion bodies associated with PAC overexpression tended to arrest cell growth, leading to potential cell lysis.

At harvest, the whole non-clarified culture broth was applied directly to a tangential flow filtration anion-exchange membrane chromatography system. One-step purification of recombinant PAC was achieved based on the dual nature of membrane chromatography (i.e. microfiltration-sized pores and anion-exchange chemistry). Due to their size, cells remained in the retentate while the extracellular medium penetrated the membrane. Most contaminate proteins were captured by the anion-exchange membrane, whereas the purified PAC was collected in the filtrate. The batch time for both cultivation and purification was less than 24 h and recombinant PAC with high purity (19 U/mg), process yield (74%), and productivity (41 mg/L) was obtained.

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Dedication

To My Parents

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AEMC	Anion-exchange membrane chromatography
AGM	Agamatine
ATPS	Aqueous two-phase system
C	Carboxyl
CV	Column volume
crAA	Poly(acrylic acid-co-methylene bisacrylamide)
DEA	Diethylamine
DEAE	Diethylaminoethyl
EA	Ethanolamine
EBA	Expanded bed adsorption
LMH	$Lm^{-2}h^{-1}$
MV	Membrane volume
NMWCO	Nominal molecular weight cut-off
PAA	Polyacrylamide
PAC	Penicillin G acylase
PDMAEMA	Poly (2-(dimethylamino)ethyl methacrylate)
PEI	Polyethyleneimine
PHMB	Polyhexamethylene biguanid
PMETAC	Poly(2-(methacryloyloxy)ethyl)trimethylamine)
PSI	Pounds per square inch
PSS	Polystyrene sulfonate
Q	Quaternary amine
QFF	Sepharose HiTrap Q Fast Flow column
S	Sulfonyl

TFF	Tangential flow filtration

List of Symbols

F_F	Feed volumetric flow rate
F_P	Permeate volumetric flow rate
F_R	Retentate volumetric flow rate

Chapter 1 – Overview

1.1 Research Background

Recent advances in the fields of proteomics have lead to a greater understanding of proteins and their roles in disease and biochemical transformation, resulting in their ever increasing incorporation into a wide variety of industries including medical sciences and industrial manufacturing. Enzymes are employed in almost every industry; from polymerases and RNAses used in molecular biology labs to lipases and proteases used in laundry detergents. Reported in 2004, biopharmaceuticals accounted for an over 50 billion dollar market [1]. However, prior to any application, proteins must be manufactured in sufficient quantities with adequate purity and sensible cost. Due to extensive downstream purification required for the production of many proteins, cost remains one of the largest barriers to their wider implementation as therapeutics and biocatalysts. Downstream purification accounts for 40-75% of the total manufacturing costs of proteins depending on the subsequent applications [2, 3]. Conventional purification schemes are long tedious cascades with many steps. Every additional step, adds processing time, material, and equipment and decreases overall process yield. Thus innovations in protein purification, particularly versatile shortened simplified schemes, which reduce the cost of purification, would have far reaching effects in many industries.

A generic protein purification scheme generally follows the broad steps of clarification, capture, intermediate purification, polishing and formulation [3]. Whether a bioprocess includes all or some of these steps will depend on its end use. Biopharmaceuticals destined for human use require the highest purity and thus will include several purification steps and extensive polishing to remove toxins and viruses, while industrial enzymes often do not strictly require high purity and

thus may employ crude or partially purified cell extracts [2, 3]. Additionally, many common purification operations require preparatory steps such as buffer exchange or centrifugation which are not amenable to processing large volumes.

A recent shift in the paradigm of research methods in the scientific community has lead to an increased awareness regarding the importance of more integrated and systematic approaches to technical obstacles. A disconnect between the optimization of upstream, midstream and downstream elements exists in the field of bioprocessing and thus, an effective solution for decreasing the costs of downstream purification may be to integrate the development of the upstream/midstream with the downstream. However, exploitation of this intrinsic connection is uncommon as most recombinant proteins are produced intracellularly. Consequently, host/strain development and cultivation conditions have few effects on downstream operations which are mainly derived from the physical properties of the culture broth such as viscosity and colloidal content. Alternatively, processing of extracellularly secreted recombinant proteins would be highly affected by the composition of the cultivation medium and hold more promise for an integrated bioprocess. While extracellular production of recombinant proteins is often proposed as a possible strategy for facilitating downstream purification of proteins produced in *Escherichia coli;* due to the low level of host impurities found in this compartment, these bioprocesses have hitherto required tedious preparatory steps that may be just as costly as the additional purification steps required for an equivalent intracellular bioprocess. In conjunction with the low proteins titers often found in extracellular secretion systems in *E. coli*, the majority of recombinant proteins continue to be manufactured using intracellular methods [1].

Liquid chromatography is the most heavily relied upon technique for increasing protein purity. Conventionally, this employs a packed bed column which uses the inherent properties of the

protein to selectively separate it from other biomolecules. However, traditional columns have several well known limitations. When scaled to large volumes, slow binding kinetics dependent on intra-particle diffusion limits column operation to low flow rates, which translates to long down times for cleaning and regeneration, and causes large pressure drops, which necessitates expensive hardware [4-6]. Chromatographic membranes offer numerous advantages over traditional packed bed columns as convective transport of the materials to the ligands results in faster binding kinetics and as a result can be operated at much higher flow rates than packed columns [4, 5]. Additionally, they experience minimal pressure drop which decreases the equipment costs and hardware footprint. Membranes are easily manufactured on a large scale, and can be disposable, drastically decreasing down time and resulting in a faster overall bioprocess. Low binding capacity of commercially available membranes has prevented their wide spread implementation in applications other than flow through purification where the target concentration is low and the drawback of low binding capacity is outweighed by the benefits of increased productivity [5]. Recent improvements in membrane chemistry has resulted in more commercially available membrane adsorbers with binding capacities comparable to currently available resins creating a renewed interest in this technology. Furthermore, membranes can be manufactured in non-conventional filtration formats, potentially allowing processing of crude feed stocks that has thus far been the domain of expanded bed chromatography.

Accordingly, interfacing tangential flow filtration membrane chromatography with extracellular protein secretion using an integrated development approach can be used to create a fast and highly simplified *E. coli* recombinant protein expression and purification system that simultaneously clarifies and purifies recombinant protein. This rationale resulted in the development of a novel simplified bioprocess, which due to the non-selective nature of the extracellular secretion

system employed, and the versatility of ion-exchange membrane chromatography, can be widely applied for the production and purification of many recombinant proteins.

1.2 Research Objectives

The overall objectives of this thesis were to:

1. develop an extracellular secretion system for the production of a suitable model protein (penicillin G acylase) with sufficient productivity to accurately study its purification;
2. develop a cultivation medium with sufficiently low conductivity to allow subsequent protein binding to the ion-exchange membrane without any sample alterations;
3. assess the binding behavior of chromatographic membranes in comparison to traditional columns and identify factors affecting membrane performance under the developed conditions; and
4. develop a process scale application for tangential flow filtration ion-exchange membrane chromatography for the simultaneous clarification and purification of the extracellular recombinant PAC.

1.3 Outline of Thesis

A review of the current research in the extracellular secretion of recombinant proteins in *E. coli*, the uses and methods of purification for penicillin G acylase (PAC), the properties of chromatographic membranes and the current applications of membrane chromatography for the purification of proteins are presented in Chapter 2. Chapter 3 presents the development of an extracellular secretion system for the purification of PAC and the brief optimization of the culture conditions which were devised with the purpose of facilitating protein binding to the ion-exchange stationary phase. Chapter 4 presents the development of a one-step purification scheme for the simultaneous

clarification and purification of PAC using tangential flow filtration anion-exchange membrane chromatography (TFF-AEMC). Finally, Chapter 5 examines the potential applications of the developed bioprocess and areas requiring further investigation.

Chapter 2 – Literature Review

Prior to the advent of recombinant DNA technology, proteins were undervalued due to their limited availability and the poor understanding of their roles in cellular processes. The fields of biochemical engineering and molecular biology have developed from an endeavor to address these issues and we are now capable of producing proteins on a sufficient scale for both the study of their function and for medical or industrial applications. In turn, increased understanding of the advantages of proteins in manufacturing applications and their role in disease has lead to their ever greater demand. Hence, proteins have and will continue to impact modern society profoundly.

While protein manufacturing methods are well established and high productivities are often possible, their adoption is often limited by their cost. Cost is mainly dictated by the downstream purification, which can account for 40-65% of the total manufacturing costs of industrial enzymes and may even be as high as 80% of the total manufacturing costs for highly purified therapeutic proteins [2]. Several well known approaches for the reduction of manufacturing costs include increasing protein titer through host engineering and/or optimization of cultivation to support high cell densities and the optimization of downstream operations to reduce process length, materials costs, and protein degradation.

2.1 Expression Host

Several expression systems for the recombinant production of proteins have been developed to meet the varying cellular processing needs of prokaryotic and eukaryotic proteins, and the industry's need for more economical high level expression. As each system has its own advantages and disadvantages, the choice of host is often determined by the protein of interest itself as no one system is compatible with all proteins or all technical product requirements. The main

characteristics for the most popular bacterial expression system (*E. coli*) and common eukaryotic expression systems (yeast and mammalian) are summarized and compared in **Table 2-1** [1, 7]. Despite of the limitations of bacterial hosts, *E. coli* remains one of the most commonly used expression hosts for the production of recombinant proteins due to the extensive knowledge of its genome, its amenability to genetic manipulation, its short generation time, its fastidiousness, and for its inexpensive cultivation requirements [1, 8, 9]. However, *E. coli* is often not a viable option for protein expression due to incompatible gene expression, protein misfolding, host/protein toxicity, formation of inclusion bodies, glycosylation, other posttranslational requirements, or regulatory concerns such as the presence of endotoxin [1, 7]. Regardless, the productivity of *E. coli* cultivation outstrips many of the other systems making it the most economical system and it continues to remain the workhorse of choice whenever possible.

Table 2-1 - Comparison of common protein expression systems

Characteristic	***E. coli***	**Yeast**	**Mammalian**
Growth Rate	Fast	Moderately Fast	Slow
Protein Productivity	High	Moderately High	Low
Cost	Low	Low	High
Secretion	Poor	Moderate	Good
Inclusion Bodies*	+	+	-
Glycosylation*	-	+	+
Genetic manipulation	Easy	Easy	Difficult
Toxins	Endotoxin	Generally regarded as safe (GRAS)	Human pathogens
Ease of protein recovery	Easy with well developed protein purification tags available	Easy with well developed protein purification tags available	Difficult
Scalability	Easy and inexpensive	Easy and inexpensive	Difficult and costly

* '+' present, '-'absent

2.2 Extracellular Secretion

Very few proteins are naturally secreted in *E. coli,* and translocation into the extracellular space is complicated by the need to traverse two membrane barriers. Despite this, targeting of recombinant proteins to be secreted has long been a strategy for simplifying downstream recovery due to the low level of contamination of host cell proteins in the extracellular medium. Generally, targeting of extracellular proteins into the extracellular space in *E. coli* is a matter of weakening the outer membrane or using a transporter system [10-12]. Strategies that have been successfully employed include addition of extracellular transport signals [13], fusion with naturally secreted proteins such as hemolysin toxin [14], coexpression of release proteins such as the *kil* gene product or bacterial release protein [15, 16], use of outer membrane less L-form *E. coli* [17], use of outer membrane mutant strains [18, 19], and addition of chemicals that can permeabilize the cellular membranes such as glycine and Triton X-100 [20]. These strategies are summarized in **Figure 2-1**. Each of these strategies has their own drawbacks and one universally applicable high-level extracellular expression approach in *E. coli* has yet to be found. Some of the drawbacks include low level expression, non native protein structures, cell lysis, stress sensitivity, and growth impairment [10-12]. Alternatively, extracellular secretion can also be employed as a strategy to improve production of difficult to express proteins. Expression in the extracellular or periplasmic space can often result in higher titers of soluble protein, possibly due to the larger presence of chaperone proteins and disulfide bond promoting proteins or from dilution into a compartment with lower protein concentration [11]. Additionally, secretion can provide increased protein stability of recombinant proteins probably due to the lower concentration of host cell proteases in this space [11, 12].

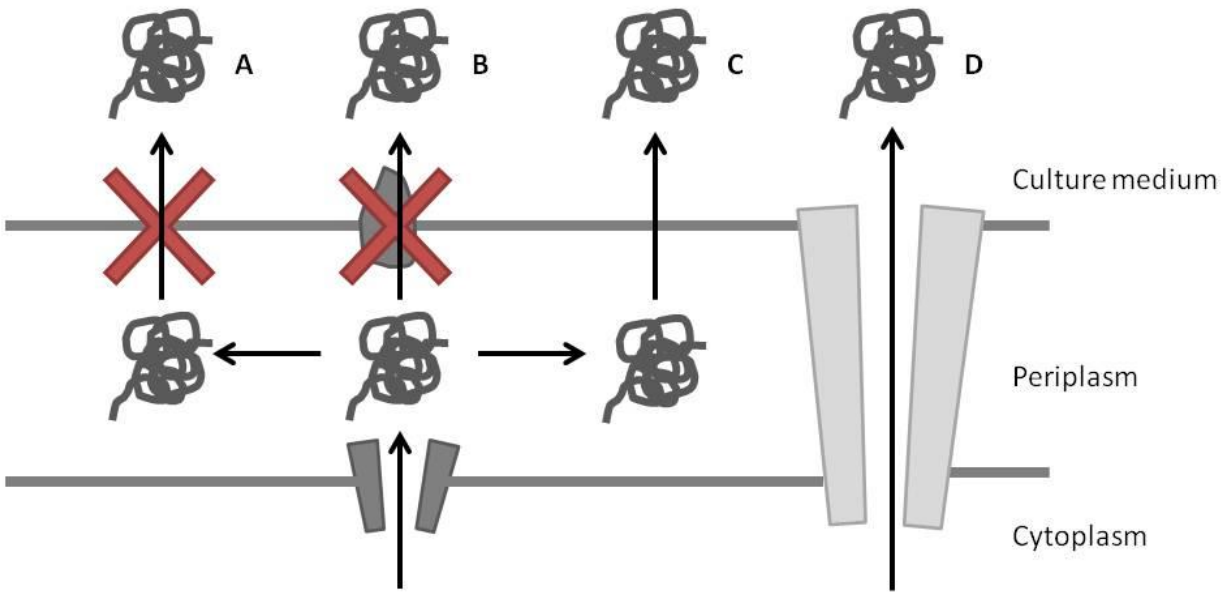

Figure 2-1 - Summary of extracellular secretion methods. Proteins are either directly transported through both membranes or first must be transported into the periplasm by transport machinery then released from the periplasmic space. (A) Outer membrane less L-form strains. (B) Mutations in major outer membrane components. (C) Permeabilization of the outer membrane through mechanical, chemical, or enzymatic treatments. (D) Specific extracellular transporters such as the hemolysin transport system (tolC/hlyA).

Approaches where recombinant proteins reach the extracellular medium through non-specific periplasmic leakage have value as versatile secretion strategies. Many of these strategies do not involve extensive genetic alterations and may be highly influenced by the composition of the culture medium giving the operator greater control over protein expression [12, 21]. Leakage is achieved by increasing the permeability of the outer membrane through mechanical treatment (ultrasound), chemical treatment (EDTA, chelating agents, magnesium, calcium, glycine, and Trition X-100), enzymatic treatment (lysozyme), or by alterations in the host cell physiology through genetic means or adjustment of cultivation conditions [10, 11]. However, many of these methods are damaging to the host cells leaving them overly sensitive to their environment or causing significant cell lysis, as in the case of lysozyme treatment or ultrasound treatment respectively.

Recently, some success has been reported with the extracellular secretion of recombinant proteins by outer membrane mutant strains. The most extreme application of this is the use of L-form strains which completely lack an outer membrane. While protein secretion is adequate in these strains, they are extremely sensitive to environmental conditions and cannot withstand the high shear stress experienced upon bioreactor cultivation making them unsuitable for industrial high-density cultivation purposes [10, 17]. Less extreme cases of non-specific leaky strains employ cell envelope mutant strains with deletions or mutations in one or more outer membrane component such as lipoproteins (*lpp*), outer membrane proteins (*ompF*) or transporter machinery (*tolA*, *tolB*) [18, 22-24]. Particularly, murein lipoprotein deletion strains (*Δlpp*) show promise in the production of recombinant proteins as this deletion has been found not to significantly affect carbon metabolism or impair cell growth when compared to the parental strain [18, 19, 25-27]. Translocation into the extracellular compartment in these systems can be very high (>90% secretion

efficiency) with relatively little culture optimization and was found to be independent of cell lysis [18, 19, 26]. This method of extracellular secretion has been commercialized by Wacker Chemicals who now offer a patented modified outer membrane mutant strain and vector systems for the extracellular secretion of recombinant proteins in *E. coli*. However, it should be noted that strict cultivation conditions must be adhered to for high level secretion [28, 29].

2.3 Properties of proteins

Development of a bioprocess starts with the determination of the criteria necessary for the product's end use and an analysis of the composition of the purification feed stock. This includes criteria such as the purity, the quantity, the cost benefit, and any additional special requirements. Characterization of the feed; such as endotoxin, proteins, and nucleic acids, and determining which impurities within the stock are absolutely prohibited in the end product can greatly facilitate process design. With these criteria in mind, it is important to understand that proteins are biopolymers consisting of a non-random sequence of amino acids joined by peptide bonds. Accordingly, each is unique and differ in terms of molecular weight, electrostatic charge, solubility, and partitioning behavior, all of which can be exploited for their purification [2, 3, 30]. Additional characteristics such as fluorescence, catalysis or color while not useful for separations are often used for monitoring purification [2, 3, 30].

2.4 Penicillin G Acylase

Penicillin G acylase (PAC) was chosen as the model protein in this study as it is an industrially significant enzyme whose annual consumption was estimated at 10-30 million tons in 2008 and an enzymatic assay, which is both highly sensitive and accurate, is available [31, 32]. PAC catalyzes

the conversion of penicillin G or cephalosporin G and related antibiotics to 6-amino penicillanic acid (6-APA) or acetoxy cephalosporanic acid (7-ADCA), respectively, with phenyl acetic acid (PAA) as a byproduct [31, 33]. These precursors are then used to synthesize semi-synthetic β-lactam antibiotics. PAC from *E. coli* ATCC 11105 is a heterodimeric protein composed of a small ~25 kDa α-subunit and large ~65 kDa β-subunit which are formed through post-translational processing of a single polypeptide [15, 34, 35]. Many technical bottle necks occur in the production of this enzyme due to its unique protein formation pathway. PAC precursor protein is synthesized in the cytoplasm and must undergo translocation into the periplasmic space, periplasmic processing, and folding before becoming fully functional [34, 35]. Formation of inclusion bodies composed primarily of PAC precursor protein is a key obstacle upon the overexpression of the PAC operon and consequently, refolding is not possible and PAC must be manufactured in its soluble form [36]. Extracellular secretion has previously been proposed as a potential strategy for alleviating the metabolic pressure of PAC overexpression and increase the flux of soluble PAC. This strategy has seen some success via coexpression with the *kil* gene, which increased the amount of PAC produced three fold [15].

Practically, PAC can be recovered after cellular disruption and isolation from the intracellular protein fraction or by first selectively extracting the periplasmic protein fraction through freeze-thaw, osmotic shock, enzymatic or other mechanical methods [37]. After clarification of the crude lysate, purification is generally achieved by selective ammonium sulphate precipitation followed by liquid chromatography [37, 38]. PAC has been successfully purified using almost all common chromatographic methods including ion-exchange, affinity, and hydrophobic interaction; however, anion-exchange is the most commonly employed chromatographic technique [37-41].

2.5 Cultivation Conditions

Cultivation conditions often play a significant role in the success of recombinant protein production and must be carefully optimized for each new protein product. Temperature, pH, osmotic pressure, medium composition (particularly carbon and nitrogen sources), and aeration can all have significant effects on growth rate, cellular physiology and gene expression [9, 42, 43]. Particularly, gene overexpression, oxygen limitation, nutrient limitation, and heat accumulation are all known to trigger cellular stress-responses which can cause growth impairment and increased protease activity [9, 44]. As *E. coli* is typically not sensitive to the shear stress experienced in a bioreactor, mixing and aeration are typically optimized to promote oxygen transfer and are balanced with the costs of power requirements. Cultivation temperature also dictates power requirements of a cultivation, and consequently because lower temperatures can increase productivity despite a decrease in growth rate, many industrial cultivations of *E. coli* are conducted below 30°C [8, 43].

Medium composition is crucial to achieving high cell density. Many nutrients must be controlled as they can inhibit growth at high concentrations, and as a result high cell densities can only be achieved through nutrient feeding during either fed-batch, chemostat or perfusion operations [8, 45]. Micronutrients such as magnesium and phosphate are essential and trace elements may be included to reduce the culture lag phase and increase protein yield [45]. While defined media are preferred for their reproducibility and higher degree of control, they are usually more expensive than complex or semi-defined media using complex components such as peptone or yeast extract [8]. Additionally, complex medium components have been known to increase protein yields and may promote soluble protein formation and therefore may be required to boost protein production at the expense of reproducibility [8].

2.6 Bioprocess Design

Rational bioprocess design must first start with the characterization of the starting material including the target and its associated impurities. Following this, several criteria must then be defined to guide the development of the purification process based on the market need and end-use including: (i) how much protein is needed, (ii) how fast is it needed, and (iii) what purity is needed [2, 3]. The second criterion is of particular importance, not only due to the universal equation of $time = money$, but because proteins are subject to protease and denaturation at separation interfaces during purification [3]. Although, there is no universal bioseparation protocol that can be applied to every protein, generally purification is approached with the heuristic presented in **Figure 2-2** [2, 3]. Typically, the first step involves the separation of the cells from the cultivation medium. In the case of intracellular proteins, following concentration, cells are disrupted, cellular debris is removed via filtration or centrifugation, and the clarified lysate is subject to liquid chromatography for a primary capture step. Intracellular proteins will then be subject to multiple chromatographic steps depending on the level of purity required which is dictated by the protein's end-use. Recovery is greatly simplified for extracellular proteins; after a solid/liquid separation step for the removal of cells and large debris the protein containing spent medium is concentrated using precipitation or another analogous technique followed by intermediate purification, due to the less complex nature of extracellular feed stocks. Following purification, therapeutic proteins require extensive polishing to remove viruses and endotoxin, while industrial enzymes may skip this step and proceed directly to formulation to preserve protein stability for product storage.

While extracellular secretion has the potential to greatly reduce the costs of product recovery, it is not often employed in *E. coli* as the benefits of simplified purification are outweighed by the greater possible efficiency when processing feed stocks with higher protein titers. Regardless,

extracellular secretion may have the added benefits of not requiring the addition of stabilizers such as protease inhibitors due to the low level of cellular proteases present in the extracellular medium. Furthermore, it will not require enzymatic treatments such as treatment with DNAse, which is often used to degrade DNA and lower the viscosity of the feed, and which must later be removed as a contaminant [3].

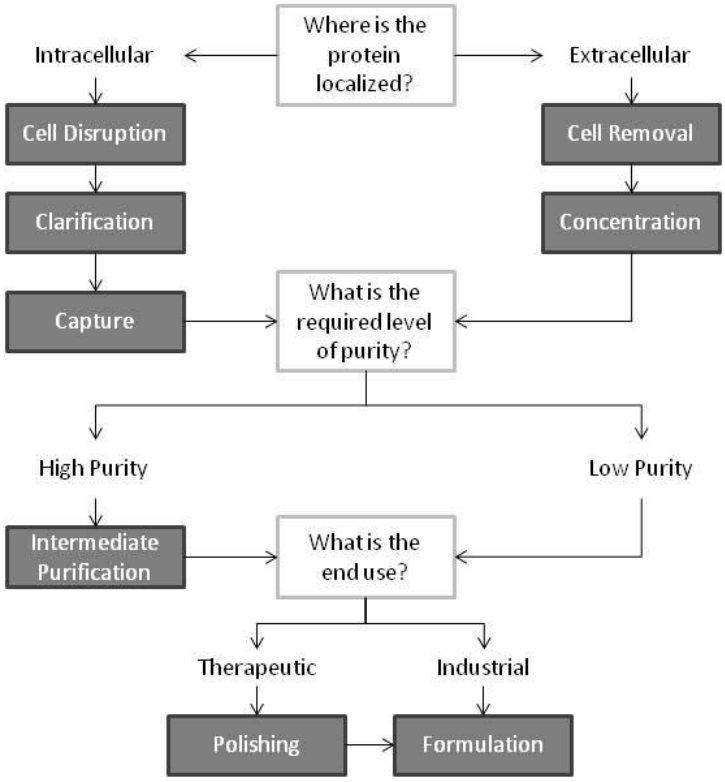

Figure 2-2 - Generic protein purification cascade

Increasing attention is being paid to the benefits of integrated process design as complementary upstream, midstream, and downstream processes may improve process efficiency and yield. This principle was demonstrated for the production of human chymotrypsin B from *P. pastoris* [46, 47]. Cultivation conditions were selected to interface cultivation and purification in such as way as to directly transfer the product at the end of cultivation to the primary recovery operation with as little alterations to the spent medium composition as possible. The cell containing culture was processed directly using cation-exchange expanded bed adsorption (EBA) chromatography and compared to extraction of chymotrypsin B from the cell containing suspension using an aqueous two phase system (ATPS) [46, 47]. In the case of EBA, a single isolation step was sufficient for purification; however, this system requires large amounts of water and as the effluent contained recombinant organisms, the discharge required inactivation [47]. ATPS yielded similar protein purity as cation-exchange EBA; however, it required an additional operation for the recovery of the product from the poly(ethylene glycol) phase [47]. Regardless, Thommes et al. [47] elegantly demonstrated the intrinsic connection between cultivation conditions and purification and found that integrated process design was an effective solution for overcoming the difficult solid-liquid separation step.

2.7 Membrane chromatography

Liquid chromatography is the most relied upon technique in protein purification. Traditionally, chromatography is conducted using functionalized cellulose based resins in tightly packed beds. Conventional columns have many well known limitations that make chromatographic operations expensive and time consuming. Thus, development of alternative stationary phases such as membranes, monoliths and mixed matrix membranes (resins embedded within a membrane support), and alternative techniques such as expanded bed chromatography, which may overcome

the limitations of packed-bed chromatography are of great interest to industry [48]. These limitations are partially a result of the slow intra-particle diffusion required for adequate binding in resin beads [4]. This imposes additional operational limitation such as slow flow rates, large pressure drops and long processing times [4, 5, 49]. Membrane adsorbers or chromatographic membranes offer a solution to these common technical problems as binding kinetics are independent of flow rate over a large range as transport of materials is dominated by convection [4, 5, 50]. A comparison of mass transport experienced in resin beads and membranes is illustrated in **Figure 2-3** [4]. Consequently, this allows membranes to operate under higher flow rates, with lower pressure drops, and may significantly shorten processing times [4, 5, 30, 49]. Additionally, membrane chromatography is directly scalable from lab to process scale reducing process development time. Several membrane geometries are available such as spiral wound devices or cross flow cassettes, and better large scale membrane manufacturing techniques are available making membranes less expensive than resins and potentially disposable, rendering regeneration and cleaning unnecessary [4, 5, 30, 49].

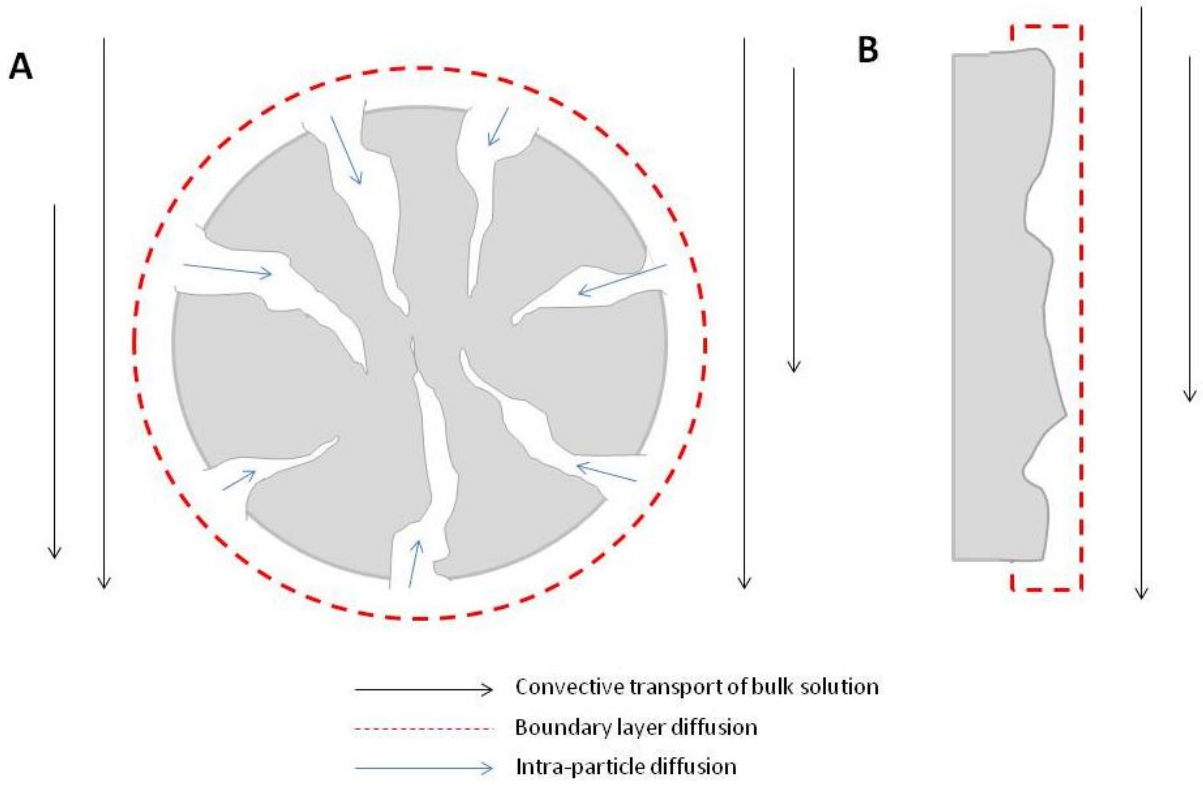

Figure 2-3 – Comparison of mass transport in (A) resin bead, (B) membrane

Membranes are now commercially available in all commonly applied chemical configurations including strong and weak cation and anion-exchange, immobilized metal affinity, protein A and G affinity, epoxy and aldehyde coupling affinity, and hydrophobic interactions [4, 5, 49]. However, the majority of recent literature published on membrane chromatography continues to be focused on development of membrane chemistry to improve binding capacities, salt tolerance, and other physical properties of chromatographic membranes, and these studies model separation performance using single or binary mixtures of proteins, consequently, the amount of literature available on the separation of complex feed stocks is relatively poor compared to traditional columns [4, 51]. Due to low binding capacities, membrane adsorbers have found somewhat of a niche in the purification of large biomolecules (where binding of these molecules mainly takes place on the outer surface of the resin bead due to steric interference which impedes entry into the pores and severely reduces the resin's binding capacity) and in flow-through polishing applications (such as the removal of virus and endotoxin in monoclonal antibody purification where the concentration of impurities is low) [51-55].

A review of current ion-exchange membrane chromatography literature summarized in **Table 2-2** shows that the majority of literature using ion-exchange membranes focuses on processing dilute feed stocks such as whey, plasma or culture supernatant. Affinity membranes are more commonly used for cell lysates [4]. This is likely due to three reasons, (i) the ability of ion-exchange chromatography to bind and elute proteins in a more concentrated form, (ii) the high number of competing protein impurities for ion-exchange ligands in high protein content cell lysates necessitating high binding capacities for efficient purification, and (iii) the reduced level of competing impurities for the highly specific affinity ligands rendering the low binding capacities of affinity membranes acceptable.

Recently, an anion-exchange hollow fiber membrane was evaluated for its ability to separate common impurities in biopharmaceutical feed stocks such as DNA, host cell protein and viruses and found to be relatively more salt tolerant compared to the commercially available Mustang Q and Sartobind Q [56]. Cross-flow chromatographic membranes represent an advantage over traditional dead-end flow membranes devices as they allow simultaneous filtration and purification. However, examples of cross-flow operation of chromatographic membranes are rare and only a few studies chronicle the dynamic binding capacities of these membranes and the separation of complex media. Wolmann et al. [57] recently demonstrated the use of a hollow fiber membrane for the purification of lactoferrin from whey using immobilized Red HE 3B dye affinity chromatography. Their membrane had a dynamic binding capacity of 110 mg/mL of membrane volume and showed good separation ability. Whey processing could greatly benefit from membrane chromatography due to the dilute nature of the target and large sample volumes that must be processed. Accordingly, recovery of lactoferrin from whey and the separation of β-lactoglobulin and α-lactalbumin from whey have also been demonstrated using cross-flow mixed matrix ion-exchange membrane chromatography [58, 59]. Immobilized metal affinity hollow-fiber membranes have also been applied for the isolation of human IgGs from plasma with high purity (98%) and good resolution between different antibody subclasses and albumin [60].

Major differences in the support and ligand chemistries of the chromatographic membranes presented in the literature make these membranes difficult to compare with each other and with traditional columns; however, many of the advantages list above have become well established knowledge and membrane adsorbers are becoming increasingly popular. The largest advantage is the potential for faster, more efficient processing and thus process economics is the driving force for

the application of these membranes and consequently, chromatographic membranes are finding their greatest advantage in polishing and single-use applications [6, 61].

Table 2-2 - Summary of ion-exchange membrane chromatography in the literature since 2005

Membrane material	Ligand ^	Adsorption Capacity*	Recovery	Membrane Geometry	Refs.
Bromomethylated Poly(2,6-dimethyl,-1,4-phenylene oxide)	DEA & C	4 mg/g for lysozyme	n/d	Amphoteric hollow fiber	[62]
Carbon nanofibres	C	>200 mg/g lysozyme SBC	>92% desorption	Flat sheets	[63]
Chitosan/ carboxymethylcellulose blend	Chitosan amino groups & CM	~250mg/g lysozyme SBC	90% desorption	Amphoteric flat sheets	[64, 65]
Nylon, polyethersulfone, regenerated cellulose & Polyvinyldifluoride	PSS	11-16mg/mL lysozyme DBC	<2% desorption	Flat sheets	[66]
Nylon/ poly(2-(methacryloyloxy)ethyl succinate)	C	122 mg/cm^3 lysozyme	>90%	Flat sheets	[67]
Polyethylene	DEA	57 mg/mL BSA DBC ~30mg/mL DNA DBC >5 LRV for PPV	90% clearance of HCP in simulated CHO supernatant spiked with IgGs & PPV virus	Hollow fibre	[56]
Polyethylene	DEA-EA	8.7 mg/mL gelsolin from bovine plasma DBC	54% recovery of gelsolin from bovine plasma	Hollow fiber	[68]
Polyethersulfone (Mustang)	Q	57 mg/mL BSA DBC	n/d	Flat sheets	[69]
Polyethersulfone (Mustang)	Q	16.1 mg/mL urease SBC 12.4 mg/mL urease DBC 2-6 mg/mL soybean proteins 10-30 mg/mL corn proteins	>80% recovery of urease from extracted soya or corn proteins spiked with urease	Flat sheets	[70]

Polyethersulfone (Mustang)	S	22 mg/mL MAb1 5% DBC	>90% recovery of MAbs, 70-80% clearance of HCP from CHO cell supernatant	Flat sheets	[71]
Polyethersulfone	S	n/d	Separtion of BSA & hemoglobin mixture	Dual layer hollow fiber	[72]
Polyimide	DEA	n/d	Separation of myoglobin & lysozyme mixture	Flat sheets	[73]
Polypropylene	Q (PMETAC)	>80 mg/mL BSA SBC >120mg/mL trypsin inhibitor SBC	>96% desorption for BSA	Flat sheets	[74]
Polypropylene	C (PAA)	56 mg/ SBC for lysozyme DBC		Flat sheets	[75]
Polypropylene	DEA	120 mg/g BSA SBC 102 mg/g BSA DBC		Flat sheets	[76]
Polysulfone	S	140 mg/mL lysozyme SBC 88 mg/mL ovalbumin SBC 66 mg/mL conalbumin SBC	95% recovery of lysozyme from egg white	Hollow fibre	[77]
Regenerated Cellulose (Vivaspin)	Q & S	11.4 mg/mL lysozyme for S 15.8 mg/mL BSA for Q	91% desorption of lysozyme for S 74% desorption of BSA for Q	Spin columns	[78]
Regenerated Cellulose (Vivaspin)	Q & S	HSA and IgG mixture Pencillin G acylase from *E. coli* lysate hGH from CHO cell culture supernatant	100% purity for hGH from CHO cells 57% recovery	Spin column	[79]
Regenerated Cellulose (Vivaspin)	Q	n/d	87.6% pure β-lactoglobulin from whey	Spin column	[80]
Regenerated Cellulose (Sartobind)	Q & S	1.02 mg/cm^2 BSA for Q 0.91 mg/cm^2 lysozyme for S	92% desorption for BSA 79% desorption for lysozyme	Spin column	[81]

Regenerated Cellulose (Vivascience)	Q & S	n/d	93% recovery of human growth hormone (hGH) in CHO cell culture supernantant	96 well plate	[82]
Regenerated Cellulose (Sartobind)	S	0.65 mg/m^2 for lactoferrin DBC	>90% recovery of lactoferrin from whey 95% purity for lactoferrin fractions 85% purity for lactoperoxidase fractions	Flat sheet	[83]
Regenerated Cellulose (Sartobind)	S	11 mg/mL lysozme DBC 24.6 mg/mL mono-PEG-lysozyme DBC	n/d	Flat sheets	[84]
Regenerated Cellulose (Sartobind)	S	n/d	Separation of Mal d1 & Mal d2 allergens	Flat sheets	[85]
Regenerated Cellulose (Sartobind)	DEA	55 mg/mL BSA SBC ~25mg/mL BSA DBC	100% desorption for BSA 75% recovery of anthrax protective antigen from *E. coli* cell lysate with 65% purity	Flat sheets	[86]
Regenerated Cellulose (Sartobind)	Q	n/d	>99% clearance of endotoxin >98% recovery of Fibroblast growth factor 2 from *E. coli* cell lysate	Flat sheets	[87]
Regenerated Cellulose (Sartorius Hydrosart)	C : PAA C: crAA	66 mg/mL IgG SBC for PAA 89.5 mg/mL lysozyme SBC for crAA	n/d	Flat sheets	[88]
Regenerated Cellulose	PDMAEMA	66.3 mg/mL BSA SBC 47-49 mg/mL BSA DBC		Flat sheets	[89, 90]
Regenerated Cellulose	PDMAEMA	130 mg/mL BSA DBC	>98% recovery and >97% purity Hb/BSA solution	Flat sheets	[91]

Regenerated Cellulose	Q: PMETAC	>130mg/mL IgG DBC		Flat sheets	[92]
Regenerated Cellulose	DEAE	135 mg/mL BSA SBC ~80mg/mL BSA DBC	65% recovery of APA, 85% purity	Flat sheets	[86]
Regenerated Cellulose	DEAE	40 mg/g BSA SBC 26.9 mg/g BSA DBC	n/d	Flat sheets	[93]
Regenerated Cellulose	C: PAA	98.5 mg/mL lysozyme SBC 72.1 mg/mL lysozyme DBC	n/d	Flat sheets	[94]
Regenerated Cellulose	Q AGM TAEA PHMB PEI	Q: 2.9 mg/mL BSA DBC| AGM: 4.9 mg/mL BSA DBC TAEA: 4.2 mg/mL BSA DBC PHMB: 8 mg/mL BSA DBC PEI: 6.1 mg/mL BSA DBC	n/d	Flat sheets	[69]
Regenerated Cellulose (Sartobind)	Q	n/d	> 4.5 LVR DNA > 4.5 LVR endotoxin > 5 LVR minute mouse viruses from clarified 324K cell lysate		[95]
Polyethersulfone (Mustang)	Q	n/d	< 1 LVR DNA > 4 LVR endotoxin		[95]
Polypropylene (ChromaSorb)	Q	67-73 mg/mL BSA DBC	> 4.5 LVR DNA > 7 LVR endotoxin >3.5 LVR minute mouse viruses from clarified 324K cell lysate		[95]

^ Q: Quaternary amine, S: Sulfonyl, C: Carboxyl, DEAE: Diethylaminoethyl, DEA: Diethylamine, EA: Ethanolamine, crAA: Poly(acrylic acid-co-methylene bisacrylamide), TAEA: Tris-2-aminoethyl amine, PHMB: Polyhexamethylene biguanide, AGM: Agamatine, PEI: Polyethyleneimine, PAA: Polyacrylamide, PMETAC: Poly(2-(methacryloyloxy)ethyl)trimethylamine), PDMAEMA: Poly (2-(dimethylamino)ethyl methacrylate), PSS: Polystyrene sulfonate

* SBC: static binding capacity; DBC: dynamic binding capacity at 10% breakthrough unless indicated otherwise

Chapter 3 – Integrated Development of an Effective Bioprocess for the Extracellular Production of Penicillin G Acylase in *Escherichia coli* and its Subsequent One-Step Purification

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The research presented in this chapter was submitted for publication to the Journal of Biotechnology. Declaration: I initiated and conducted all experiments presented herein under the supervision of Dr. C. P. Chou, Dr. J. Scharer and Dr. M. Moo-Young. Drs. Honeymann and Crossley, and Mr. Fenner were consulted for technical aspects of chromatographic operation. Dr. Suen was consulted as an expert on chromatography.

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3.1 Introduction

Bioprocess design for recombinant protein production is driven by the needs for high-level gene expression (developed in the upstream stage for strain design), large-scale production (conducted in the midstream stage for cultivation), and acceptable protein quality (addressed in the downstream stage for purification). Typically, downstream purification represents the most significant expense in protein manufacturing by accounting for 40-65% of the total manufacturing cost [2, 3]. Technological experience accumulated over the past few decades has highlighted the importance of more systematic and integrated approaches for bioprocess development. As a result, tackling technical issues in the upstream and/or midstream stages might be an effective solution to the reduction of the high cost associated with the downstream processing stage. However, such an intrinsic connection among all bioprocessing stages is rarely exploited. Hence, the focus of this study is to use this integrated approach to demonstrate a novel bioprocessing application for recombinant protein production, whereby recombinant protein was produced extracellularly under cultivation conditions that allow direct protein capture and single step purification from the cell-free medium.

E. coli secrets few proteins extracellularly and, consequently, targeting of recombinant proteins into the extracellular compartment becomes difficult and is better achieved in Gram-positive species, which lack the outer membrane, or yeasts, which are better equipped for excretion [43]. However, the lack of native protein secretion in *E. coli* makes the extracellular production of recombinant proteins using this common host system highly desirable as a method of simplifying downstream processing. By targeting recombinant proteins into the extracellular compartment, the level of contaminant proteins from the host cells can be significantly reduced and the foreign gene products are sequestered away from intracellular proteases [10, 11]. Strategies for protein secretion

in *E. coli* have been extensively reviewed and generally involve either weakening the cellular envelope or using a transporter system [9-11, 28]. Practical approaches that have been explored include the addition of extracellular transport signals, fusion with naturally secreted proteins such as hemolysin toxin [14], coexpression of release proteins [15], use of L-form *E. coli* [17], and addition of chemicals that can cause cellular permeation [20]. Each of these approaches has individual drawbacks, such as low productivity, non-native protein structure, cell lysis, stress sensitivity, and growth arrest [11, 43]. Strains with mutation(s) in the major outer membrane components were identified as potential platforms for secretion of periplasmic proteins into the extracellular medium [10, 11]. However, they were not thoroughly investigated as it was generally believed that these strains were growth-impaired and cannot be cultured to a high cell density for industrial applications. Recently, interest in these strains has been renewed by partially overcoming the growth issue. For example, knocking out the *lpp* gene encoding murein lipoprotein allows the secretion of recombinant proteins targeted in the periplasm into the extracellular medium without severely retarding cell growth [18, 19]. Furthermore, the use of outer membrane mutants as expression hosts could potentially enhance recombinant protein production due to the increased solubility of proteins in the periplasm and could potentially be combined with other genetic strategies such as coexpression of periplasmic chaperones and knock-out of periplasmic proteases [10, 11].

In this study, using penicillin G acylase (PAC) as the target protein, an *Escherichia coli* host/vector system was developed for high-level expression and extracellular secretion of recombinant PAC. A cultivation medium was specifically developed to enhance culture performance and facilitate harvest and purification of secreted recombinant PAC directly from unaltered cell-free medium by anion-exchange chromatography. PAC was selected as a target

protein due to its industrial significance in the production of β-lactam antibiotics [31]. It is a heterodimeric protein maturing through posttranslational modification in the periplasm of *E. coli* [15, 96]. PAC precursors tend to misfold and form inclusion bodies upon the overexpression of the *pac* gene in *E. coli* and this has been identified as one of the major limitations on the production of this industrial enzyme [96]. Practically, PAC is purified primarily through chromatographic techniques, including anion-exchange chromatography, cation-exchange chromatography, and even affinity chromatography [38-40]. Due to the nature of the intracellular production of PAC, these chromatographic techniques often represent a major purification effort after a tedious multi-step pretreatment, including cell harvest, cell disruption, protein precipitation, and desalting [38].

The extracellular production of PAC developed in this study offers an attractive scheme to significantly reduce bioprocessing complexity and operating cost. Downstream purification of PAC was facilitated by direct loading of cell-free medium for one-step anion-exchange chromatography. While it is not uncommon for extracellular proteins to be purified using anion-exchange chromatography, the spent medium is subject to various pretreatments, such as centrifugation, ultrafiltration, dialysis, and precipitation for the removal of excess salts and large debris prior to its application. Typical media for high-cell-density cultivation of *E. coli* contain a relatively high concentration of salts and/or various complex and charged components, such as oligopeptides, which may complicate the operation of anion-exchange chromatography [8]. Hence, a major challenge for direct capture and purification of PAC from the spent medium is to develop an appropriate medium that optimizes both cultivation and purification.

3.2 Materials and Methods

3.2.1 Bacterial strains and plasmids

JE5505 (*F⁻, Δ(gpt-proA)62, lacY1, tsx- 29, glnV44(AS), galK2(Oc), & λ⁻, Δlpp-254, pps-6,hisG4(Oc), xylA5, mtl-1, argE3(Oc), thi-1)* [97], HB101 (*F⁻, hsdS20, leuB6, recA13, ara-14, proA2, lacY1, thi-1, galK2, rpsL20, xyl-5, mtl-1, supE44, λ⁻*) [98], and JM109 (*F' [traD36 proAB⁺ lacI^q lacZΔM15], recA1, supE44, endA1, hsdR17, gyrA96, relA1, thi^-1, mcrA, Δ[lac-proAB]*) obtained from the CCRC, Taiwan were used as the hosts for the production of recombinant PAC. The plasmid pTrcKnPAC2902 containing the *pac* operon from *E. coli* ATCC11105 regulated by the *trc* promoter was previously constructed [96].

3.2.2 Cultivation

The recombinant strain was stored at -80°C as an LB/glycerol stock. The cells were revived by streaking on LB agar plates with kanamycin (50 μg/mL). A single colony was inoculated into a flask with 50 mL of LB medium (5 g/L yeast extract, 10 g/L tryptone, 0.5 g/L NaCl, and 50 μg/mL kanamycin) and incubated at 30°C in a rotary shaker for 16 h at 200 rpm. Fifty milliliters of this seed culture (2%) was used to inoculate a bench-top bioreactor (Omni-Culture, VirTis, Gardiner, NY) containing 1 L working volume of medium with 20 μL/L Antifoam 204 at 28°C (Sigma, St. Louis,MO). Various media were prepared with composition listed in **Table 3-1**. Chemicals such as yeast extract, Tryptone, and casamino acids were purchased from BD (Franklin Lakes, NJ, USA), while chemicals such as 3-(N-morphlino)propanesulfonic acid (MOPS) and NaCl were purchased from Fisher Scientific (Waltman, MA, USA). Trace elements were added to a final concentration of 2 mM $MgSO_4 \cdot 7H_2O$, 2 mM KH_2PO_4, 0.2 mM $CaCl_2 \cdot 2H_2O$, 1.5 mg/L of $FeSO_4 \cdot 7H_2O$ and 0.6 mg/L of $ZnSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 7H_2O$, and 3 mg/mL thiamine where indicated. Protein

expression was induced with the indicated concentration of isopropyl β-D-thiogalactopyranoside (IPTG) when the cell density reached approximately 0.5 OD_{600}. The bioreactor was purged with filter-sterilized air and agitated at the rates given in **Table 3-1**. The pH was regulated at 7.0 ± 0.1 by the addition of 1 N NH_4OH or 1 N HCl using a combination pH electrode (Mettler-Toledo, Switzerland), a pH controller (PC310, Suntex, Taipei, Taiwan), and two MasterFlex peristaltic pumps (Cole-Palmer,Vernon Hills, IL, USA).

3.2.3 Treatment of culture sample

The culture sample was appropriately diluted with 15 mM NaCl for measuring cell density at 600 nm with a spectrophotometer (DU520, Beckman Coulter, Fullerton, CA, USA). For the preparation of the cell extracts, 20 OD units were centrifuged at 2°C and 6000 g for 6 min in a Hettich Universal 320 R centrifuge (Hettich Instruments, Beverly, MA, USA). The supernatant containing extracellular proteins was analyzed for secreted PAC. The cell pellet was resuspended in 1 mL PBS (0.05 M, pH 7.4) and sonicated for 5 min using an ultrasonic processor (Misonix, Farmingdale, NY, USA). The cell lysate was centrifuged at 2°C for 10 min at 10000 g. The supernatant was analyzed for soluble intracellular PAC activity. The pellet containing insoluble proteins was resuspended in TE/SDS buffer (10 mM Tris HCl pH 8, 1 mM EDTA, 1% SDS) and heated at 95°C for 5 min. All fractions were analyzed by SDS-PAGE and western blotting.

3.2.4 Enzyme assay

Penicillin G acylase was assayed at 37°C using penicillin G as a substrate. The amount of enzymatic reaction product, 6-aminopenicillanic acid (6-APA), was quantified using a colorimetric method developed previously [32]. All assays were conducted in duplicate. One unit was defined as the amount of enzyme that hydrolyzed 1.0 μmol penicillin G per minute at 37°C. The specific activity

(in U/L/OD_{600}) was calculated as the quotient of the activity (in U/L) and cell density (in OD_{600}). The secretion efficiency (%) was determined as the fraction of total PAC activity that was extracellular.

3.2.5 Total protein assay

The bicinchoninic acid assay (BCA) for total protein concentration was performed using a kit (Pierce Biotechnology, Rockford, IL, USA) in the microplate assay format. Samples were all performed in duplicate and were appropriately diluted in 25 mM Tris-HCl (pH 8) to fall in the linear range of the kit. The standard curve was performed in duplicate for each plate with bovine serum albumin. Absorbance was measured at 562 nm with a Thermo Labsystems Multiskan Ascent photometric plate reader (Thermo Scientific, Wilmington, USA).

3.2.6 SDS-PAGE and Western blot

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a Mini-PROTEAN II electrophoresis cell (Bio-Rad, Hercules, CA, USA) using a 12.5% polyacrylamide separating gel stacked by a 4% polyacrylamide stacking gel. Final loading amount for total protein (soluble and insoluble) was 0.1 OD_{600} units, while 10 μL was loaded for extracellular supernatant and for chromatography fractions. Electrophoresis was conducted under a constant voltage of 200 V for 60 min. Gels were either stained using silver nitrate and dried, or used for western blot analysis. For western blots, gels were electroblotted on to a PVDF membrane (Pall Corporation, Port Washington, NY, USA) using a Mini Trans-Blot Cell (BioRad, Hercules, CA, USA) according to a standard protocol [99]. The electrophoretic transfer was conducted at a constant voltage of 100 V for 1 h. Protein-antibody hybridization was performed as described by the membrane manufacturer [100]. The primary antibody was a polyclonal rabbit anti-PAC β-subunit IgG and the secondary

antibody was a goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP) (Sigma, St. Louis, MO, USA). HRP was detected by colorimetric visualization using 3-3-diaminobenzidine tetrahydrochloride (DAB) as the substrate. Dried SDS-PAGE gels and western blots were scanned using an HP laserjet scanner 3020.

3.2.7 Anion-exchange chromatography

Anion-exchange chromatography was performed using a low pressure liquid chromatography system (Bio-Logic LP, Biorad, Hercules, CA) which allowed online UV absorbance (280 nm) and conductivity monitoring using the accompanying software (LP Logic, Biorad, Hercules, CA). A 5mL Q Sepharose™ Fast Flow Column (GE Healthcare, Buckinghamshire, UK), herein refered to as QFF columns, with a reported dynamic binding capacity of 120 mg of HSA per milliliter of resin was attached upstream of the indicators and downstream of the sample loading device. The column was equilibrated for 15 min at the indicated flow rate (either 1 mL/min or 4 mL/min) with the appropriate loading buffer (either 25mM Bis-Tris-HCl pH 7 or 25mM Tris-HCl pH 8). Samples of 2.5, 30, 50, 60, 300 mL of culture broth were harvested from cultivation of JE5505 harboring pTrcKnPAC2902 in LCM3 medium after 12 h of induction with 0.15 mM IPTG. The samples were centrifuged at 6000 g for 10 min and filtered with 0.22 μm polyethersulfone syringe filters (VWR, Radnor, PA, USA) to remove cell debris. These samples were either directly loaded at their current pH of 7 or adjusted to a pH of 8 by addition of 3 N NaOH before loading. After loading, weakly bound protein was removed from the column by washing with loading buffer for 10 min at the indicated flow-rate. Following the washing step, either a 60 mL linear salt gradient from 0-1 M NaCl was used to elute proteins from the column or a step-wise elution using 10 mL of 1 M NaCl was performed as indicated by the chromatogram conductivity. All steps were performed at the same flow-rate. Eight milliliter fractions were collected with a fraction collector (BioLogic

BioFrac, Biorad, Hercules, CA) and pooled into loading flow-through, wash flow-through, and elution peaks. All fractions were assayed using the BCA and PAC enzyme assay and analyzed using SDS-PAGE. Pooled fractions were desalted by diafiltration over 25 fold to remove oligopeptides using 3 kDa cut-off ultrafiltration centrifugal devices (Palls Corporation, St.Louis, MO, USA). Chromatography was performed in duplicate under identical chromatography conditions with broth from different cultivations.

3.3 Results and Discussion

3.3.1 Extracellular PAC expression by leaky outer-membrane mutant

Industrial bioprocesses require strains which can attain a high cell density with a relatively high growth rate and are less sensitive to harsh bioreactor environment. To determine whether the *lpp*-deletion mutant, JE5505, would be a suitable strain for extracellular PAC production, the plasmid pTrcKnPAC2902 was transformed into this strain. HB101 with an uncompromised outer membrane was used as a control strain for comparison. Initially, cultivation of JE5505 harboring pTrcKnPAC2902 in LB medium induced with 0.1 mM IPTG was performed to verify its capacity in the extracellular production of PAC under bioreactor conditions (**Table 3-1**). The specific PAC activity in the extracellular fraction was significantly higher than that exhibited by the control strain HB101 harboring pTrcKnPAC2902 (40% vs. 11%) under similar culture conditions. It should be noted that a low level extracellular secretion is expected for the control strain due to the natural leakage of periplasmic proteins during growth [11]. However, due to its high oligopeptide content, LB was not compatible with the subsequent step of anion-exchange chromatography. The sample medium loaded for ion-exchange chromatography must also have a low ionic strength to allow proper binding of proteins to the chromatographic matrix. Additionally, the extracellular production

of PAC from cultivation in LB was limited by the low final cell density. Therefore, more than twelve medium recipes were formulated for cultivation under different bioreactor conditions and the culture performance of four distinct media is summarized in **Table 3-1**. For most cultivations, the PAC expression level and secretion efficiency increased with cultivation time and reached a plateau by 20 h after induction. Hence, culture samples taken at this time point were used for comparison.

Table 3-1 - Summary of culture performance for extracellular production of penicillin G acylase in various media formulated for subsequent ion-exchange chromatography

Strain	**JE5505**					**HB101**
Medium Name	**LB**	**LCM1**	**LCM2**	**LCM3**	**LCM3**	**LCM3**
Medium Composition						
Glucose [g/L]	-	6	6	6	6	6
NaCl [g/L]	10	0.1	0.1	0.1	0.1	0.1
Casamino acids [g/L]	-	2.5	2.5	5	5	5
Yeast extract [g/L]	5	-	-	2.5	2.5	2.5
Tryptone [g/L]	10	-	-	-	-	-
MOPS buffer [mM]	-	-	20	20	20	20
Trace element solution*	-	+	+	+	+	+
Cultivation Conditions						
Agitation [rpm]	250	500	250	250	250	250
Aeration [vvm]	1	2	1	2	2	2
IPTG induction [mM]	0.1	0.05	0.1	0.1	0.15	0.15
Culture Performance						
Cell density [OD_{600}]	1.3	1.6^	1.6	1.9	2.6	5.1
Conductivity [mS/cm]	>30	4.7	4.4	6.7	6.8	6.6
Extracellular PAC activity [$U/L/OD_{600}$]	206	58	85	199	442	128
Total PAC activity [$U/L/OD_{600}$]	512	102	88	206	487	556
Secretion efficiency	40%	57%	96%	97%	91%	23%

***** + indicates addition of trace element solution describes in materials and methods
^ Growth rate of this culture was severely impaired due to increased sensitivity of JE5505 to low medium osmolarity

3.3.2 Formulation of medium with a low ionic strength

Typically, the ionic strength permissible for ion-exchange chromatography operation should be less than 5-7 mS/cm [101]. Common complex medium components, such as yeast extract, contain oligopeptides that can interfere with protein binding for ion-exchange chromatography. Consequently, a defined medium using low conductivity components was developed not only to reduce the ionic strength and the oligopeptide content, but also to sustain simultaneous cell growth and high-level production of extracellular PAC. Several medium components for *E. coli* cultivation [102] were screened using shaker-flask cultivation by evaluating cell growth and the conductivity of spent medium. A medium recipe consisting of mainly glucose, casamino acids, and a minimal amount of salts was formulated. This medium had a conductivity of 3.3 mS/cm before cultivation and a final conductivity of less than 5 mS/cm after cultivation. The increase in the ionic strength during cultivation was associated with the addition of base for pH control. The growth of JE5505 harboring pTrcKnPAC2902 in LCM1 was severely impaired in the bioreactor. Surprisingly, the growth of one of the control strain, JM109 harboring pTrcKnPAC2902 in LCM1 medium, was not retarded under a similar bioreactor condition (reached a density of 3.3 OD_{600} within 4 h post-induction). It was further observed that the cell growth of JE5505 harboring pTrcKnPAC2902 was not improved by decreasing the agitation speed in the bioreactor, but such growth retardation no longer existed when cultivating in shaker flasks (reached a density of 0.8 OD_{600} within 6 h of inoculation). Based on these observations, it was postulated that the host strain of JE5505 could be particularly sensitive to shear stress under low osmolarity conditions due to its destabilized outer membrane. In fact, it was previously reported that JE5505 had a decreased growth rate in low ionic-strength media and other *lpp* deletion strains were also found to exhibit decreased growth with the exclusion of Ca^{2+} and Mg^{2+} ions from the medium [21, 103]. Consequently, the medium (named

LCM2) was altered to include MOPS, which has a low conductivity and can provide a buffering capacity at pH 7 during cultivation. As a result, the amount of ions added for pH control during the cultivation was significantly reduced and the unaltered spent medium could be directly loaded for anion-exchange chromatography [104]. The application of LCM2 could alleviate the growth impairment of JE5505 harboring pTrcKnPAC2902 in the bioreactor while maintaining a low conductivity and suitable pH of the spent medium. Most importantly, the level of the extracellular secreted PAC increased to 96%, although the specific and total PAC activity remained relatively low. Alternatively, M9 medium supplemented with 1% casamino acids and 1% glucose could be used to achieve high secretion efficiency. It has been shown that 95% secretion efficiency was obtained upon expressing recombinant human secretory pancreatic trypsin inhibitor protein [26]. A similar M9 medium was also successfully applied to achieve over 90% secretion efficiency upon the expression of recombinant xylanase and cellulase using another *lpp* deletion strain of C609Y [18]. However, the relatively high salt concentrations in the M9 medium precluded its application in the current study.

3.3.3 Effect of medium composition on the production of PAC

It was reported that the inclusion of complex medium components during cultivation may enhance recombinant protein production [8, 105]. Thus, the effects of complex medium components (specifically, yeast extract and casamino acids) and induction conditions on the production of PAC were investigated. JE5505 harboring pTrcKnPAC2902 was cultivated in LCM3 medium and the production of PAC was induced by 0.1 mM IPTG. This resulted in an increased total PAC activity with an increased final cell density, unaltered secretion efficiency, and a permissible conductivity of the spent medium. The increased supplementation of amino acids could potentially decrease protease activity induced by a stringent-like stress response [44] associated with amino acid

limitation, resulting in a reduced degradation of PAC. In addition, the supplementation of non-essential nutrients such as vitamins and nucleotides in the complex medium components may be critical for enhancing the production of PAC. While complex medium components are generally not preferred for large scale cultivations, the supplementation of yeast extract appears to be critical for effective extracellular production of PAC. The total specific PAC activity was further increased without any adverse effect on cell growth and PAC secretion efficiency when the IPTG concentration was increased to 0.15 mM, implying the importance of determining an appropriate induction condition for optimal culture performance.

3.3.4 Effect of inclusion body formation

The culture performance of both JE5505 harboring pTrcKnPAC2902 and HB101 harboring pTrcKnPAC2902 using LCM3 medium is summarized in **Figure 3-1** and **3-2**, respectively. The production of PAC by JE5505 harboring pTrcKnPAC2902 was primarily formed during the growth phase as more than 90% of the total recombinant PAC was synthesized within the first 8 h of induction with an effective extracellular secretion. After this production period, cell growth was arrested and then the culture pH became basic, suggesting possible depletion of glucose [106]. Although the recombinant host strain of JE5505 exhibited poor growth in this study (1 OD_{600} = ~0.5 g/L dry cell weight [107]), it was previously shown to reach a high cell density of 26 g/L dry cell weight using fedbatch cultivation for the production of interferon [108]. Nevertheless, a low ionic strength might not be easily maintained in a fedbatch culture, due to the addition of acid and base for pH control. In that regard, chemostat cultivation could be an alternative approach for this type of operation due to the dilution effect of the continuous feed. On the other hand, HB101 harboring pTrcKnPAC2902 had more prolonged cell growth than JE5505 harboring pTrcKnPAC2902 with approximately the same *pac* gene expression level under identical bioreactor conditions, possibly

due to a healthier cell physiology. The improved cell growth resulted in an increased volumetric PAC activity. Note that, compared to the traditional approach for intracellular production of PAC, the secretion strategy adopted in this study appears to hardly affect the *pac* gene expression level. The insoluble fraction of cell lysate was also analyzed by Western blotting (**Figure 3-1B and 3-2B**). It was rather surprising that a significant amount of inclusion bodies composed of PAC precursors accumulated in JE5505 harboring pTrcKnPAC2902, but not HB101 harboring pTrcKnPAC2902; and this might potentially result in the difference in cell physiology. HB101 harboring pTrcKnPAC2902 was previously reported to form inclusion bodies when cultivated in LB medium under similar cultivating conditions [96]. These previous and current results suggest that both host strain and culture conditions could potentially influence the formation of inclusion bodies.

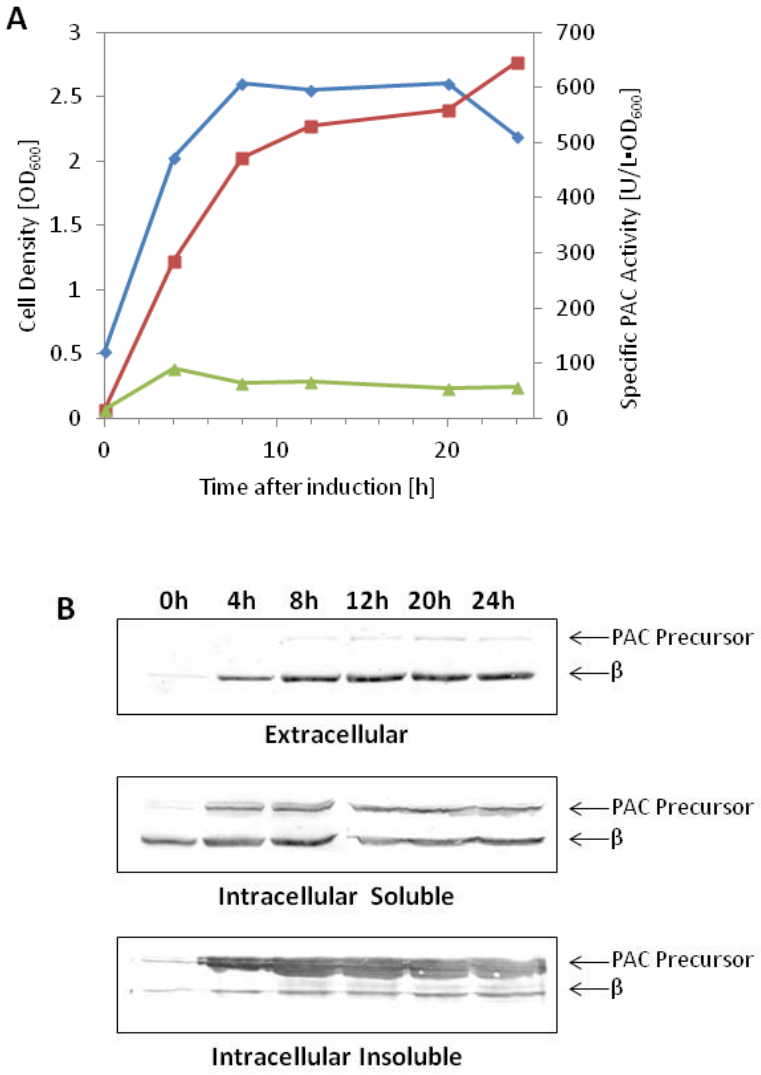


Figure 3-1 - Culture performance for the production of PAC using JE5505 harboring pTrcKnPAC2902 in LCM3 medium induced with 0.15 mM IPTG in a bioreactor. (A) Time profile showing the cell density (diamond), specific intracellular PAC activity (triangle) and extracellular PAC activity (square). (B) Immunological analysis showing the localization of PAC. Samples of the extracellular, intracellular soluble protein and intracellular insoluble protein fractions taken at different time points from 0-24 h after induction were analyzed. PAC precursor and mature PAC beta subunit are indicated

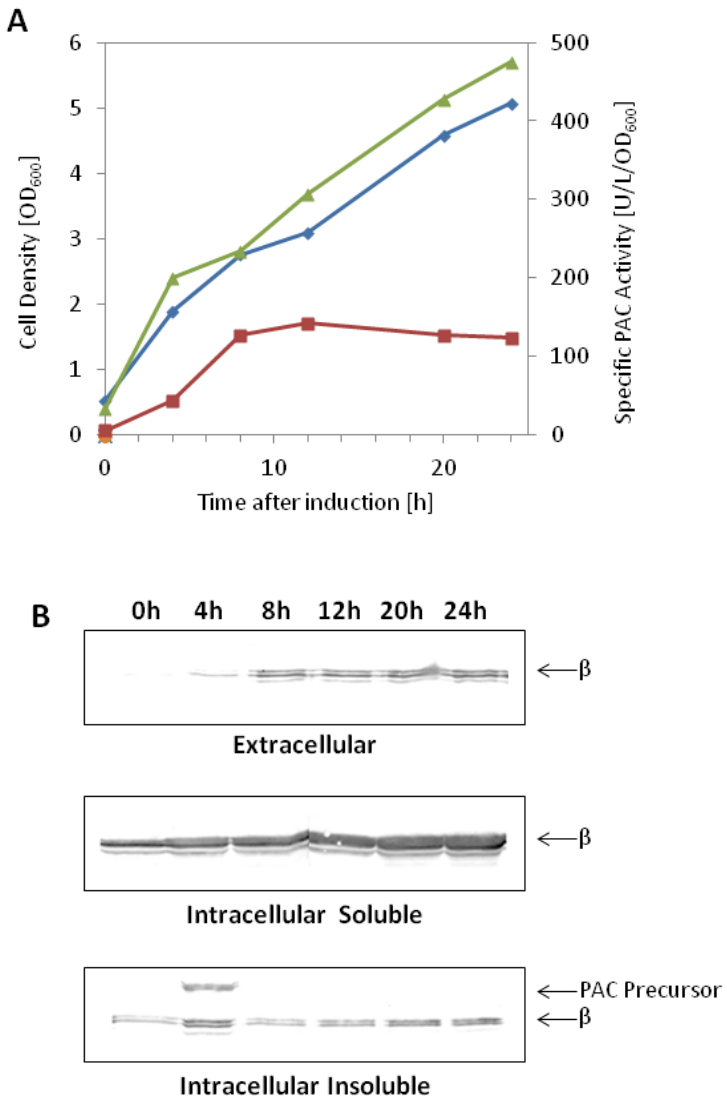


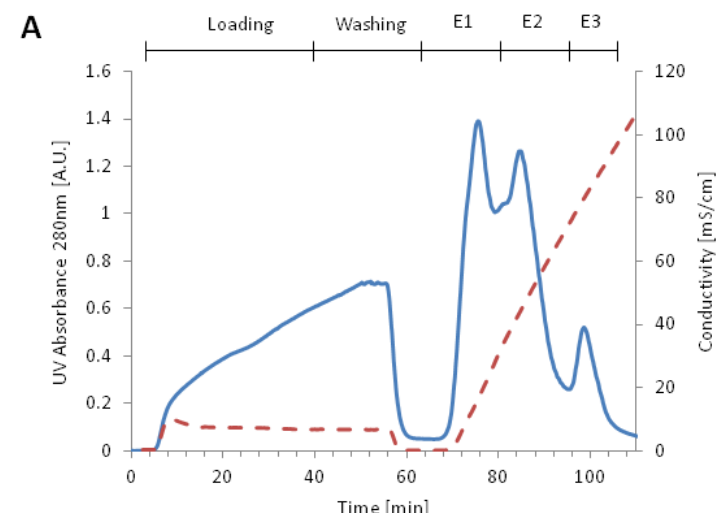
Figure 3-2 - Culture performance for the production of PAC using HB101 harboring pTrcKnPAC2902 in LCM3 medium induced with 0.15 mM IPTG in a bioreactor. (A) Time profile showing the cell density (diamond), specific intracellular PAC activity (triangle) and extracellular PAC activity (square). (B) Immunological analysis showing the localization of PAC. Samples of the extracellular, intracellular soluble protein and intracellular insoluble protein fractions taken at different time points from 0-24 h after induction were analyzed. PAC precursor and mature PAC beta subunit are indicated.

Several effects were identified to be associated with the formation of inclusion bodies upon the *pac* gene overexpression in *E. coli*, including the toxicity of inclusion bodies (deteriorating cell physiology), misfolding of PAC precursors in the cytoplasm and/or periplasm, and ineffective posttranslational processing (i.e. autoproteolysis) of PAC precursors in the periplasm [15, 36, 96, 109]. Extracellular secretion of intracellular proteins was proposed to have the potential to alleviate inclusion body formation due to the release and dilution of the accumulated proteins inside cells [10, 15]. Such general knowledge for extracellular protein release was apparently in contradiction to the current results, i.e. effective expression/secretion of PAC accompanied significant inclusion body formation (in JE5505 harboring pTrcKnPAC2902) whereas intracellular overexpression of PAC resulted in hardly any inclusion body formation (in HB101 harboring pTrcKnPAC2902). While medium composition was proposed to be a potential factor, poor cell physiology associated with the leaky nature of JE5505 could be critical for inclusion body formation. The characterization of such an effect will improve the understanding of the formation mechanism of PAC inclusion bodies.

3.3.5 Downstream purification of PAC

Based on the isoelectric point of PAC (pI ~ 6) [110], anion-exchange chromatography was used as a major purification step for the downstream processing of PAC. The low ionic strength of the clarified spent LCM3 medium containing extracellularly secreted PAC produced by JE5505 harboring pTrcKnPAC2902 allowed its direct loading onto the chromatographic column. The purification results are summarized in **Figure 3-3**. While the purification performance under the two pH values investigated was rather indistinguishable, the operation under pH 8 had a higher peak resolution possibly due to the decreased volumetric flow rate used in that experiment. PAC was harvested and purified through a “negative” separation scheme since, according to the SDS-PAGE

analysis, PAC was the major species present in the flow-through fraction and the majority of the contaminant proteins were adsorbed onto the chromatographic column and then eluted in the fractions corresponding to the first or second peak. Only 2.5% of the total PAC activity loaded to the column was detected in the washing flow-through and elution fractions. The fractions corresponding to the last peak eluted at a high salt concentration were primarily composed of RNAs (**Figure 3-4**) due to a low level of cell lysis during the cultivation. A pH value of 7.0 was however selected as the operating pH to minimize the pH fluctuation during the chromatographic operation as the cultivation was also conducted under this pH. A relatively high flow rate of 4 mL/min was used to facilitate the chromatographic operation given that the resolution associated with the elution step is not critical for a "negative" separation. Purification of PAC under these operating conditions is summarized in **Table 3-2**. While the purified PAC was still contaminated by oligopeptides which were not removed during the chromatographic operation, the purity was justified to be high enough for use as an industrial enzyme.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E2
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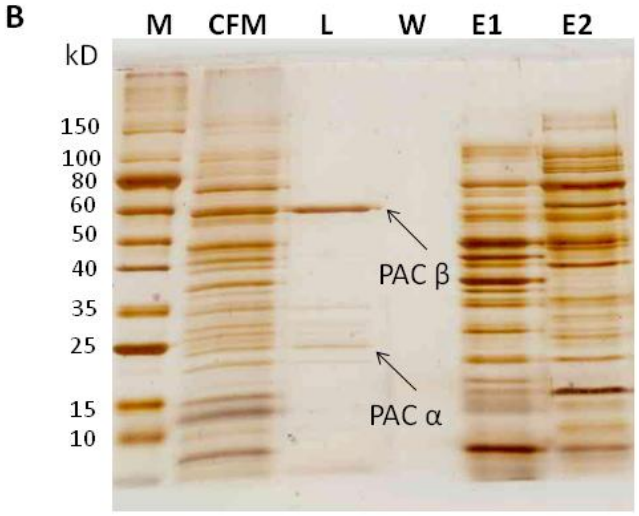
UV Absorbance 280nm [A.U.]
Conductivity [mS/cm]
Time [min]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PAC β
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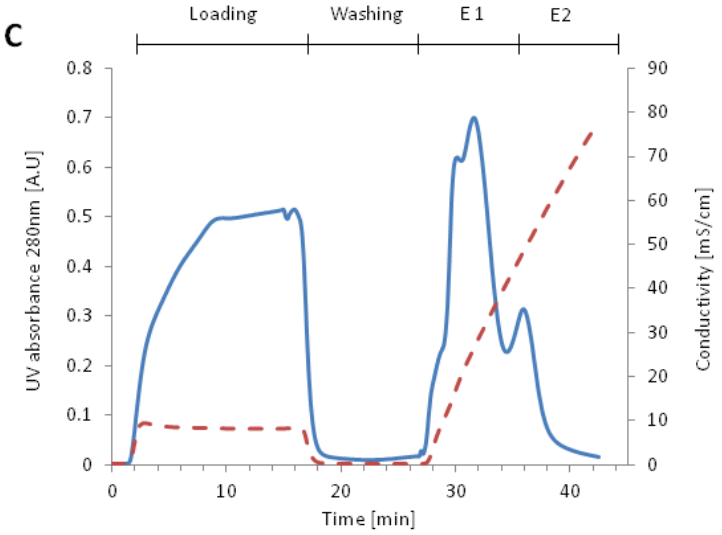
C
Loading
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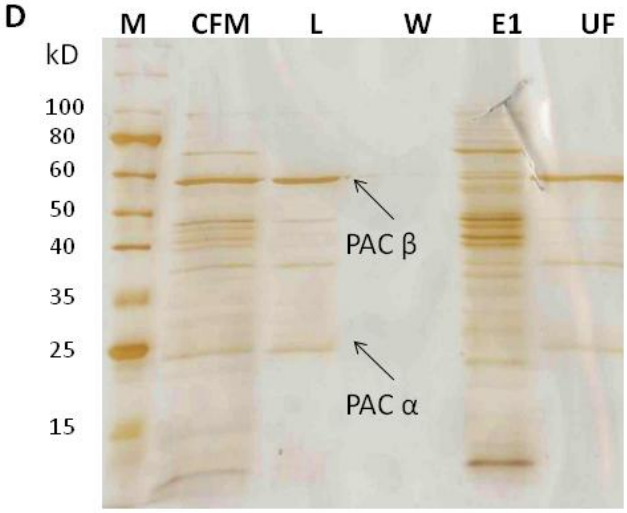
UV absorbance 280nm [A.U]
Conductivity [mS/cm]
Time [min]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PAC β
PAC α

Figure 3-3 - (A) Chromatogram when 50 mL of spent LCM3 medium from the cultivation of JE5505 (pTrcKnPAC2902) adjusted to pH 8 was loaded to a 5 mL QFF anion-exchange column at a flow rate of 1 mL/min. UV absorbance is indicated with a solid line and conductivity is indicated with a dashed line. Major steps are indicated above the plot including: loading flow-through (L), washing flow-through (W), elution 1 (E1), elution 2 (E2), and elution 3 (E3) if applicable. (B) SDS-PAGE analysis of the loaded cell free medium (CFM), the pooled fractions from the chromatogram shown in panel A, and a protein marker (M). Note: Elution 3 (E3) was not analyzed as it did not contain any protein and had high salt content. (C) Chromatogram when 60 mL of unadjusted spent LCM3 medium (pH 7) from the cultivation of JE5505 (pTrcKnPAC2902) was loaded to a 5 mL QFF column at flow rate of 4 mL/min. (D) SDS-PAGE analysis of pooled fractions from the chromatogram shown in panel C. Note: Elution 2 (E2) was not analyzed as it did not contain protein and had high salt content.

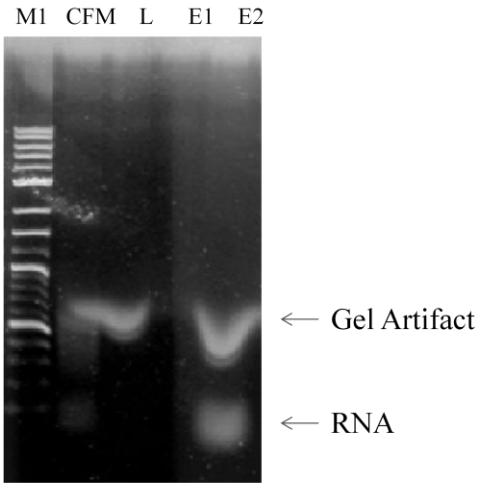

Figure 3-4 –One percent agarose gel electrophoresis of chromatography fractions after separation of 50mL of spent LCM3 medium adjusted to pH8 by a 5 mL QFF anion-exchange column at a flow rate of 1 mL/min. A 1 Kbp marker from Fermentas (M1), cell free medium (CFM), loading flow-through (L), elution peak 1 (E1) and elution peak 2 (E2) are indicated.

Table 3-2 – Quantification of the separation performance of PAC purification using the QFF column. Sixty millilitres of cell free LCM3 culture broth was loaded to the Q column at pH 7 at 4 mL/min

		Protein Concentration [µg/mL]	Volumetric PAC Activity [U/L]	Specific Activity [U/mg]	PAC Recovery	Purification [Fold]
Cell Free Medium	Total protein including oligopeptides	633	666	1.05	-	-
	Total protein excluding oligopeptides	133	678	5.10	-	-
Anion-Exchange Flow-through	Total protein including oligopeptides	493	649	1.32	97%	1.3
	Total protein excluding oligopeptides	39	635	16.3	98%	3.2

The observation that the majority of PAC was localized in the flow-through fraction without binding to the chromatographic medium was surprising considering its isoelectric point. It is believed to be caused by the competitive binding of various biomolecules, including contaminant proteins and oligopeptides. To verify this, different loading volumes of cell-free medium (i.e. 2.5, 30, 60, and 300 mL) were applied to the chromatographic column under identical operating conditions (**Figure. 3-5**). No PAC breakthrough was detected when a small volume of 2.5 mL was loaded and PAC was entirely eluted in the fractions with a low ionic strength between 20-30 mM NaCl. On the other hand, when 30 mL of the cell-free medium (with 76% oligopeptide content based on the BCA assay) was loaded, highly purified PAC was collected in the flow-through fractions with most contaminant proteins being retained by the chromatographic column. However, oligopeptides cannot be completely eliminated from the product (with 32% oligopeptides content

based on the BCA assay). Increasing the loading volume to more than 60 mL will further release oligopeptides into the flow-through fractions (up to ~90% oligopeptides content based on the BCA assay). The results suggest a competitive binding scenario during the loading stage of the chromatographic operation, particularly under the condition of high loading volume. PAC appeared to be the major species in the cell-free medium with a low binding affinity (or surface charge) to the chromatographic medium and, as a result, increasing loading volume above 1 column volume could result in elution of the bound PAC by proceeding highly charged impurities. Thus, the loading volume will affect the purification performance of anion-exchange chromatography. While the presence of oligopeptides won't affect PAC catalytic activity upon industrial applications, they can be easily removed if necessary using ultrafiltration, as demonstrated in this study.

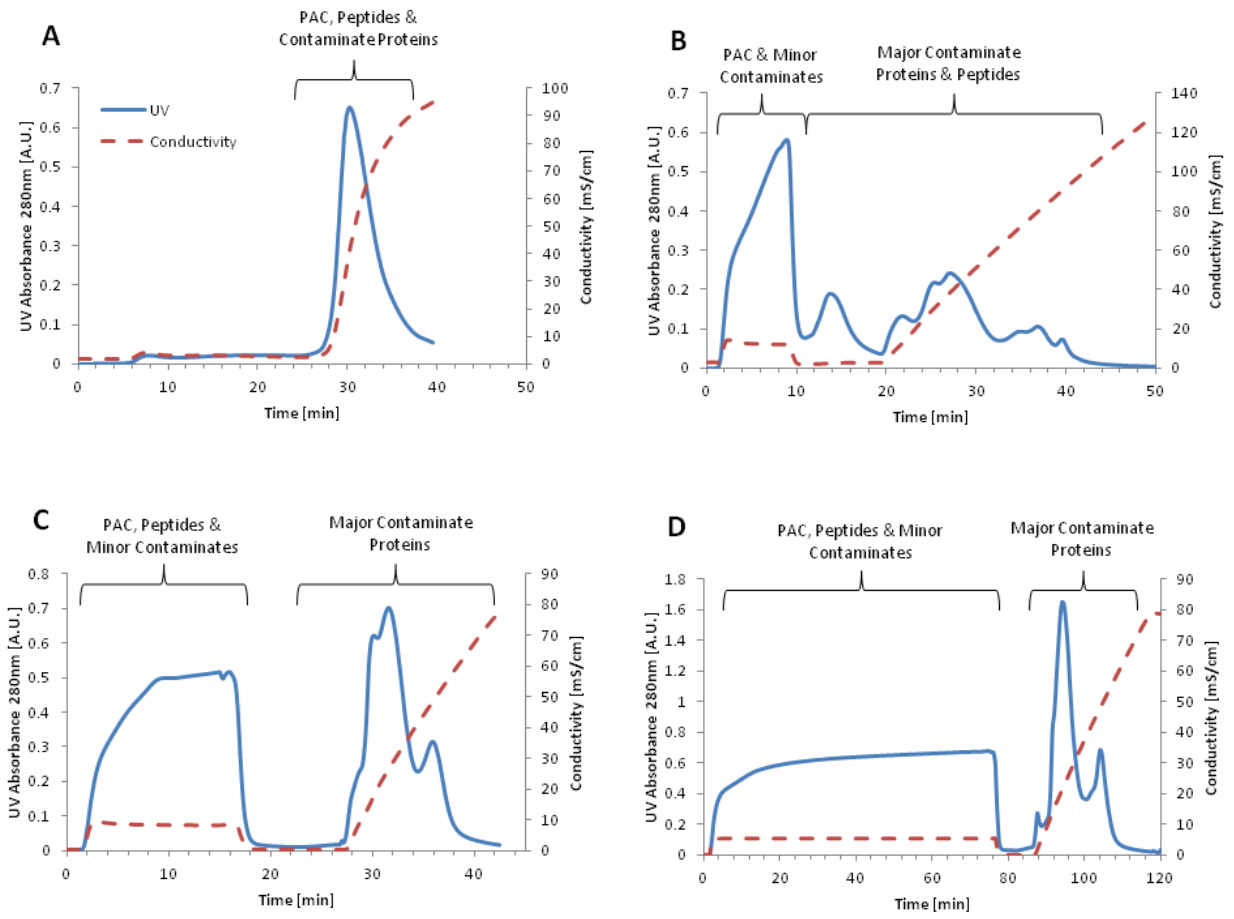

Figure 3-5 - Effect of loading volume on separation performance for 5mL QFF column operates at pH 7 and 4 mL/min. Location of major proteins of interest are indicated above the plot. UV absorbance is indicated with a solid line and conductivity is indicated with a dashed line. (A) 2.5 mL (B) 30 mL (C) 60 mL (D) 300 mL. Note: a step-wise elution was used when 2.5 mL was loaded to the column.

Chapter 4 – Simultaneous clarification of *Escherichia coli* culture and purification of extracellularly produced penicillin G acylase using tangential flow filtration and anion-exchange membrane chromatography (TFF-AEMC)

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The research presented in this chapter was prepared for submission to the Journal of Chromatography B. Declaration: I initiated and conducted all experiments presented herein under the supervision of Dr. C. P. Chou, Dr. J. Scharer and Dr. M. Moo-Young Drs. Honeymann and Crossley, and Mr. Fenner were consulted for technical aspects of chromatographic operation. Dr. Suen was consulted as an expert on chromatography.

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4.1 Introduction

Downstream processing of bioprocesses for recombinant protein production includes several major preparative and purifying steps, i.e. clarification, capture, intermediate purification, polishing, and formulation [2, 3]. It often accounts for the most significant portion of protein manufacturing costs. Technological experience accumulated over the past few decades has highlighted the importance of more systematic and integrated approaches for optimal bioprocess development. In light of this, tackling technical issues in the upstream (i.e. strain construction) and/or midstream (i.e. cultivation) stages might become an effective solution to the reduction of the high costs associated with the downstream processing stage. However, practical demonstration of this well-perceived knowledge is still uncommon up to now because most recombinant proteins are produced intracellularly and, as a result, bioprocessing operations in the cultivation and downstream processing stages are rather independent. While strategies for extracellular production of recombinant proteins [11] have been proposed, downstream processing might remain tedious without excluding extensive preparative steps due to the composition incompatibility of the spent culture medium and chromatographic buffer.

Among various techniques associated with downstream processing for recombinant protein production, membrane filtration and chromatography are commonly performed [2]. While traditional chromatographic columns are well characterized and widely used, they have many limitations, such as lengthy cleaning and packing steps, low diffusive binding of materials, low flow rate, high pressure drop, and long processing time [5]. Chromatographic membranes, on the other hand, offer numerous advantages over chromatographic columns, including high convective binding of materials, high flow rate, low pressure drop, short processing time, being disposable rendering regeneration and cleaning unnecessary, and being easy to scale up [4, 5, 30]. Moreover,

improvements in membrane chemistry have yielded better membrane products with various chromatographic functionalities, such as ion-exchange, affinity, and hydrophobic interaction, and high binding capacities comparable to resins [4, 5]. In particular, ion-exchange membrane chromatography is gaining popularity as a polishing step for the removal of virus particles and endotoxins upon the production of monoclonal antibodies [6]. Additionally, chromatographic membranes can be manufactured in a non-conventional format of cross-flow geometry, such as hollow-fiber, plate and frame or spiral wound devices, which was originally designed to reduce fouling during ultrafiltration [3-5]. These tangential flow devices give chromatographic membranes an extra dimension of applicability that has not been fully explored.

Chromatography is usually the key step to increase the purity of recombinant protein during downstream processing. Prior to this step, extensive preparative steps, which may be as time-consuming and costly as chromatographic operations, are required. For recombinant proteins produced intracellularly in *E. coli*, the preparative steps typically include centrifugation, cell disruption, filtration, precipitation, and desalting to formulate the lysate in a buffer compatible with the subsequent chromatographic operation (**Figure 4-1**). While the strategies for extracellular production of recombinant proteins have the major advantage of separating the recombinant protein product from most intracellular contaminant proteins, further purification of the secreted recombinant protein product will most likely require several of the above preparative steps for buffer exchange. Accordingly, simplification or even elimination of these preparative steps would substantially reduce the processing time and manufacturing costs.

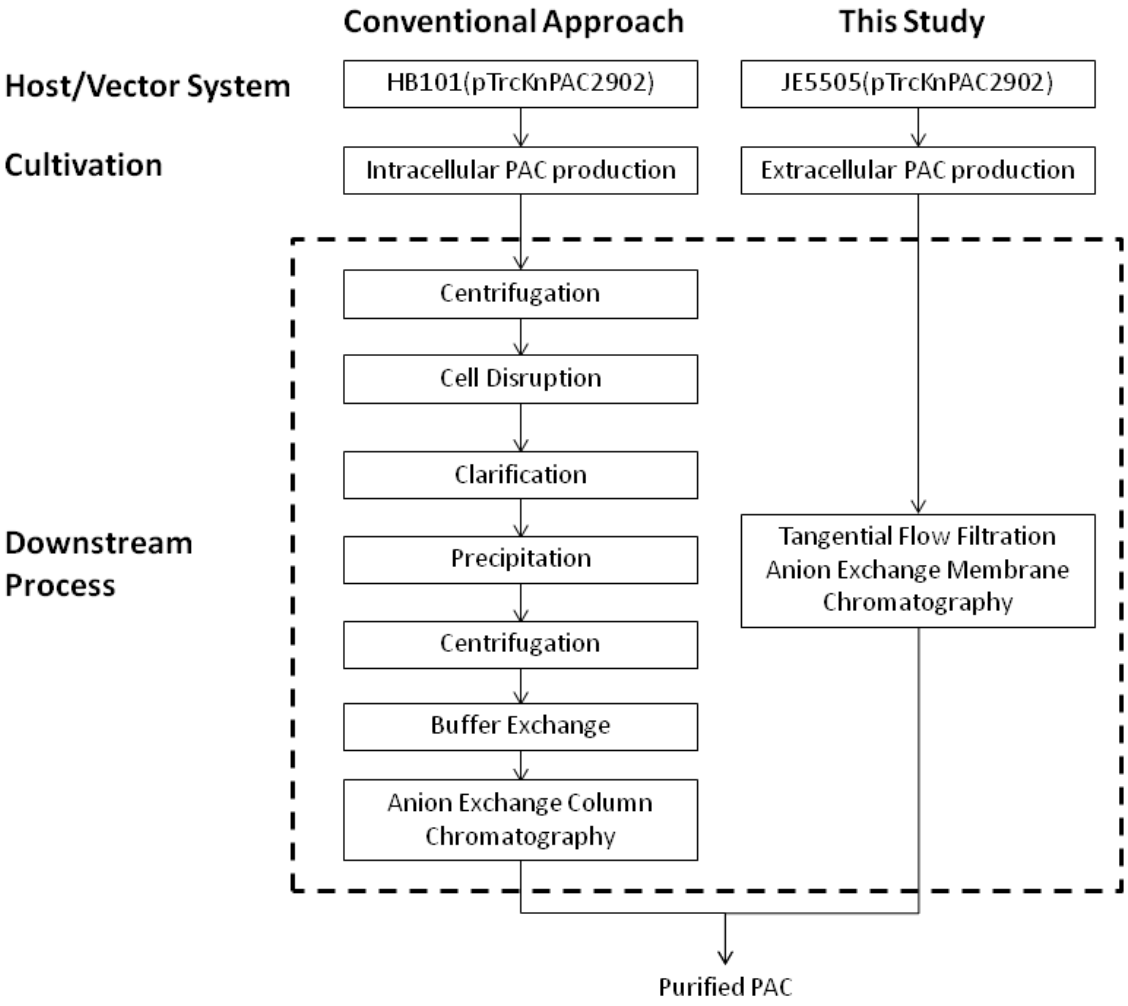


Figure 4-1 - The proposed purification scheme investigated herein using tangential flow filtration (TFF) anion-exchange membrane chromatography (AEMC) to simultaneously clarify and purify extracellular PAC compared to an equivalent scheme using traditional AEC column chromatography for purification of intracellular PAC.

In this study, we demonstrate a novel and effective bioprocess for extracellular production of recombinant protein in *Escherichia coli* and its immediate purification through the seamless integration of cultivation and downstream processing steps. The integrated system combined simultaneous operations of *tangential flow filtration* for culture clarification and *anion-exchange membrane chromatography* (TFF-AEMC) for PAC harvest/purification. Penicillin G acylase (PAC), an industrial enzyme for the production of β-lactam antibiotics [31], was used as a target protein for the demonstration since it is normally expressed as a periplasmic protein in *E. coli*. Extracellular production of recombinant PAC was achieved through the use of an outer-membrane mutant with a defective murein lipoprotein [10, 18, 26] as an expression host. The mutation resulted in the leakage of periplasmic proteins, including PAC, through the compromised outer-membrane into the extracellular medium with a minimal effect on cell growth. Compared to the traditional downstream processing for purification of intracellularly produced PAC using anion-exchange chromatography as the key purification step, the developed integrated system greatly reduced the length, complexity, and manufacturing costs of this bioprocess (**Figure 4-1**). The demonstration of TFF-AEMC for simultaneous culture clarification and recombinant protein purification significantly extends the benefits and applicability of the biochemical and genetic strategies for extracellular production of recombinant proteins.

4.2 Materials and Methods

4.2.1 Plasmids and strains

The strain used for PAC expression in this study was *E. coli* JE5505 (Coli Genetic Stock Centre, Yale, USA) genotype (*F^-, Δ(gpt-proA)62, lacY1, tsx- 29, glnV44(AS), galK2(Oc), & λ^-, Δlpp-254, pps-6,hisG4(Oc), xylA5, mtl-1, argE3(Oc), thi-1)* [97]. Transformation was done by electroporation

using the *E. coli* Pulser (Bio-Rad, Hercules, CA). The plasmid pTrcKnPAC2902 containing the *pac* operon from *E. coli* ATCC11105 regulated by the *trc* promoter was previously constructed [96].

4.2.2 Cultivation methods

The recombinant strain was stored at -80°C in a LB/glycerol stock. The cells were revived by plating on LB agar supplemented with kanamycin (50 μg/mL). A single colony was inoculated into a flask of 50 mL of LB broth (5 g/L yeast extract, 10 g/L tryptone, 0.5 g/L NaCl, and 50 μg/mL kanamycin) and incubated in a rotary shaker for 16 h at 30°C and 200 rpm. The 50 mL seed culture was inoculated into a bench-top bioreactor (Omni-Culture, VirTis, Gardiner, NY) containing 1 L working volume of LCM3 medium (5 g/L casamino acids, 2.5 g/L yeast extract, 20 mM 3-(N-morpholino)propanesulfonic acid (MOPS) buffer, 2 mM NaCl, 2 mM $MgSO_4 \cdot 7H_2O$, 2 mM K_2HPO_4, 0.2 mM $CaCl_2 \cdot 2H_2O$, 1.5 mg/L $FeSO_4 \cdot 7H_2O$, 0.6 mg/L of $MnSO_4$, 0.6 mg/L $CuSO_4 \cdot 5H_2O$, 0.6 mg/L $ZnSO_4 \cdot 7H_2O$) with 20 μL/L Antifoam 204 (Sigma, St. Louis, MO). The bioreactor was purged with filter-sterilized air at 2 vvm and agitated at 250 rpm. The pH was regulated at 7.0 ± 0.1 by the addition of 1 N NH_4OH using a combination pH electrode (Mettler-Toledo, Switzerland) a pH controller (PC310, Suntex, Taipei, Taiwan), and two MasterFlex peristaltic pumps (Cole-Palmer,Vernon Hills, IL, USA). The reactor was operated at 28°C for approximately 4 h until the cell density reached a density of 0.5 OD_{600} and protein expression was induced with 0.15 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and the broth was harvested 12 h post induction for purification.

4.2.3 Chromatography materials

The strong anion-exchange (Q) membranes were provided by Natrix Separations Inc. (Burlington, ON, Canada). The typical mean dynamic binding capacity for BSA is 200 mg/mL of membrane volume at 10% break-through. Membranes were installed into a either a 25 or 47 mm stainless steel holder NX9100 series (Natrix Separations Inc., Burlington, Canada). One 25 mm membrane comprises 0.1 mL membrane volume while two 47 mm membranes comprise 0.75 mL of membrane volume. A 0.02 m^2 cross-flow cassette comprises 5 mL of membrane. The loading/wash buffers were composed of either 25 mM Tris-HCl (pH 8) or 25 mM Bis-Tris-HCl (pH 7) where appropriate. The elution buffer consisted of the appropriate loading buffer containing 1 M NaCl. All buffers were prepared with 18 MΩ deionized water and filtered with 0.45 μm polypropylene filters (VWR, Radnor, PA, USA).

4.2.4 Dead end chromatography

Dead end chromatography was performed using low pressure liquid chromatography system (Bio-Logic LP, Biorad, Hercules, CA) with online UV absorbance at 280 nm and conductivity meters. These metrics were recorded using the accompanying software LP Logic (Biorad, Hercules, CA). Two 47 mm Q membrane cut discs in the stainless steel holder was attached upstream of the indicators and the inlet was downstream of a sample injection device. All runs were operated at a flow rate of 4 mL/min. The membrane was equilibrated for 15 min with the appropriate loading buffer. Culture broth was centrifuged at 6000 g for 10 min and adjusted to the appropriate pH then filtered using 0.22 μm polypropylene syringe filters (VWR, Radnor, PA, USA) prior to loading. Twenty milliliters of filtered cell free medium was loaded onto the membrane at a flow rate of 4 mL/min. After loading, 60 mL of loading buffer was used to wash weakly bound protein from the membrane. After washing, a 60 mL linear salt elution gradient was applied to the membranes. Eight

millilitre fractions were collected with a fraction collector (BioLogic BioFrac, Biorad, Hercules, CA). Fractions were pooled into loading flow-through, washing flow-through, and two elution peaks. All fractions were assayed using the BCA and PAC enzymatic assay and analyzed for protein composition by SDS-PAGE. All dead-end experiments were performed in duplicate.

4.2.5 Determination of Dynamic Binding Capacity

Determination of the dynamic binding capacity was performed using the low pressure chromatography BioLogic system previously described. One 25 mm Q membrane cut disc in a stainless steel holder was equilibrated with 15 mL of loading buffer (25 mM Bis-Tris-HCl pH 7.0) at 1 mL/min prior to sample loading. One hundred milliliters of clarified spent medium with unadjusted pH (7.0 ± 0.1) was loaded to the membrane at 1 mL/min. Fractions were collected every 1 min and analyzed for the relative purity of PAC in the flow-through by SDS-PAGE stained with silver nitrate and analyzed using Image J software (National Institute for Health). Effluent UV absorbance at 280 nm and conductivity were monitored as previously described.

4.2.6 TFF-AEMC

The cross-flow system was assembled using TangenX pressure gauges, TangenX cross flow apparatus (TangenX, Shrewsbury, MA, USA), Masterflex L/S 25 silicone tubing, Masterflex 7550-60 computerized peristaltic pump (Cole-Palmer, Vernon Hills, IL, USA), 1 L pyrex reservoir bottle with bottom hose outlet, 1-L pyrex flask (Corning, Corning, NY, USA), and Explorer Pro EP2102 balance (Parsippany, NJ, USA) as seen in **Figure 4-2**. A 0.02 m^2 membrane cassette was hydrated by pumping 500 mL of 25 mM Bis-Tris-HCl pH 7 at a feed flow rate (F_F) = 600 L/h·m^2 (LHM) and permeate flow rate (F_P) = 60 LHM for 15 min. The membrane back-pressure was maintained at 5

psi for all subsequent steps using a pressure valve. After equilibration, the buffer from the reservoir and permeate was removed and discarded. One liter of either clarified (supernatant collected after centrifugation at 6000g for 6 min at 4°C) or non-clarified culture broth was added to the reservoir. The loading stage was conducted at F_F = 600 LHM until 750 mL of permeate was collected. The remaining sample was removed from the reservoir and 500 mL of 25 mM Bis-Tris-HCl pH 7 was used to wash the membrane for 30 min at F_F = 600 LHM. The wash buffer was removed and 250 mL of 1 M NaCl in 25 mM Bis-Tris-HCl pH 7 was used to elute the contaminating proteins from the membrane for 30 min at F_F = 600 LHM. The permeate and reservoir protein content was monitored by the BCA assay and PAC assay approximately every 30 min. The permeate flow rate was monitored during the loading stage using the balance. The UV absorbance at 280 nm was recorded using the Biologic LPLC system.

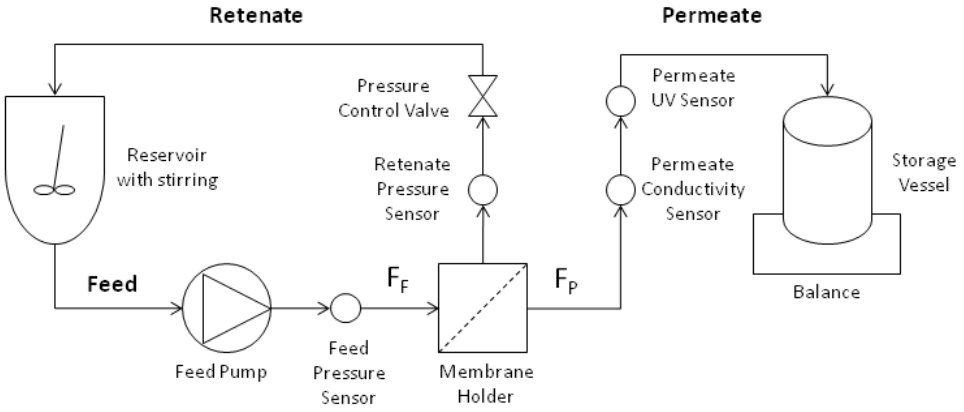


Figure 4-2 - Schematic of cross-flow system. F_F – feed flow rate; F_P – permeate flow rate.

4.2.7 Ultrafiltration

A 25 fold reduction in oligopeptides was achieved by diafiltration using 3 kD nominal molecular weight cut-off (NMWCO) centrifugal devices and samples were resuspended in loading buffer to their original volume and analyzed using BCA assay, PAC assay and SDS-PAGE (Pall's Corp., St. Louis, USA). Permeate was also subjected to 25 fold diafiltration using a 10 kD NMWCO cellulose membrane and a 200 mL stirred cell device (Millipore, Billerica, USA). Liquid was driven through the membrane by nitrogen that was used to pressurize the device to 25 psi.

4.2.8 Enzymatic activity

Penicillin G acylase was assayed at 37°C using penicillin G as a substrate. The amount of enzymatic reaction product of 6-aminopenicillanic acid (6-APA) was quantified using a colorimetric method developed previously [32]. All assays were conducted in duplicate. One unit was defined as the amount of enzyme that hydrolyzed 1.0 μmol penicillin G per minute at 37°C.

4.2.9 Total protein determination

The BCA assay for total protein concentration was performed using a kit (Pierce Biotechnology, Rockford, IL, USA) in a microplate assay format. Samples were all performed in duplicate and were appropriately diluted using the appropriate loading buffer to fall in the linear range of the kit; 20-2000 μg/mL. The standard curve was performed in duplicate on each plate with BSA. Absorbance was measured at 562 nm with Thermo Labsystems Multiskan Ascent photometric plate reader (Thermo Scientific, Wilmington, USA).

4.2.10 SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a Mini-PROTEAN II electrophoresis cell (Bio-Rad, Hercules, CA, USA) using a 12.5% polyacrylamide separating gel stacked by a 4% polyacrylamide stacking gel. The loading amount was 10 μL for chromatography fractions. Electrophoresis was conducted under a constant voltage of 200 V for 60 min. Gels were stained using silver nitrate and dried. Dried gels were scanned using an HP laserjet scanner 3020.

4.3 Results and Discussion

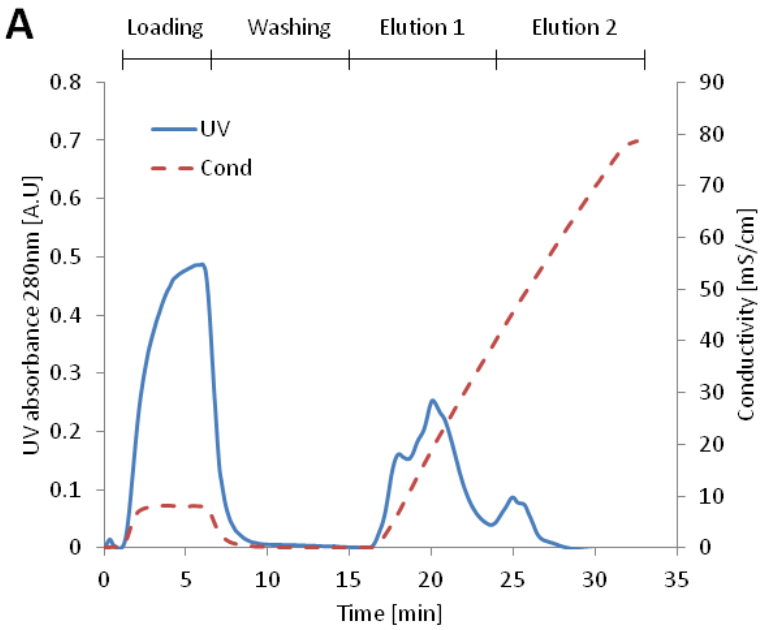
4.3.1 Binding of PAC on anion-exchange membrane

The rationale of the present study was to create an integrated downstream processing scheme with minimum preparative steps for direct harvest and purification of the extracellularly produced recombinant PAC using TFF-AEMC. To ensure the functional operation of anion-exchange membrane chromatography, the spent medium should have a sufficiently low conductivity for its direct processing without buffer exchange. Thus, medium composition and cultivation conditions were previously determined for effective extracellular production of recombinant PAC with minimum growth impairment. Using a bench-top bioreactor, JE5505 harboring pTrcKnPAC2902 was cultivated in LCM3 media for extracellular production of PAC. The spent cell-free medium was collected at 12 h post-induction as protein expression had reached a plateau and extended cultivation would result in significant cell lysis which complicated downstream processing. At this time, the culture had a cell density of 2.8±0.3 OD_{600} with a specific extracellular PAC activity of 434±86 $U/L/OD_{600}$. Most importantly, the secretion efficiency was as high as 90% and the spent medium had a sufficiently low conductivity (5.9±0.8 mS/cm) to allow its direct loading for subsequent

anion-exchange chromatography. Recombinant PAC in the spent medium was purified based on a "negative" separation through which the majority of contaminant proteins bound to the anion-exchanger and PAC was recovered in the flow-through fraction with a significantly increased purity.

Theoretically, the operating conditions for ion-exchange chromatography should not differ remarkably between a column and a membrane. Nevertheless, binding of PAC onto the anion-exchange membrane chromatography was characterized in this study with a dead-end membrane device to ensure its functional operation and optimize the purification performance. Using anion-exchange membrane cut discs in a stainless steel holder with a low hold-up volume, the spent cell-free medium at pH 7 containing recombinant PAC was loaded for chromatographic operation and the results are summarized in **Figure 4-3**. Various fractions were collected, pooled, and analyzed, including the loading flow-through, washing flow-through, and elution. These samples were also subjected to ultrafiltration (3 kD NMWCO) to remove residual oligopeptides in order to properly quantify the protein concentration. It was observed that recombinant PAC hardly bound to the anion-exchange membrane and the majority was collected in the flow-through fraction with significantly improved purity (**Figure 4-3B**). Two distinct peaks were shown upon the elution with salt and they were determined to primarily consist of contaminant proteins and RNA, respectively (**Figure 4-3C**). The results suggest that most contaminant proteins were removed through their binding onto the anion-exchange membrane and, as a result, PAC purity was significantly increased. The low level of RNA and plasmid DNA detected in the spent medium suggest minor cell lysis during the cultivation and the released nucleic acids were completely adsorbed by the anion-exchange membrane and eluted at a high salt concentration (**Figure 4-3C**) without contaminating the purified PAC. PAC purification performance for anion-exchange membrane chromatography was found to be excellent under both pH 7 and pH 8 operating conditions (**Table 4-1**), implying a

precise pH control during the cultivation might not be critical. Because of the high costs associated with extensively purifying enzymes, industrial processes tend to use crude extracts or partially purified enzymes for catalysis. Hence, the purified PAC based on anion-exchange membrane chromatography in this study should be suitable for industrial applications even though it is not extensively purified.


A
Loading
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Elution 1
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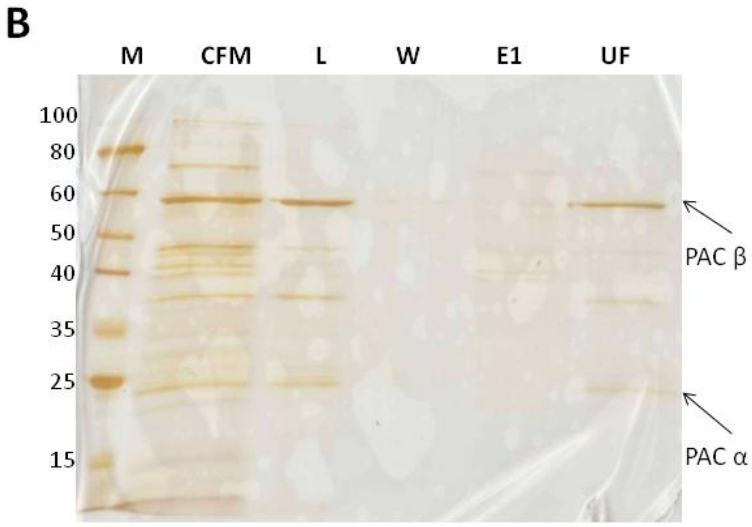
Cond
UV absorbance 280nm [A.U]
Conductivity [mS/cm]
Time [min]
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35


B
M
CFM
L
W
E1
UF
100
80
60
50
40
35
25
15
PAC β
PAC α

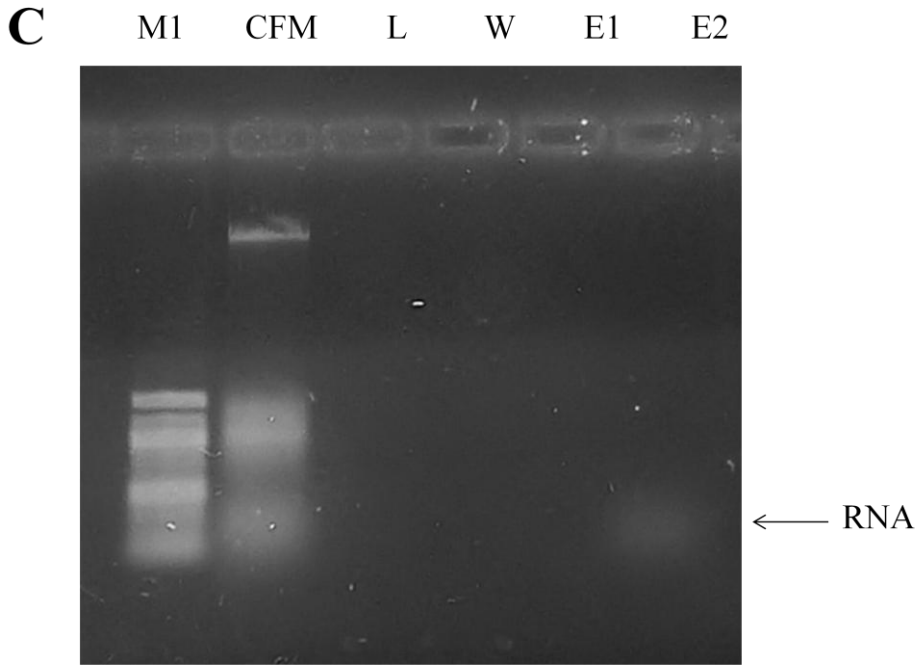


Figure 4-3 -Purification of extracellular PAC from 20 mL of cell free medium using Q membranes in dead-end flow format at pH 7 at 4 mL/min. [A] Chromatogram indicating the UV absorbance (solid line) and conductivity (dashed line). Fractions were pooled according to the 4 steps shown above the plot. [B] Pooled fractions from the separation in panel A were analyzed using SDS-PAGE. Lanes contain protein markers (M), cell free medium (CFM), loading flow-through (L), washing flow-through (W), elution 1 (E1) and the ultrafiltrate of the loading flow-through (UF). PAC subunits are indicated with arrows. [C] One percent agarose gel electrophoresis of chromatography fractions. Lanes contain Fermentas 100 bp marker (M1), cell free medium (CFM), loading flow-through (L), washing flow-through (W), elution peak 1 (E1) and elution peak 2 (E2).

Table 4-1 - Performance of PAC purification by AEMC by direct loading of 20 mL of clarified culture supernatant to two 47 mm dead-end Q membranes operated at pH 7 and pH 8 with a flow-rate of 4 mL/min.

		Protein Concentration [mg/mL]	Volumetric PAC Activity [U/mL]	Specific Activity [U/mg]	PAC Recovery	Purification [Fold]
Cell Free Medium	Total protein including oligopeptides	0.63 ± 0.05	0.67 ± 0.04	1.1 ± 0.1	-	-
	Total protein excluding oligopeptides	0.133 ± 0.007	0.69 ± 0.02	5.2 ± 0.3	-	-
Anion-exchange (pH 8)	Total protein including oligopeptides	0.357 ± 0.009	0.50 ± 0.01	1.40 ± 0.05	75%	1.3
	Total protein excluding oligopeptides	0.03 ± 0.01	0.516 ± 0.008	17 ± 5	103%*	3.3
Anion-exchange (pH 7)	Total protein including oligopeptides	0.396 ± 0.001	0.503 ± 0.004	1.27 ± 0.01	75%	1.2
	Total protein excluding oligopeptides	0.03 ± 0.01	0.45 ± 0.02	15 ± 6	89%*	2.9

*PAC recovery during ultrafiltration step.

In addition, the dynamic binding capacity of the anion-exchange membrane for binding of the contaminant proteins was evaluated by continuous loading of 100 mL spent cell-free medium through a 25 mm disc membrane and the results are summarized in **Figure 4**. No protein breakthrough occurred after loading the first 6 mL. SDS-PAGE analysis indicates that PAC was the first major protein to break through the membrane with a few minor contaminant proteins at the loading volume of 8 mL, suggesting that PAC might carry the least surface charge under these loading conditions. As expected, the break-through of the contaminant proteins increased with the loading volume and the PAC purity dropped slightly after a loading of 20 mL of the spent medium, resulting in approximately 85% PAC purity according to the densitometric analysis of the SDS-PAGE gel. The purity decreased significantly when the loading volume was more than 35 mL. However, complete protein break-through was never achieved due to a serious membrane fouling which developed upon a loading of more than 50 mL of the spent medium. The results suggest that the loading volume for AEMC critically affected the PAC purity and must be carefully assessed.

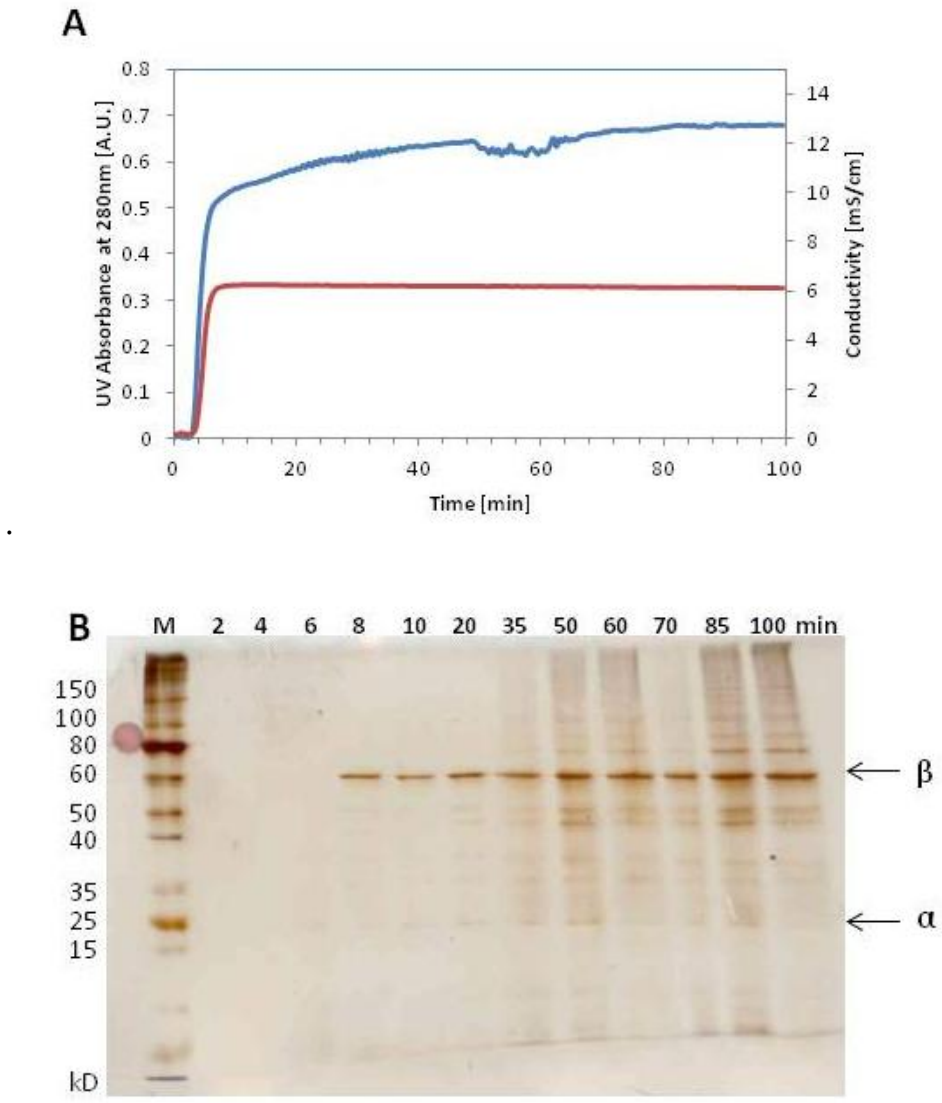
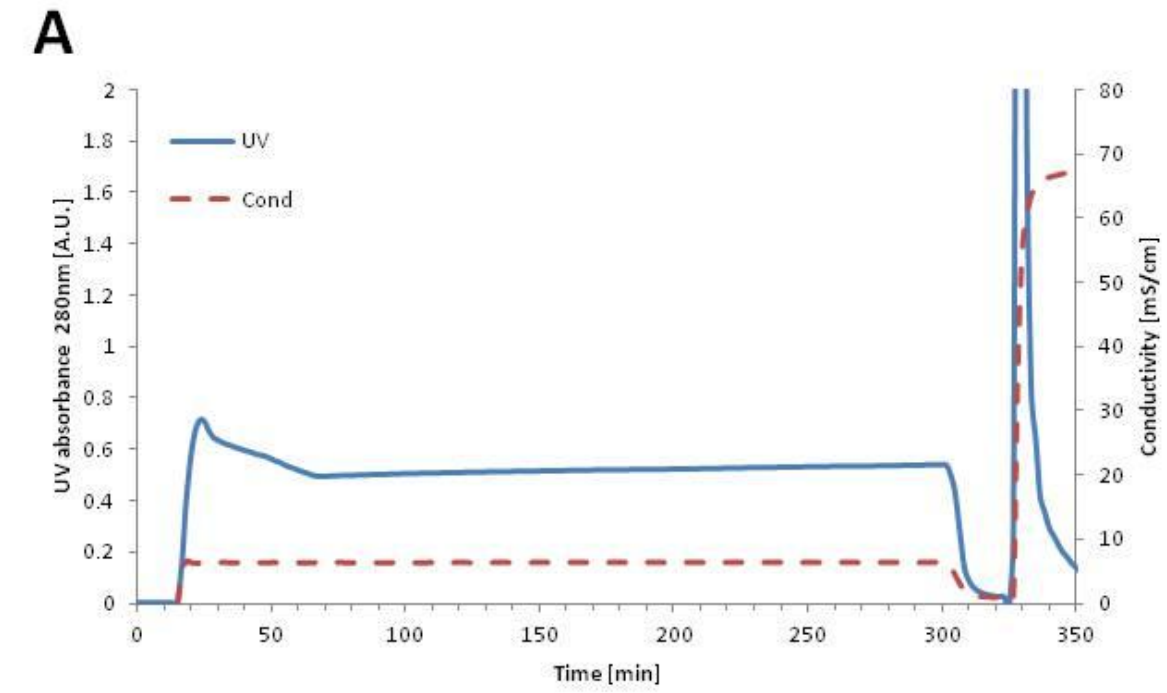
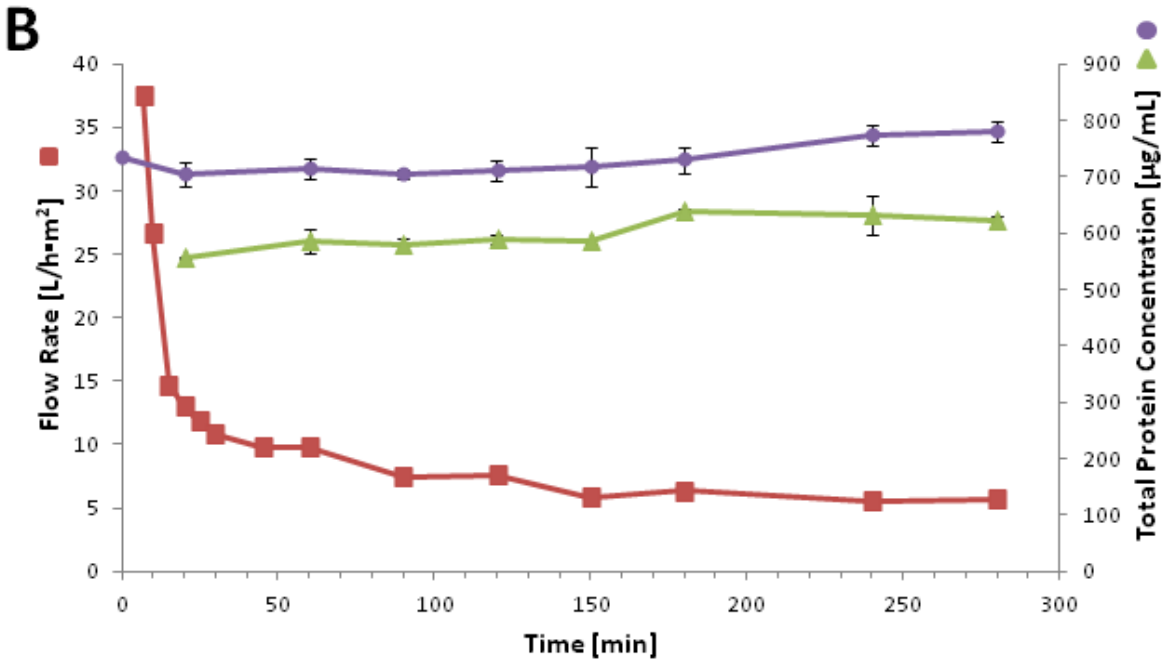


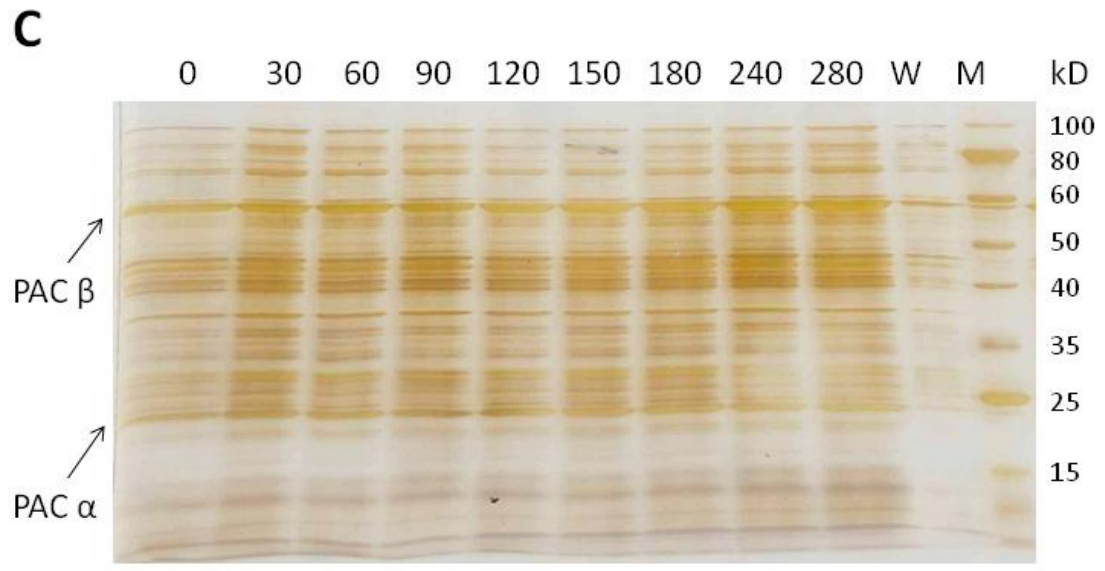
Figure 4-4 -Determination of dynamic binding capacity of Q membrane when 100 mL of clarified spent medium was loaded to one 25 mm cut disc operated at a volumetric flow rate of 1 mL/min and pH of 7.0. (A) Chromatogram of protein breakthrough. (B) SDS-PAGE analysis of corresponding protein breakthrough. PAC subunits, the protein marker molecular weights (M) and the time at which the chromatography fraction was taken are indicated.

4.3.2 Simultaneous culture clarification and PAC purification with TFF-AEMC

Using the developed system of TFF-AEMC (**Figure 4-2**), one liter of the whole *E. coli* culture broth at pH 7 containing the extracellularly secreted recombinant PAC was processed and the results of which are summarized in **Figure 4-5**. The culture in the reservoir was circulated at a rate of 600 LHM for approximately 280 min with the back-pressure being maintained at 5 psi. The flow rate of the permeate was monitored to ensure no fouling of this system. The chromatogram associated with the TFF-AEMC operation is shown in **Figure 4-5A**. The total protein concentration of the permeate, in terms of the UV absorbance at 280nm, peaked upon the initial loading of the culture, then dropped slightly due to the binding of contaminant proteins onto the membrane, and remained relatively constant up to the washing step. The constant total protein concentration in the permeate leads to two observations. First, cell lysis was not aggravated during the TFF-AEMC processing. This is important, since further cell lysis would contaminate the extracellular medium and complicate PAC purification. Second, the binding of the extracellular contaminant proteins onto the membrane was effective without exceeding the chromatographic capacity of the membrane. Because PAC was unable to bind onto the anion-change membrane under the spent-medium conditions, the majority of it was collected in the permeate with a small amount of contaminant protein. As a result, its purity has been significantly increased through this “negative” separation.


A
UV
Cond
UV absorbance 280nm [A.U.]
Conductivity [mS/cm]
Time [min]
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
0
10
20
30
40
50
60
70
80
0
50
100
150
200
250
300
350


B
Flow Rate [L/h•m²]
Total Protein Concentration [µg/mL]
Time [min]
0
5
10
15
20
25
30
35
40
0
100
200
300
400
500
600
700
800
900
0
50
100
150
200
250
300


C
0
30
60
90
120
150
180
240
280
W
M
kD
100
80
60
50
40
35
25
15
PAC β
PAC α

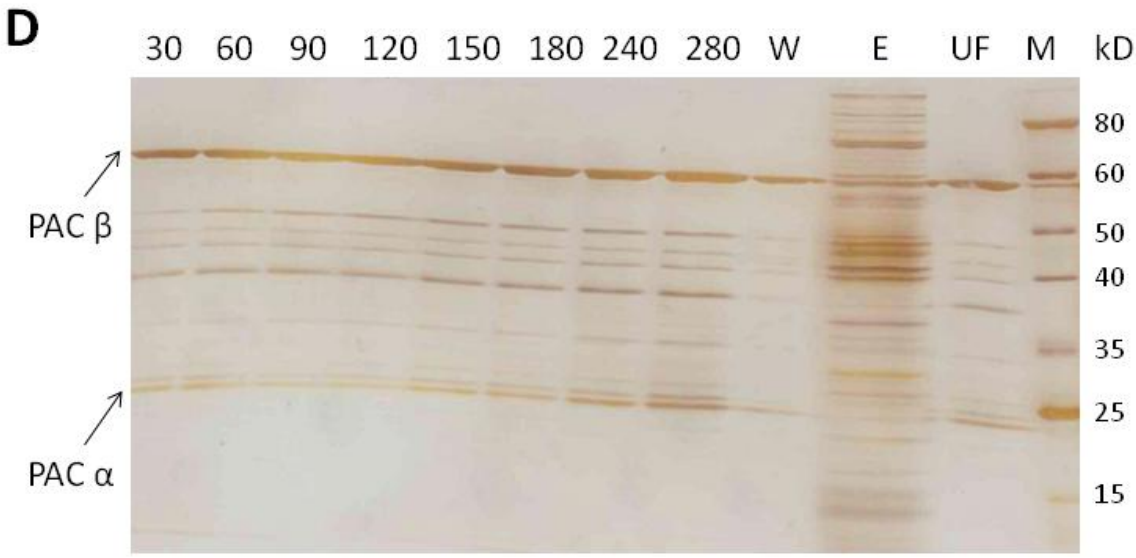

D
30
60
90
120
150
180
240
280
W
E
UF
M
kD
80
60
50
40
35
25
15
PAC β
PAC α

Figure 4-5 -Purification of extracellular PAC using non-clarified cultivation broth and Q anion-exchange cross-flow membranes. (A) Typical chromatogram of PAC purification using non-clarified cultivation broth containing extracellular PAC. UV absorbance is indicated with a solid line, conductivity is indicated with a dashed line. (B) Monitoring for fouling and cell lysis during loading stage of cross-flow purification. Permeate flow rate in squares was used to monitor fouling, retentate total protein in circles was used to monitor the amount of lysis, and permeate total protein concentration in triangles was used to monitor the final product. (C) SDS-PAGE analyses of retentate protein content during cross-flow purification. Samples were collected from the reservoir from 0-280 min. The washing step retentate (W) and a protein marker (M) were also analyzed. (D) SDS-PAGE analysis of permeate samples taken during cross-flow operation. Samples were collected from the permeate effluent from 30-280 min. A protein marker (M), a sample of the washing step permeate (W), the elution step permeate (E) and the ultrafiltrate of the permeate (UF) were also analyzed

Reduction in the volume of the retentate due to the draining of the permeate resulted in an increase in the cell density of the retentate. Out of the 1-L whole culture broth being processed, approximately 750 mL was collected as permeate containing purified PAC. The cell density in the retentate increased 4 fold. Because of the "negative" separation mode associated with PAC purification, the permeate containing the purified product was continuously drained from the processing stream. Hence, the system required a minimum operating volume (~ 100 mL in this study to cover the tubing volume and minimum working volume of the reservoir) for continuous operation. Such a minimum volume is not required for "positive" separations, for which the product to be purified binds onto the membrane and the processing is typically operated in a recycle mode though which the permeate is recycled back to the reservoir without being drained from the process stream [3, 58]. A major technical issue for the TFF-AEMC processing, particularly for that with a "negative" separation mode, is that the increasing cell density of the retentate tends to aggravate membrane fouling and consequently prevents product harvest/purification. The circulation rate of the whole culture broth has to be high enough to minimize the fouling without causing cell lysis. In this study, while the flow rate of the permeate began at a reasonably high level of approximately 38 LHM, it quickly decreased to 10 LHM within the first 50 min of the processing and eventually reached a constant flow rate of approximately 6 LHM till the end (**Figure 4-5B**), suggesting membrane fouling was minimal.

4.3.3 Analysis of protein compositions

The total protein concentrations (analyzed by the BCA assay) for both cell-free retentate and permeate were maintained relatively constant at 700 μg/mL and 570 μg/mL, respectively, before 200 min (**Figure 4-5B**), suggesting the processing resulted in minimum cell lysis during this time period. Note that oligopeptides were included when these two concentrations were analyzed with

the BCA assay and, after excluding oligopeptides based on the data in **Table 4-2**, the actual total protein concentrations for both cell-free retentate and permeate were estimated to be 140 μg/mL and 50 μg/mL, respectively. The difference between these two concentrations, i.e. 90 μg/mL, reflects the amount of contaminant proteins binding onto the membrane. Such binding behavior (internal fouling) was suspected to be the major cause of reduced permeate flow rate since a similar decrease in the permeate flow rate of the permeate was observed when clarified broth was applied under the same operating conditions a final constant flow rate of 6.0 LHM was reached after 80 min of processing. While the total protein concentration of the permeate was rather constant according to UV monitoring (**Figure 4-4A**), the BCA assay revealed low-level cell lysis towards the end of the processing, particularly after 200 min, when a slight increase in the total protein concentration for both the permeate and cell-free retentate were observed (**Figure 4-4B**). The protein composition of the cell-free retentate was also monitored by SDS-PAGE and low-level cell lysis could be detected by visualizing new bands in the late samples (i.e. 240-and 280-min) (**Figure 4-4C**). While no major difference in the protein composition of the cell-free retentate was observed, the volumetric PAC activity in this fraction increased over the time, i.e. from 1.08 U/mL at 0 min to 1.58 U/mL at 280 min. Such concentration polarization is not unusual for ultrafiltration and is indicative of membrane fouling [3, 111] though the flow rate of the permeate was not fully stopped. If necessary, the fouling can be relieved by reversing the retentate flow direction or increasing the tangential flow rate to improve the flux of the permeate [3].

Table 4-2 - Summary of purification performance by TFF-AEMC

		Volume [L]	Total Protein [mg/mL]	PAC Activity [U/mL]	Specific Activity [U/mg]	Purification [Fold]	Step Recovery[a]	Overall Recovery[b]
Cell Free Medium	Total protein including oligopeptides	1.00	0.734 ± 0.00	1.07 ± 0.03	1.46	-	-	-
	Total protein excluding oligopeptides	1.00	0.146 ± 0.01	1.07 ± 0.03	7.33	-	-	-
Anion-exchange Permeate	Total protein including oligopeptides	0.75	0.599 ± 0.03	1.03 ± 0.04	1.72	1.2	96%	72%
	Total protein excluding oligopeptides	0.75	0.055 ± 0.01	1.05 ± 0.04	19.1	2.6	102%	74%

[a] Recovery of PAC based on the actual volume processed
[b] Recovery of PAC based on the original volume processed

Compared to the crude protein composition of the cell-free medium (i.e. the 0-min lane in **Figure 5C**), all permeate samples contained relatively pure PAC, for which both α and β subunits were clearly visible as the major bands (**Figure 4-5D**). Such a high PAC purity in the permeate was stably maintained throughout the entire period though there appears to be cell lysis towards the end of the processing. After approximately 750 mL of permeate was drained, the remaining retentate was collected and a wash buffer was used for brief circulation to remove any unbound proteins and cells from the system. All bound proteins on the anion-exchange membrane were eluted through a brief circulation of the loading buffer supplemented with 1 M NaCl. A large elution peak was visible on the chromatogram and the eluted proteins were collected from the permeate side. Both wash and elution fractions contained minimum PAC activities (less than 8% and 1% of the total PAC activity respectively) and were analyzed by SDS-PAGE (**Figure 4-4D**). The results suggest most contaminant proteins bound onto the anion-exchange membrane, resulting in a significant

increase of PAC purity in the permeate. Additionally, the purity of PAC in the permeate could be potentially affected by the conductivity of the spent medium, which determined the binding efficiency of contaminant proteins.

4.3.4 PAC purification performance

The performance for the one-step purification of PAC by TFF-AEMC is summarized in **Table 4-2**. A minor technical issue associated with the purification of PAC in this study (i.e. a "negative" separation mode) was the presence of oligopeptides in the spent medium. Because oligopeptides hardly bound onto the anion-exchange membrane, they coexisted with purified PAC in the permeate. Such a technical issue would not exist for a "positive" separation mode, for which oligopeptides could be easily separated from the target protein binding onto the ion-exchange membrane. While the oligopeptides will not affect the catalytic activity of PAC upon industrial applications, if necessary, they can be easily removed by ultrafiltration during the concentration step given that the permeate was already pre-filtered by microfiltration upon the TFF-AEMC processing. The specific PAC activity for the crude cell-free spent medium was already as high as 7.33 U/mg protein (excluding oligopeptides). With the TFF-AEMC processing, it reached an extremely high level of 19.1 U/mg protein (excluding oligopeptides), resulting in a 3.4 fold purification. Note that the specific activity of PAC from SIGMA-ALDRICH (catalog no. 76427) was only 5-10 U/mg protein. In addition to the high purification fold, the recovery of PAC activity for the TFF-AEMC processing was as high as 96%. This means 72% of PAC in the 1-L cell-free medium was harvested and purified in the 750-mL permeate. This overall recovery was limited by the volume of permeate that could be separated/drained from the whole culture during the TFF-AEMC processing. Regardless, 72% is still considered an excellent yield and is often higher than the yields associated with the traditional multi-step processes for purifying intracellular PAC. Also importantly, the

overall batch cycle time involved in the developed bioprocess, including both cultivation and TFF-AEMC processing, was less than 24 h, significantly enhancing the overall productivity of this bioprocess.

Chapter 5 - Conclusions and Recommendations

5.1 Conclusions

The integrated bioprocess developed in this work is a highly simplified, inexpensive, and easily implemented system for recombinant protein production and purification. The outer-membrane mutant strain of JE5505 was demonstrated to be an effective cell factory for the extracellular production of periplasmic PAC under proper culture conditions. While growth impairment was detected under certain low osmolarity conditions, the expression host strain exhibited a high level of *pac* expression with excellent secretion efficiency. The extracellularly secreted PAC was subsequently harvested and purified by "negative" separation upon direct loading of the spent medium to TFF-AEMC. This one-step downstream purification simultaneously clarified and purified recombinant PAC based on the dual nature of the anion-exchange membrane as a filter and a chromatographic medium. The decreased number of steps for downstream processing and excellent purification performance could represent a significant cost saving for manufacturing of recombinant PAC. Due to the non-selective nature of the secretion system (i.e. any periplasmic proteins can be potentially secreted) and the versatility of ion-exchange chromatography, the developed strategy could be easily implemented for the production of many recombinant proteins making the developed bioprocess and its associated equipment extremely versatile an advantage rarely encountered in protein manufacturing. Overall, a high purity of PAC was achieved based on this simple one-step purification. The current study has demonstrated that perceptive design in the upstream (i.e. strain construction) and midstream (i.e. cultivation) stages can greatly enhance the performance of the downstream processing, particularly the often overlooked preparatory steps. While such an integrated approach potentially represents an effective strategy for novel bioprocess

design, it also provides a major challenge in identification of key factors and/or bioprocess variables for systematic optimization of the overall bioprocess. For example, with respect to the currently developed host/vector system effective in the extracellular production of PAC, further optimization of the culture medium to sustain both high-cell-density cultivation and high-level *pac* expression without losing its compatibility for downstream processing remains a challenge.

5.2 Recommendations

A highly simplified bioprocess for the production and purification of extracellular proteins in *E. coli* has been successfully demonstrated. Several factors were determined to have an effect on separation performance and secretion efficiency; however, further study is required to determine the acceptable operating range of these parameters. Consequently, the following investigations are recommended:
Upstream Development:

1. Low cell density was found to be a significant obstacle in the endeavor to increase productivity of the developed bioprocess. While this problem may be partially tackled through biochemical optimization of the midstream cultivation, the results upon the cultivation of HB101 harboring pTrcKnPAC2902 suggest additional upstream development may be a more effective solution. Under identical conditions, HB101 (pTrcKnPAC2902) out-performed JE5505 (pTrcKnPAC2902) as this culture reached a higher cell density and all PAC expressed was found to be in its soluble active form. Taking this into consideration, low cell density during the cultivation of JE5505 (pTrcKnPAC2902) may simply be a result of growth arrest due to the physiological stress of inclusion body formation, or may be due to differences in host genetic background. As it is known that some *E. coli* strains are capable of higher cell densities, knockout of the *lpp* gene in HB101 could allow HB101 to

acquire the “leaky” phenotype while maintaining the high level expression of soluble PAC and high cell density and should be explored [43].

2. Inclusion body formation during cultivation of JE5505 harboring pTrcKnPAC2902 was suspected to be a major impediment in the efficiency of PAC expression and extended cultivation lead to cell lysis resulting in low cell density. Inclusion body formation in this case may be a result of the dilution of periplasmic folding modulators and disulfide bond forming proteins upon leakage into the extracellular medium, consequently, coexpression of folding chaperones may alleviate inclusion body formation. This strategy has previously been demonstrated to successfully increase soluble PAC productivity in the periplasm in this lab and successful resolution of this problem could help our understanding of the mechanism of inclusion body formation in *E. coli* and requires further study [112, 113].
3. Finally, inclusion body formation may be due solely to the nature of the posttranslational modifications required for formation of functional PAC; therefore, this issue may not exist upon the expression of other recombinant proteins with straightforward posttranslational processing. This may be investigated by examining this host under the developed conditions upon the expression of other recombinant proteins.

Midstream Development:

1. Cultivation conditions for the production of PAC was only briefly explored in this work, and as a result, both cell density and PAC productivity could greatly benefit from a full optimization of the culture conditions, particularly medium composition and induction conditions.
2. A significant challenge to the successful integration of the production and purification of PAC was the necessity to maintain low conductivity and high cell densities in a batch

bioreactor. Additionally, due to the acidification of the medium during culture growth, pH control significantly contributed to the conductivity of the spent medium. Thus, batch and fed-batch cultivation are poor techniques for meeting these requirements and thus chemostat cultivation should be explored. Conductivity of the spent medium would easily be maintained in a chemostat due to the continuous dilution of the culture medium by the feed. Additionally, this may provide another method of addressing the low cell density issue as higher cell densities are more readily achieved during chemostat operations.

Downstream Development:

1. Several factors affecting the separation performance of PAC by TFF-AEMC were indentified, primarily the conductivity and pH of the spent medium. However, many factors are known to affect the performance of membranes in TFF such as transmembrane pressure, flow-rate, flow direction, and thus a more throughout investigation of both the possible operating range of the feed and the TFF-AEMC should be performed.
2. Additionally, a study of the effects of flow rate and cell density on fouling and cell lysis should be undertaken to determine any correlation between these factors and thus the possible operational limitations of this particular bioprocess.
3. One major disadvantage of the developed bioprocess was the need to remove peptides remaining in the loading flow-through and concentrate the product using ultrafiltration. Ultrafiltration is an expensive time consuming technique. This particular technical issue only pertains to flow-through applications and not bind-elute strategies, thus, a bind-elute strategy could be far more productive than a flow-through operation and can be demonstrated by applying this bioprocess to another more highly charged target protein and thus concurrently prove the versatility of the developed system.

4. Finally, while this process was found to be exceedingly simple and fast, processing time could be further decreased by the direct coupling of chemostat effluent to TFF-AEMC consequently creating a truly seemless simply, versatile bioprocess.

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