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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)

Valerie Orr[a], Jeno Scharer[a], Murray Moo-Young[a], C. Howie Honeyman[b], Drew Fenner[b,1], Lisa Crossley[b,1], Shing-Yi Suen[c], C. Perry Chou[a,*]

[a] Department of Chemical Engineering, University of Waterloo, 200 University Avenue West, Waterloo, Ontario, Canada N2L 3G1
[b] Natrix Separations, Inc., 5295 John Lucas Drive, Unit 6, Burlington, Ontario, Canada L7L 6A8
[c] Department of Chemical Engineering, National Chung Hsing University, 250 Kuo Kuang Road, Taichung 402, Taiwan

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ABSTRACT

Downstream purification often represents the most cost-intensive step in the manufacturing of recombinant proteins since conventional purification processes are lengthy, technically complicated, and time-consuming. To address this issue, herein we demonstrated the simultaneous clarification and purification of the extracellularly produced recombinant protein by *Escherichia coli* using an integrated system of tangential flow filtration and anion exchange membrane chromatography (TFF-AEMC). After cultivation in a bench-top bioreactor with 1 L working volume using the developed host/vector system for high-level expression and effective secretion of recombinant penicillin G acylase (PAC), the whole culture broth was applied directly to the established 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) and cross-flow operations. Most contaminant proteins in the extracellular medium were captured by the anion-exchange membrane and cells remained in the retentate, whereas extracellular PAC was purified and 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), yield (72% recovery), and productivity (41 mg of purified PAC per liter of culture) was obtained. Due to the nature of the non-selective protein secretion system and the versatility of ion-exchange membrane chromatography, the developed system can be widely applied for effective production and purification of recombinant proteins.

1. Introduction

Traditional downstream processing for recombinant protein production includes several major preparative and purifying steps, i.e. clarification, capture, intermediate purification, polishing, and formulation [1,2]. It often accounts for the major portion of protein manufacturing costs. Technological experience accumulated over the past few decades has highlighted the importance of more systematic and integrated approach 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 in *E. coli* 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 [3] 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

* Corresponding author. Tel.: +1 519 888 4567x33310.
E-mail address: cpchou@uwaterloo.ca (C.P. Chou).
[1] Present address: BioVentures.ca, 82 Olivia Place, Ancaster, Ontario, Canada L9K 1R4.

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packing steps, low diffusive binding of materials, low flow rate, high pressure drop, and long processing time [4]. 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 [4–6]. Moreover, recent improvements in membrane chemistry have yielded better membrane products with various chromatographic functionalities, such as ion-exchange, affinity, and hydrophobic interaction, as well as high binding capacities comparable to resins [4,6]. In particular, ion-exchange membrane chromatography is gaining popularity as a polishing step for the removal of viral particles and endotoxin upon the production of monoclonal antibodies [7]. 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 [1,4,6]. 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 of the process stream during downstream processing. Prior to this step, extensive preparative steps are required, which may be as time-consuming and costly as chromatographic operations. For recombinant proteins produced intracellularly in *E. coli*, the preparative steps typically include centrifugation, filtration, precipitation, and desalting to formulate the lysate in a buffer compatible with the subsequent chromatographic operation (Fig. 1). While the strategies for extracellular production of recombinant proteins have the major advantage of the recombinant protein product being separated from most intracellular contaminant proteins, further purification of the secreted recombinant protein product will most likely require several of the above preparative steps (Fig. 1). Accordingly, simplification or even elimination of these preparative steps would substantially reduce the processing time and manufacturing costs.

In this study, we demonstrated a novel and effective bioprocess for extracellular production of recombinant protein in *Escherichia coli* and its immediate purification through the seamless integration of all 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 [8,9], was used as a target protein for the demonstration because it is normally expressed in the periplasm of *E. coli* with a heterodimeric structure consisting of a 23-kDa α-subunit and a 63-kDa β-subunit through rather unique post-translational processing [10,11]. The industrial importance of PAC also leads to the development of various expression strategies for its large-scale production. Extracellular production of recombinant PAC was achieved through the use of an outer-membrane mutant with a defective murein lipoprotein [12–14] 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 (Fig. 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.

2. Materials and methods

2.1. Plasmids and strains

The strain used for PAC expression in this study were *E. coli* JE5505 (Coli Genetic Stock Centre, Yale, USA) genotype (*F*$^-$, *Δ(gpt-proA)62*, *lacY1*, *tsx-29*, *glnV44(AS)*, *galK2(Oc)*, and λ$^-$, *Δlpp-254*, *pps-6*,*hisG4(Oc)*, *xylA5*, *mtl-1*, *argE3(Oc)*, *thi-1)* [15]. 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 [10].

2.2. Cultivation methods

The recombinant strain was stored at −80 °C in a lysogeny broth (LB)/glycerol (85% (v/v) LB broth described below, 15% (v/v) glycerol) stock. The cells were revived by plating on LB agar (15 g/L agar dissolved in LB broth) 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 sparged with filter-sterilized air at 2 vvm and agitated at 250 rpm. The pH was regulated 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) at pH = 7.0 ± 0.1. The reactor was operated at 28 °C. Protein expression was induced with 0.15 mM isopropyl-β-D-thiogalactopyranoside (IPTG) after approximately 4 h when the cell density reached 0.5 OD600. The broth was harvested after 12 h of induction for purification.

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 bovine serum albumin (BSA) is 200 mg/mL of membrane volume at 10% break-through. Membranes were installed into either a 25 or 47 mm stainless steel holder NX9100 series (Natrix Separations Inc., Burlington, Canada). One 25 mm membrane comprises 0.1 mL of membrane volume, two 47 mm membranes comprise 0.75 mL of membrane volume, and one 0.02 m^2 cross-flow cassette comprises 5 mL of membrane volume. The loading/wash buffers were composed of 25 mM Tris–HCl (pH 8) or 25 mM Bis-Tris–HCl (pH 7). 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).

2.4. Dead end filtration anion-exchange membrane chromatography (DEF-AEMC)

DEF-AEMC, a process where the anion-exchange filter membrane is oriented perpendicular to the flow direction of the feed solution passing through the membrane, 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