Boston Mountain Biotech: Lotus® Platform

The Lotus platform satisfies the need of CMOs to reduce the cost of biopharmaceutical manufacturing by providing genetically modified host cells that improve the efficiency of drug purification, which are compatible with existing validated manufacturing equipment and processes, and which do not negatively affect upstream expression efficiencies. A major cost associated with biotherapeutic production is the chromatography capture of the protein of interest. Loss of the final product is a common complication and is due in part to reduced column capacity (mg bound / volume column) which drives a reduced overall column efficiency (mg target protein bound / total mg bound protein). This problem is further exacerbated by the multiple rounds of purification necessary to meet regulatory standards. The foremost cause of reduced yield is binding of the resin by host cell proteins. Aside from competing for binding sites with the biotherapeutic, the presence of these proteins necessitates additional rounds of column separations to achieve the required product purity (e.g., six rounds for recombinant production of insulin from *E. coli*). The Lotus platform addresses the negative impact of host cell proteins through two steps: identifying host cell proteins that account for the largest reduction in capacity for a given column, and then disrupting the associated genes **(Figure 1)**. Boston Mountain Biotech (BMB) has completed the identification step for multiple chromatography resins, including ion exchange, the most affordable and widely used type of resin.

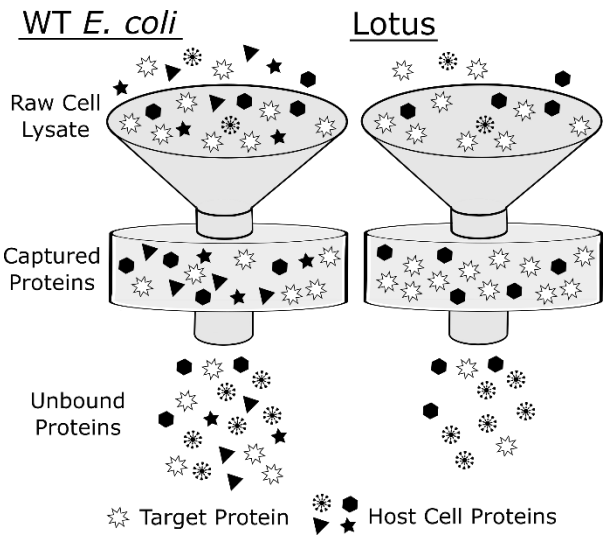


Figure 1. The Lotus platform. Proteins that represent the greatest barriers to purification are reduced in the production strain, simplifying downstream processing.

We have cataloged *E. coli*'s ion exchange separatome—the collection of proteins that bind to the purification column – by subjecting clarified *E. coli* lysates, in the absence of target protein, to a diethyl-aminoethyl (DEAE) anion-exchange column. Protein fractions, eluted at 12 different NaCl concentrations, were then subjected to LC-MS/MS based on established techniques. The resulting dataset revealed the constituents and their relative abundance under each elution condition in each eluted fraction, independent of an overexpressed target. We then formulated an "importance score" (IS) for each constituent based on the following equation:

$$\mathrm{IS}_i = \sum_j \left[b_1 \left(\frac{y_{c,j}}{y_{max}}\right)\left(\frac{h_{i,j}}{h_{i,total}}\right)\left(\frac{h_{i,j}}{h_{j,total}}\right)\left(\frac{MW_\mathrm{i}}{MW_{ref}}\right)^{\alpha} \right]_i$$

where i is the protein constituent, j is the elution window, b_1 is a chromatography-specific adjustment factor (a scaling factor reflecting protein i metabolic necessity), $y_{c,j}$ is the concentration of the elution solvent in elution window j, y_{max} is the maximum solvent concentration, $h_{i,j}$ is the amount of protein i in the elution window, $h_{j,total}$ is the total amount of protein in the window, $h_{i,total}$ is the total amountof protein i bound across all windows, MW_i is the molecular weight of protein i, MW_{ref} is the molecular weight of a reference protein within the host cell proteome, and α is a steric factor, which is 1 when the column is saturated. The importance score provides a ranking based on the adsorption strength, adsorption specificity, adsorption abundance, protein size (steric effect), metabolic necessity, and extent of column saturation. The summation can be adjusted to account for the windows in which the therapeutic protein will be bound. The resulting rankings indicate which proteins represent the greatest barriers to maximizing the column binding and

purity of the eluted biotherapeutic. Using this strategy, we identified the top-ranked *E. coli* proteins based on loading in 10 mM NaCl (Top 12 in **Table 1**), which are target-independent.

Six of the top-ranked non-essential genes (*rfaD, usg, rraA, cutA, nagD, speA*) from *E. coli* MG1655 (bolded genes in **Table 1**) were then sequentially removed via chromosomal deletions. While this is not the only way to eliminate these gene products, this method is well established in the literature. When loading at a more industrially standard condition of 100 mM NaCl, the deletion strain showed a 16% decrease in total host cell protein adsorbed to the DEAE column when compared to the parent MG1655 strain. Furthermore, despite these deletions, host cell growth was minimally diminished (**Figure 2**). Testing was then conducted to ascertain the increase in initial capture following overexpression of afGFPuv in the deletion strain when compared to MG1655. After conducting multiple experiments to test various industrial loading parameters (equal amounts of afGFPuv, equal concentrations of afGFPuv, and equal amounts of total protein), a minumum of 35% increase in capture efficiency was observed for the deletion strain over MG1655, demonstrating the effectiveness of this technology.

Table 1. Top-ranked E. coli proteins based on Importance score (IS). Essential (E), non-essential (N) from Ecocyc. Genes deleted in Lotus strain in bold

Gene	IS	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

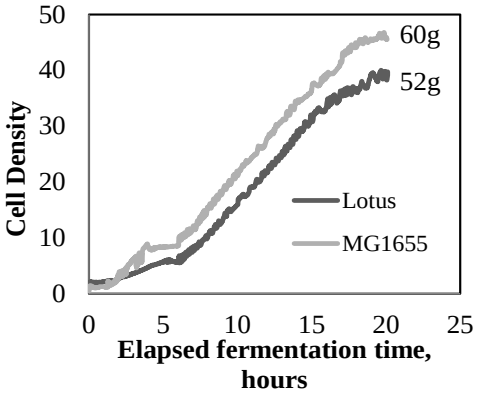


Figure 2. Growth of the wild-type strain compared to the knockout strain, both strains expressed afGFPuv. Final cell pellet mass indicated in grams.

The Lotus strain described above demonstrated a remarkable improvement in target protein capture. BMB decided to explore the use of CRISPR-mediated gene silencing to see if it would be able to provide a single construct that can coordinately silence multiple genes.

Summary of work most recently completed

The overall goal of the Lotus platform development was to create a means of boosting chromatographic protein capture as part of the production and purification of therapeutic proteins. BMB previously developed the patented importance equation approach to identify the most problematic host cell proteins (HCPs) that preferentially bind or otherwise compromise common purification columns, referred to as "interfering" or "nuisance" proteins. Eliminating the genes encoding these interfering proteins from a protein production host such as *Escherichia coli* by gene knockout greatly boosted the chromatographic capture efficiency of overexpressed recombinant target proteins, thereby reducing overall costs of the process. These strains are referred to as the "Lotus" cells.

Strain evaluation of Lotus BL21 and MG1655 indicates that increases in protein capture of at least 50% are achievable. BMB evaluated expression and purification of mcpSRP43 in the previously developed Lotus BL21 and MG1655 recombinant deletion strains. For the remainder of this document, LTSF06 refers to MG1655 *ΔrfaD Δusg ΔrraA ΔcutA ΔnagD ΔspeA* and LTSB06 refers to BL21 *ΔrfaD Δusg ΔrraA ΔcutA ΔnagD ΔspeA*, each containing the same six gene knockouts. After fermentation, lysates were subjected to ion exchange chromatography. For this set of experiments, the selected basis was one gram of cell pellet. The data from these experiments are shown in **Figure 3.** An increase in recombinant

target protein (mcpSRP43) capture from 2.8 to 4.3 mg was observed when using Lotus in MG1655 cells compared to that in wild-type cells, representing an improvement of 54% (calculated using a standard percent error calculation). Similarly, for BL21 strains, the Lotus strain outperformed the wild-type strain by increasing capture by 74%.

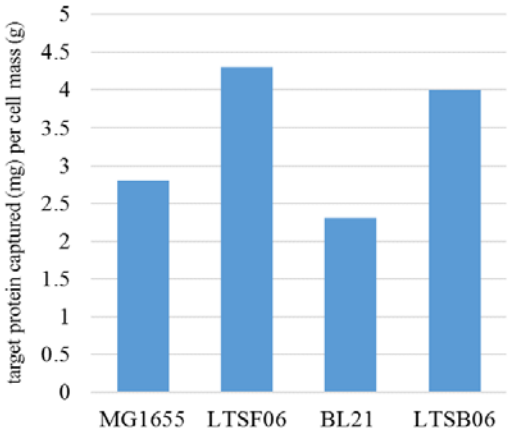
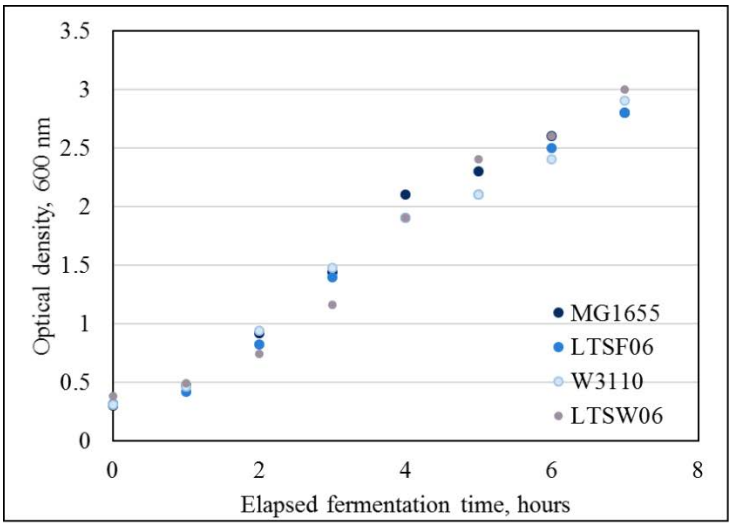


Figure 3: An increase in capture per gram of cells was observed in lysates from Lotus MG1655 and BL21. Purification of LTSF06 lysates resulted in a 54% increase in capture of target protein. Similarly, purification of LTSB06 lysates resulted in increases of capture by 74%.

Development of an additional industrially relevant *Escherichia coli* K-12 strain that exhibits similar titers and growth compared to wild-type strain. BMB also decided to complete gene knockouts via recombineering techniques in *Escherichia coli* W3110. A commercial collaborator (Gene Bridges, Germany) suggested using this strain as an additional validation test of the Lotus technology. Also, BiologicsMD's plasmid BMD26 was redesigned and was industrially expressed in W3110. Knockouts that were previously performed in MG1655 were repeated in W3110 (the six genes are *rfaD*, *usg*, *rraA*, *cutA*, *nagD*, and *speA*). Growth and expression studies were completed and shown with control data from MG1655 strains (**Figure 4**). Capture data is currently under evaluation as part of the Phase 1B funded work. For the remainder of this report, LTSW06 refers to W3110 Δ*rfaD* Δ*usg* Δ*rraA* Δ*cutA* Δ*nagD* Δ*speA*.



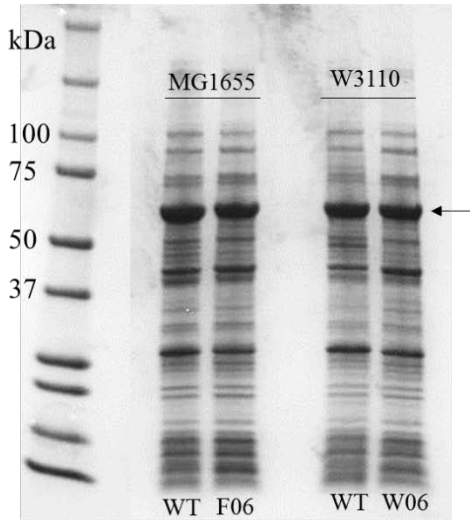


Figure 4. Growth studies and SDS-PAGE indicate that cell growth and (right) expression of target protein, as indicated with the arrow, are not inhibited by the removal of six genes from the chromosome of MG1655 or W3110.

As part of continued work, analysis of endotoxin and host cell protein (HCP) concentration was completed. For determination of endotoxin concentration, a chromogenic LAL endotoxin kit was used. Similarly, an ELISA assay for HCP concentration was completed. The samples that were tested were the same samples that indicated that there was an increase in capture from Figure 3 above. It was determined that there was no difference on final endotoxin concentrations between eluted fractions from Lotus MG1655 cells and wild-type cells. However, the assay indicates that there was a 14% reduction in endotoxin concentrations in eluted fractions from Lotus BL21 cells compared to wild-type BL21 cells. While these initial results are interesting, these experiments are being repeated to confirm the results.