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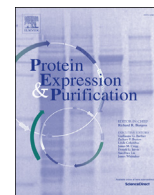

Development of a novel engineered *E. coli* host cell line platform with improved column capacity performance for ion-exchange chromatography

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ABSTRACT

This article reports on the analysis of an engineered *Escherichia coli* designed to reduce the host cell protein (HCP) burden on recombinant protein purification by column chromatography. Since downstream purification accounts for a major portion of production costs when using a recombinant platform, minimization of HCPs that are initially captured or otherwise interfere during chromatography will positively impact the entire purification process. Such a strategy, of course, would also require the cell line to grow, and express recombinant proteins, at levels comparable to, or better than, its parent strain. An *E. coli* strain with a small number of strategic deletions (LTSF06) was transformed to produce three different recombinant biologics to examine growth and expression, and with another model protein to assess growth and the effect of selectively reduced HCPs on target product capture on DEAE ion exchange medium. Cell growth levels were maintained or increased for all constructs, and a significant reduction in HCP adsorption was realized. Indeed, a breakthrough analysis indicated that as a result of reducing adsorption of particular HCPs, a 37% increase in target protein capture was observed. This increase in product capture efficiency was achieved by focusing not on HCPs that co-elute with the recombinant target, but rather on those possessing particular column adsorption and elution characteristics.

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1. Introduction

Improvements in downstream processing occur when the number of purification steps are reduced, individual step yield is increased, or selectivity and/or capture capacity toward the target product is improved [1]. Traditionally, downstream processing consists mainly of generic unit operations that include chromatography, precipitation, and diafiltration/ultrafiltration, and as these steps decrease in number, the overall yield increases. Column chromatography, an essential step in bioseparation, has been improved by tailoring the properties of the adsorbent, optimizing elution gradients, and when possible, exploiting affinity tags (e.g., His_6, maltose binding proteins, Arg_8). The latter is representative of a molecular biology approach that increases column effectiveness by providing the necessary biochemistry to dictate selective adsorption. Indeed, there are a multitude of affinity tags and corresponding affinity resins that when deployed can provide highly enriched product and sometimes homogeneous product [2–11]. However, in commercial applications, the resolving power of such systems must be weighed against the added resin and affinity tail removal expenses, and any existing licensing fee(s).

While the use of affinity tags may be attractive, other avenues have been investigated to exploit gene modification in chromatographic purification. Specifically, deleting genes encoding host cell proteins (HCPs) that co-elute with the target protein has been touted as a method to increase purity of a given recombinant DNA product. Cai et al. first described the proteome associated with Immobilized Metal Affinity Chromatography (IMAC), with similar reporting done by other investigators for other affinity resins [12–15]. HCP reduction to improve the quality of a particular recombinant product has been proposed where three to four genes coding for proteins that co-elute with the target have been deleted from the proteome. Liu et al. describe deletions for purification of Green Fluorescent Protein via IMAC, while Caparon et al. describe deletions for purification of Apolipoprotein A 1 Milano utilizing maltose-binding as the first capture step [15, 16]. Each protocol was

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designed to enhance the purity of a specific recombinant target protein by deleting co-eluting contaminants, and neither publication reports any actual gain in product purity achieved. In the case of Liu et al. it can be calculated that should the genes identified and deemed important be deleted, an increase of significantly less than one percent (1%) in column capacity would be achieved. Lacking in these references is a means of applying quantitative metrics to prioritize deletions that lead to increases in separation efficiency independent of target proteins, and a method to interpret these data to prepare a broadly useful host cell or set of host cells that provide increases in separation efficiency for as many different target molecules as possible. Other workers have described host cell proteome modifications designed to improve cell growth and protein expression or extracellular recombinant protein excretion [16–20].

In contrast to the enhancement of expression of a specific recombinant product, improvement in upstream productivity, or deletion of co-eluting contaminants, the effect on product purification of deleting HCPs that adsorb to the column irrespective of the target protein and that adversely affect overall column performance has not been examined, especially in the case of proteins retained by non-affinity adsorbents. The present authors have hypothesized that if this set of HCPs is identified and deleted, modified, or inhibited, a more robust strategy to increase the efficiency of the capture step would be achieved (U.S. Patent No. 8,927,231; PCT International Publications WO 2013/138351 and WO 2015/042105). To our knowledge, no preceding journal report has proposed or deployed this strategy for any type of adsorbent, let alone a non-affinity resin, to improve product capture by reducing the amount of particular nuisance HCPs. We demonstrate here that a significant overall improvement in chromatographic loading and capture can be achieved by strategically eliminating the binding of highly interfering HCPS, thereby increasing target protein adsorption without sacrificing host cell performance (growth rate and protein expression), secretion characteristics, or stability of the target protein.

2. Materials and methods

2.1. Vectors, and media

E. coli MG1655 was obtained from the Yale *E. coli* genetic Stock Center (New Haven, CT). An engineered *E. coli* strain created in-house, henceforth referred to as LTSF06, has the genotype (K-12 F− λ− ilvG− rfb-50 rph-1(ΔrfaD(hldD), Δusg ΔrraA ΔcutA ΔnagD ΔspeA). Briefly, strains with single and multiple gene deletions were constructed according to the protocol described in literature [21], which utilizes the λ-Red system in conjunction with FLP-FRT recombination to remove the desired genomic regions and selection markers. Six genes (*ΔrfaD(hldD)*, *usg*, *rraA*, *cutA*, *nagD*, and *speA*) were selected for deletion based on work done by the authors as previously described in PCT International Publication WO 2015/042105. Selected genes were deleted with knockout primers based on those developed and described in the Keio collection [22]. Confirmation of gene deletions was determined by PCR. Ultimately, the mutant strain containing all six deletions was constructed and named *E. coli* LTSF06. *rfaD* is also referred to as *hldD* in the literature.

A recombinant expression vector expressing a fusion protein of an anti-*Candida* peptide and green fluorescent protein (referred to as AFP-GFP_{UV} for the rest of the article) was constructed as described in the literature [20]. Briefly, the DNA fragment with *E. coli* codon preference encoding the anti-*Candida* peptide having the amino acid sequence GYKRKFFKRKTM was encoded in the forward oligo while the reverse complement of 3′ end of green fluorescent protein (GFP_{UV}) was encoded in the reverse oligo. Using PCR, a pBAD vector containing this recombinant gene was created. Electroporation or heat shock was used for transformation of cells [23,24].

2.2. Cultivation

For shake flask cultivation, overnight cultures of an *E. coli* strain were started in LB medium. The cultures were shaken at 250 rpm and incubated at 37 °C. After cultures reached an optical density of 0.6, they were induced as described in Table 1. After an induction period of 4 h, the cells were harvested via centrifugation at 5000 × g and stored at -20 °C.

Fed-batch fermentation was completed using the method described in the literature [25] using LB medium in a 5 L Applikon bioreactor (Foster City, CA) equipped with BioXpert Advisory software. The temperature of the bioreactor during the fed batch fermentation was maintained at 37 °C using a heating jacket and cooling loop during fermentation. Further, the pH of fermentation broth was maintained at 6.8 using 7 M NH_4OH and the dissolved oxygen content was kept above 50% throughout the fermentation procedure by an external oxygen supply. For real time monitoring of the optical density, a Bugeye optical density probe (Buglab, Foster City, CA) was used that aided in the control of the glucose feed. For these experiments, anti-foam KFO673 (Emerald Foam Control, LLC, Cheyenne, WY) was delivered via peristaltic pump when a probe detected the presence of foam. The fermentation proceeded for a total of 24 h from inoculation to harvest. Cells were induced 3 h prior to harvest by centrifugation at 5000 × g, and stored at -20 °C as described in the literature [23].

2.3. Lysate preparation

To prepare lysates, 10 g of pellets were kept on ice and resuspended in 20 mL of 25 mM Tris buffer, at pH 8. The pellet suspension was sonicated on ice for a total of 100 s using a 10-s pulse followed by a 30-s rest period method. After centrifugation at 5000 × g for 3 min, extracts were clarified using a 0.45 μm syringe filter and stored at -20 °C.

2.4. Column capacity

An ÄKTA was used for all chromatographic studies. DEAE was selected as the ion exchange resin due to its prevalence of use as the initial capture step in industrial manufacturing. For all experiments, a “1-ml HiTrap” DEAE FF column from GE Healthcare was used. The loading buffer contained 25 mM Tris buffer, 10 mM NaCl, to minimize non-specific binding (Buffer A). The elution buffer contained 25 mM Tris buffer, 1 M NaCl, which is sufficient to desorb bound proteins (Buffer B). The system was equilibrated and base-lined per the manufacturer's instructions before loading the column based on the reported dynamic binding capacity of 110 mg HSA (human serum albumin)/ml resin. Breakthrough analysis for MG1655 and LTSF06 was performed using clarified lysate produced from fermentation of *E. coli* containing pAFP-GFP_{UV}. For each run, the column was initially washed with 30 column volumes (CV) of cleaning buffer (25 mM Tris-HCl, 2 M NaCl, pH 8), followed by 30 CV of deionized water, and equilibrated with 10 CV of Buffer A (25 mM Tris-HCl, 10 mM NaCl, pH 8). To generate a breakthrough curve, the column was continuously loaded with clarified lysate at 0.32 cm/s until the concentration (fluorescence) of outlet streams emerging out of the column were similar to the concentration in the clarified lysate and outlet streams emerging out of the column were similar. Fractions, 100 μL in volume, were collected to generate the breakthrough curve. Concentrations (RFU/mL) of samples exiting the

Table 1
Information on strains, plasmid and induction method used.

Strain	Plasmid	Induction
E.coli MG1655	pAFPGFP	5 mM Arabinose; 3h pre harvesting (fed batch fermentation)
E.coli LTSF06	pAFPGFP	5 mM Arabinose; 3h pre harvesting (fed batch fermentation)

column was plotted as a function of volume.

2.5. Analytical

For protein concentration determination, a Bio-Rad DC Protein Assay was used according to the manufacturer's instructions. Fluorometric measurements were performed using a RF-Mini 150 Recording Fluorometer obtained from Shimadzu (Kyoto, Japan) with excitation and emission filters of 395 nm and 510 nm, respectively. Fractions, each of 100 μl volume, were collected and analyzed.

3. Results and discussion

3.1. Fed batch characteristics and expression with LTSF06

LTSF06 is an engineered *E. coli* cell line created by deleting 6 HCP genes identified by an algorithm that considered binding conditions, elution profile, concentration, steric effects, and metabolic necessity, as described in PCT International Publication WO 2015/042105. Briefly, to construct a reduced HCP *E. coli* cell line designed to improve DEAE chromatographic efficiency, 784 proteins were ranked, and six genes were identified (Table 2) that were not considered metabolically essential. The basic form of the equation that defines the "Importance Score" (as described in PCT International Publication WO 2015/042105), referred to as IS, favors the elimination of peptides, polypeptides, or proteins that have high affinity for the adsorbent and/or broadly elute as %B increases. Use of the IS to evaluate the behavior of the proteins which interact with DEAE resin permitted an empirical assessment of this protein ensemble, termed the DEAE separatome, in contrast to describing the adsorption and elution behavior based on multicomponent (Langmuir) adsorption. *E. coli* knockout strain LTSF06 was constructed using homologous recombination, and its growth, expression capability, and lysate properties were assessed. Fed-batch culture (Fig. 1) of *E. coli* LTSF06 demonstrated that growth of this knockout strain was not compromised as similar trajectories of growth units versus elapsed fermentation time were obtained for the parent (MG1655) and mutant deletion strain (LTSF06). On average, at the end of the fermentation period, 52 g of cell pellet (wet cell weight) were obtained for the LTSF06 deletion strain as compared to 60 g for the MG1655 parent.

3.2. DEAE breakthrough analysis

AFP-GFP_{UV} was used as a model protein to continue the comparison of cell lines due to the simplicity of the fluorescence assay. Cell lysates were obtained from resuspension of an equal weight of cell pellets of LTSF06:pAFP-GFP_{UV} and MG1655:pAFP-GFP_{UV} as described earlier. Fluorometric analysis of these lysates indicated that LTSF06 exhibited about 1.8 times more fluorescence (20,000 RFU/mL) than MG1655 (10,860 RFU/mL), which suggested higher GFP_{UV} content in the former. This difference in GFP_{UV} content was taken into account when calculating the adsorption capacity of the column. Total protein analysis performed on the same cell lysates showed that LTSF06 has 1.5 times more total protein content than MG1655. Fig. 2 shows the breakthrough of the LTSF06 deletion strain compared to that of the parent MG1655 strain. In the figure, the y-axis describes the concentration of GFP_{UV} leaving the column

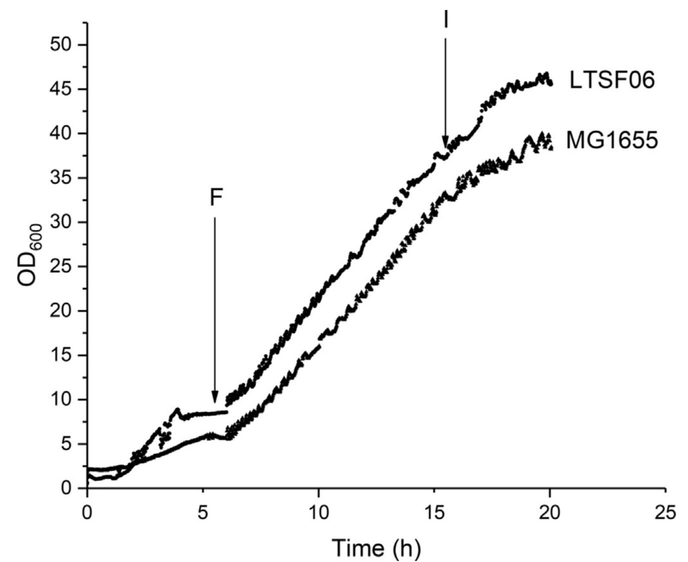

Fig. 1. Trajectories of fed-batch fermentation as monitored by Absorbance at 600 nm 'F' indicates where the Feed was started and 'I' indicates where induction occurred. The comparable optical density profiles demonstrate that growth was not compromised after gene deletions.

Table 2
Top-ranked genes based on Importance score. Essential (E), non-essential (N) from Ecocyc. Genes deleted in Lotus® strain in bold.

Gene	Importance Score	E/N	Function
hldD	**0.07259**	**N**	**synthesis of ADP-heptose precursor of core LPS**
usg	**0.01034**	**N**	**unknown**
rraA	**0.00928**	**N**	**inhibits RNase E**
rpoB	0.00876	E	RNA polymerase, β subunit
rpoC	0.00811	E	RNA polymerase, β′ subunit
tufA	0.00758	E	elongation factor Tu
cutA	**0.00736**	**N**	**copper binding protein**
ptsI	0.00724	E	PTS enzyme I
nagD	**0.00661**	**N**	**UMP phosphatase**
ycfD	0.00638	E	ribosomal protein-arginine oxygenase
speA	**0.00589**	**N**	**arginine decarboxylase, biosynthetic**
gldA	0.00550	N	L-1,2-propanediol dehydrogenase/glycerol dehydrogenase

with time. As expected, curves for both types of samples remain low and eventually rise as the column adsorbs both the target protein and other proteins in the samples. The area to the left of the curves represents the amount of GFP_{UV} adsorbed, calculated by

$$q = \frac{Q_0 C \int (1 - C) dt}{V}$$

where, q is binding capacity, C is concentration (RFU/mL), Q_0 is flowrate, V is column volume Capture efficiencies were 37–38% higher for lysates derived from the LTSF06 deletion strain than for MG1655, because columns adsorbed 15,300 RFU and 11,100 RFU of GFP_{UV}, respectively.

4. Conclusion

The data presented here demonstrate that the properties of a strategically designed *E. coli* deletion strain can address the problem of adsorption of HCPs that compromise initial capture steps from a cell lysate. Recombinant *E. coli*, an important bacterial expression platform for the manufacture of a variety of products that range from industrial enzymes to therapeutics, produce a complicated lysate mixture from which the desired material is conventionally obtained via a combination of empirical bioseparation steps. Notwithstanding centrifugation and lysis, a ubiquitous unit operation is the use of a chromatography resin to capture the desired product and concentrate it while providing some measure of purification. Effective deployment of ion-exchange mandates high capture efficiency and selectivity for the target protein not only to minimize resin use and process time, but also to reduce the number of different HCPs passed to subsequent steps in the process. Addressing this mandate by engineering the host cell to reduce the downstream burden is, therefore, an attractive option, and began with an analysis of the separatome or chromatographic sub-proteome of *E. coli* that interacted with the bioseparation medium (US 8927231; PCT International Publication WO 2015/042105). When cultured, the proof of concept deletion strain displayed minimal disparities in terms of growth characteristics or expression capabilities compared to the unmodified parent strain.

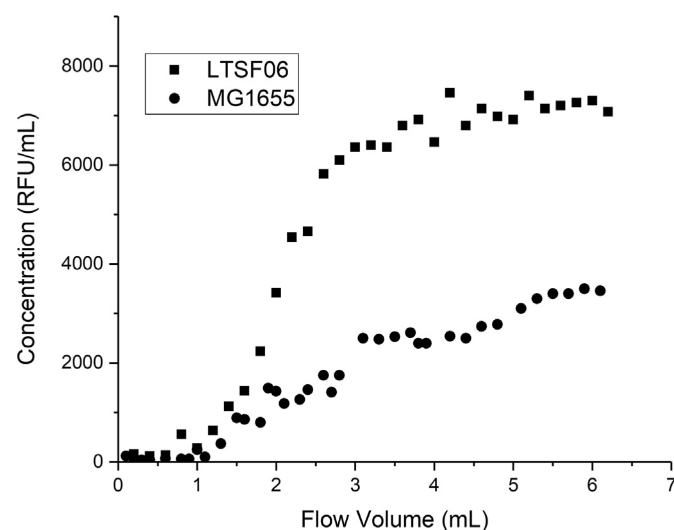

Fig. 2. Improvement in column capacity demonstrated by using AFP-GFP_{UV} as a model protein. A consistent improvement of 38% in capture efficiency was observed when equal amount of total proteins was loaded to the column.

Finally, DEAE protein capture was examined. The initial capture of the recombinant product drives both the effectiveness of the DEAE adsorption and the subsequent bioseparation recovery regimen regardless of whether the purification process consists of a single chromatography step (which is unlikely) or combinations of unit operations. The breakthrough curve for AFP-GFP_{UV} indicated a significant increase in the amount of target protein bound when deletion strain LTSF06 was used for recombinant protein expression. While it could be argued that more target protein binds because a higher level of AFP-GFP_{UV} is initially present in the extract, the shape of the breakthrough isotherm and the quantity of bound target protein cannot be discounted. The improved separation capacity reported here was achieved by selectively deleting only six genes, representing only 0.119% of the *E. coli* genome. Deletion of this small number of genes reduced HCP adsorption between 14% and 17% (reported in PCT International Publication WO 2015/042105) while disproportionately increasing separation capacity 38% without compromising either cell growth or expression capacity compared to the unmodified parent cells. These results demonstrate that the separatome concept employing a quantitative scoring method to rank HCPs is a highly effective, novel quantitative and rational means of enhancing chromatographic separation capacity, and therefore chromatographic selectivity and purity, of the final recovered protein product.

In conclusion, while the proof of concept strain displayed the requisite properties of unchanged expression and upstream characteristics, improved target capture was evident. It sets the stage for the continued improvement of the Lotus® platform and the development of similar strategies with other commercially important host cells.

Conflict of interest

Intellectual Property Rights: Brune and Beitle are listed as inventors of the technology disclosed in US 8927231 and PCT International Publication WO 2015/042105, discussed in this manuscript. Boston Mountain Biotech LLC (BMB) has licensed this intellectual property from the University of Arkansas and the University of Pittsburgh. Both Brune and Beitle will receive royalties via the U of A license agreement.

Financial relationship

Brune is the founder and CEO of BMB. Beitle is on the Advisory Board of BMB.

Employment

Fruchtl is an employee of BMB.

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