A Unique *E. coli*–Based Secretion System

Wacker Biotech's ESETEC® system enables the controlled secretion of correctly folded recombinant protein products at high titers

Marcel Thön, PhD, and Ilona Koebsch, PhD

Most monoclonal antibodies (mAbs) require glycosylation for proper biological activity. As microbial hosts, *Escherichia coli* cells do not have the ability to perform glycosylation. As a result, the use of Chinese hamster ovary (CHO) cell lines has become the industrial gold standard for expressing mAbs and other biopharmaceuticals.

CHO cells possess the machinery for post-translational modifications, thus making it possible to purify correctly folded and secreted proteins directly from the culture broth. Consequently, the booming demand for antibodies led to the success of CHO cells in biomanufacturing. Nevertheless, CHO-based systems still suffer from slow cell growth and low productivity. Moreover, process development using mammalian cells is time-consuming, taking up to five months for the selection of the optimal clone for non-antibody products.

Fast, safe, and cost-effective manufacturing solutions are necessary to speed up clinical development timelines and reduce the cost burden on public healthcare systems.

Personalized medicine and biosimilars are just two examples underlining the need for innovative expression platforms that combine adaptability to a large variety of biopharmaceutical proteins with high productivity and rapid process development.

Wacker Biotech provides an efficient *E. coli*–based secretion technology, ESETEC, which enables the controlled secretion of correctly folded recombinant protein products into the fermentation broth. This simplifies the primary recovery and purification processes. By enabling the secretion of a broad range of secreted products with molecular weights of 5–150 kDa, the best technology provides an alternative, a cost-effective means of expressing any nonglycosylated biopharmaceutical.

Key elements of ESETEC include the specifically optimized and genetically well-characterized *E. coli* K12 strains (biosafety level 1) as production hosts. These strains, which are intellectual property of Wacker, are approved by the European Medicines Agency (EMA) and the Food & Drug Administration (FDA) for clinical supply. These host strains allow secretion of the desired product across both *E. coli* membranes into the culture medium. In addition to the host strains, the ESETEC technology also includes toolbox elements to allow optimal secretory production of target proteins. These elements include:

- Plasmids with different origins of replication for fine-tuning the expression level
- The tac promoter system including the lacIq repressor for IPTG-based induction of product formation
- Components of the secretion apparatus such as plasmid-encoded proprietary signal sequences for transport of target proteins into the oxidative periplasm
- Periplasmic chaperones and isomerases that promote proper protein folding
- Specially engineered ESETEC host strains and plasmids for antibiotic-free selection and manufacture (based on plasmid-encoded complementation of deleted chromosomal essential genes)

ESETEC has successfully expressed and secreted different target proteins and peptides, including difficult-to-produce proteins. The portfolio includes proteins of prokaryotic, eukaryotic, or artificial origin; proteins with a wide range of molecular weights (5–150 kDa) and isoelectric points; monomers–tetramers–heterodimers; fusion or native proteins; proteins with authentic N-termini and different starting amino acids; proteins with multiple disulfide bridges; novel antibody formats (e.g., single-domain antibodies) and antibody fragments (e.g., Fab); scaffolds, peptides, enzymes, and growth factors such as hGH.

Newly engineered host and optimized fermentation

ESETEC is being improved continuously. Some improvements exploit the ways that the efficiency of secretion is highly dependent on the properties of the particular target protein. While some proteins

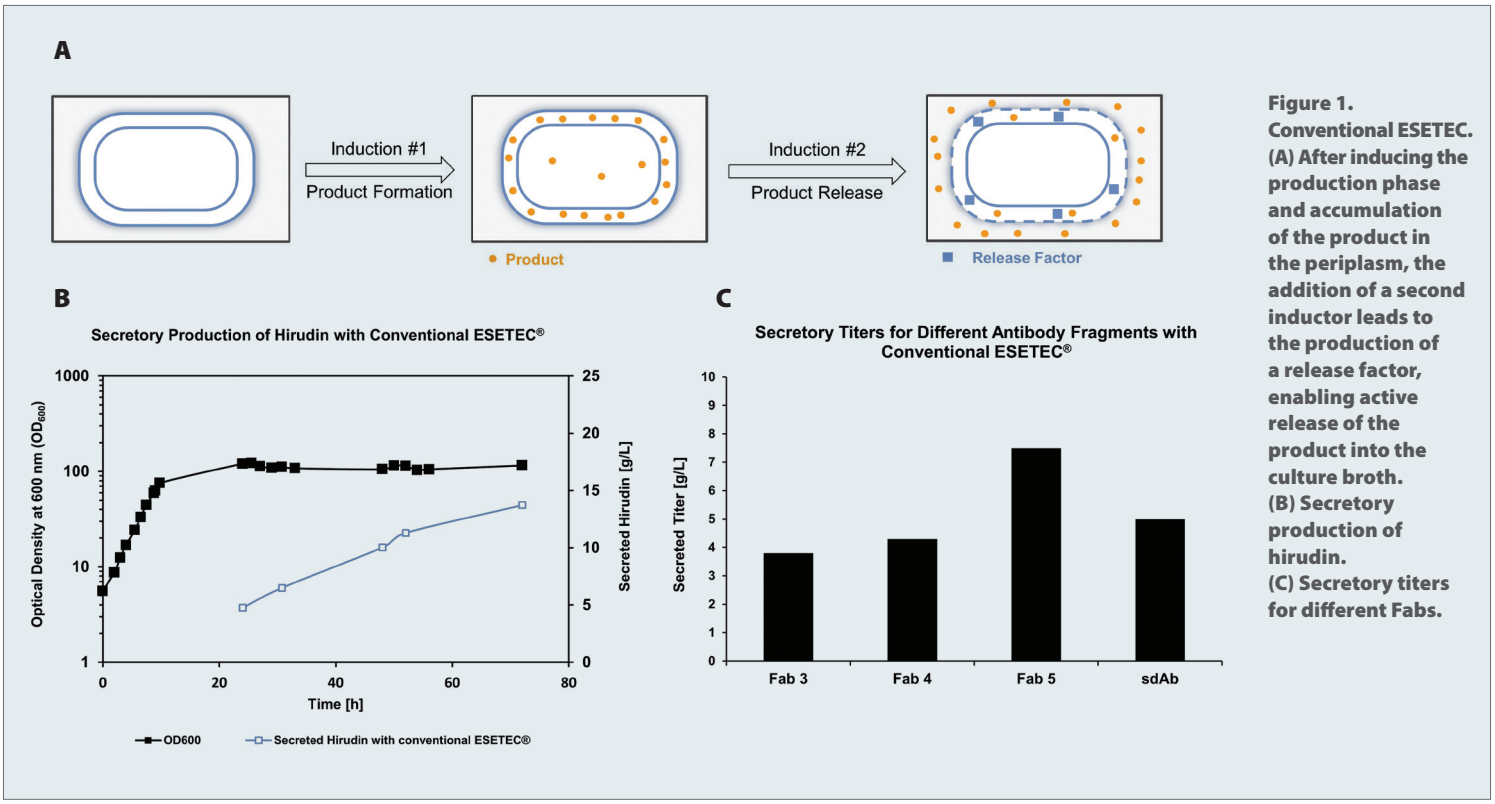

Figure 1. Conventional ESETEC. (A) After inducing the production phase and accumulation of the product in the periplasm, the addition of a second inductor leads to the production of a release factor, enabling active release of the product into the culture broth. (B) Secretory production of hirudin. (C) Secretory titers for different Fabs.

are easily secreted into the external medium, others remain trapped inside the periplasm. To overcome such limitations for hard-to-secrete proteins, additional genetic engineering was carried out to ensure both a high level of production and maximum release of the target recombinant protein.

An improved host strain is now available that combines an excellent expression rate with fully controlled product release into the culture broth. The improvement relies on two successive steps: 1) product formation and, 2) product release.

In the first step, the target protein, which accumulates in the periplasm, reaches the highest production level. In the second step, a certain release factor is produced, and it induces the secretion of the target protein into the medium (*Figure 1*). Many different inducible promoters for the production of the release factor were tested, leading to the identification of a tightly repressed version, which when induced promotes a strong release of the target protein into the culture broth.

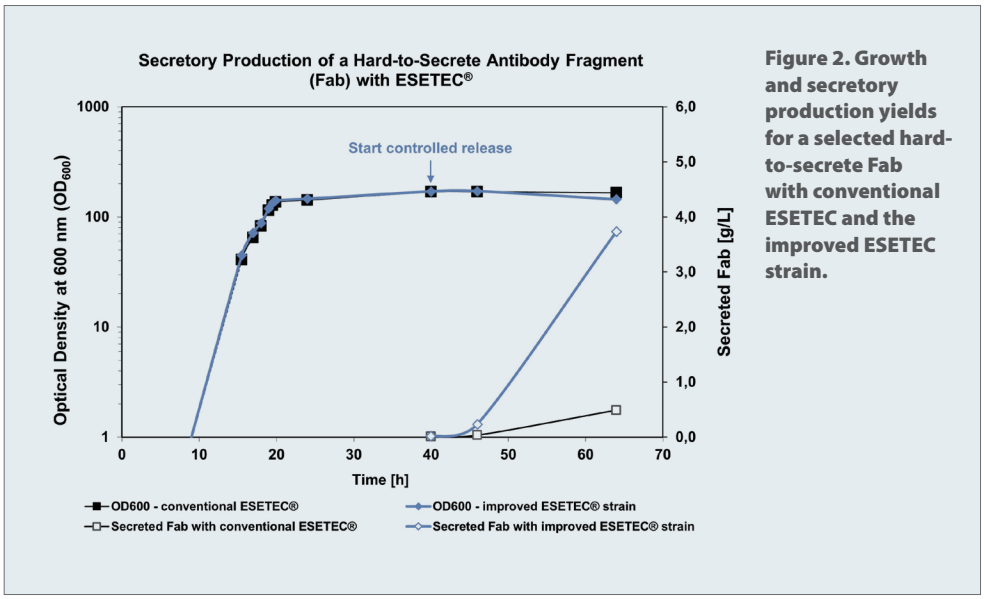

Figure 2. Growth and secretory production yields for a selected hard-to-secrete Fab with conventional ESETEC and the improved ESETEC strain.

As a result, exposure time to helper elements like chaperones and isomerases in the periplasm is now under better control, which optimizes correct folding. Additional control over the subsequently induced secretion leads to an optimum result for each target protein.

Wacker Biotech has a strong track record in the production of different proteins at high yields with conventional ESETEC. For complex molecules such as hirudin and

antibody fragments (Fabs), secretory titers of 14 g/L and >7 g/L could be achieved, respectively (*Figure 1*).

Figure 2 shows the results for a hard-to-secrete Fab produced with conventional ESETEC and the improved ESETEC strain. With the latter, secretory yields of a correctly folded, fully functional, and hard-to-secrete Fab could be achieved that were up to eight times higher.

The ability of ESETEC to export proteins enables the purification of the product without cell disruption and results in higher yields and quality. The secretion of target proteins reduces process-related impurities like host cell proteins (HCPs) and endotoxin, which need to be removed by more extensive purification in conventional *E. coli* procedures.

To figure out the effect of the controlled release mechanism on the quality of the ESETEC supernatants, measurements were carried out for viscosity, HCPs, and endotoxin.

Viscosity

For viscosity, which is an important parameter for further downstream filtration steps, the principle of controlled release for the improved ESETEC strain also led to supernatants with superior quality. Supernatants with viscosities of 1.0 – 1.5 mm^2/s can be easily processed during subsequent downstream filtration steps such as sterile filtration, microfiltration, and/or depth filtration. The measured viscosities indicate good processability of all supernatants during downstream.

HCP contaminants

The level of contamination with HCPs was evaluated by Western blot analysis for the cytoplasmic protein elongation factor thermo unstable (EF-Tu). The analysis revealed that no contamination with that representative cytoplasmic protein was detected in the supernatants of either conventional ESETEC or the improved strain. Furthermore, direct measurements for contamination with HCPs revealed a 67% decrease of HCPs per mg secreted Fab for the improved strain compared to the conventional strain (*Figure 3*).

Endotoxin

A key challenge with any *E. coli*–based system for biopharmaceutical production is the presence of lipid A, or endotoxin, which is known to contaminate protein products and can cause lethal septic shock (Stark et al., *Sci. Adv.* 2021). As a result, the amount of endotoxin in formulated biopharmaceuticals is regulated by the FDA and the EMA (Stark et al., *Sci. Adv.* 2021).

Endotoxin measurements revealed a similar picture for the contamination with HCPs. With the improved ESETEC strain, the amount of endotoxin per milligram Fab could be decreased by 71% for the hard-to-secrete Fab molecule (*Figure 3*).

Conclusion

Wacker Biotech's microbial secretion technology, ESETEC, offers a cost- and time-effective alternative for the production of any nonglycosylated therapeutic protein. With the improved strain and its principle of controlled release, it is now possible to achieve superior secretory titers also for hard-to-secrete proteins that would have remained trapped in the periplasm using conventional ESETEC strains. GEN

Marcel Thön, PhD, is senior expert downstream processing development at Wacker Biotech. Ilona Koebsch, PhD, (ilona.koebsch@wacker.com) is business development manager at Wacker Biotech. Website: www.wacker.com.

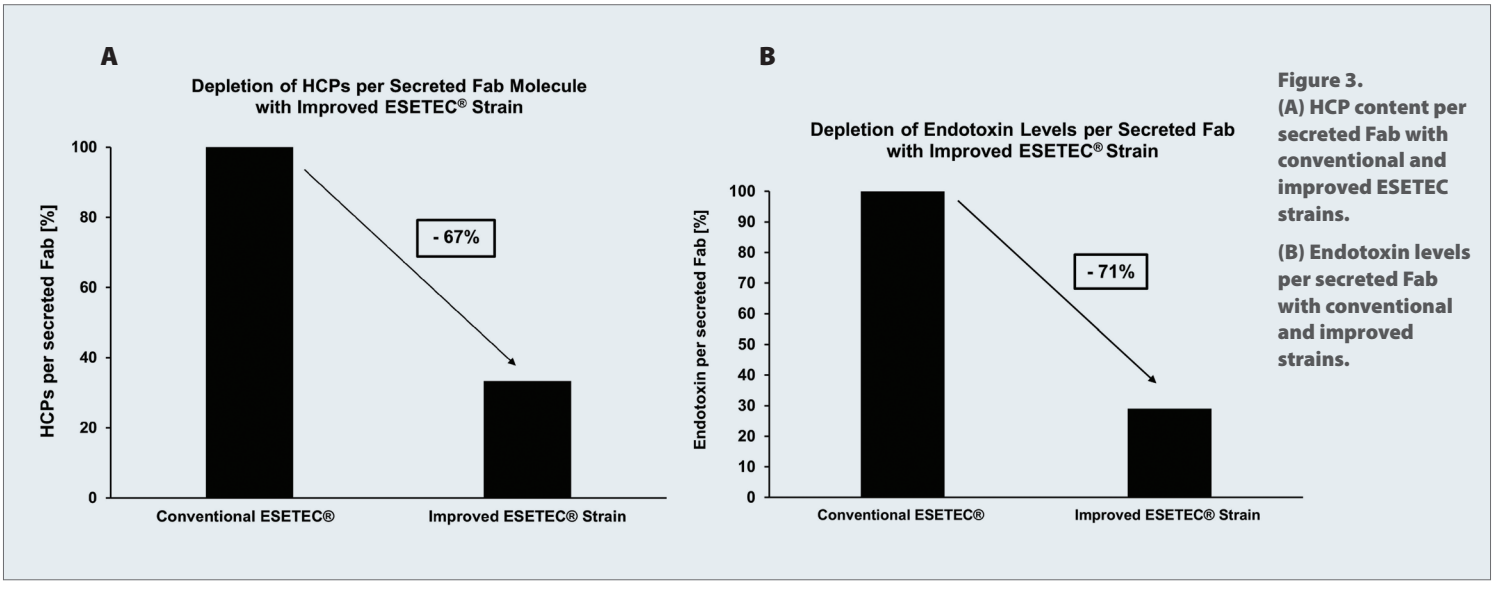

Figure 3.
(A) HCP content per secreted Fab with conventional and improved ESETEC strains.
(B) Endotoxin levels per secreted Fab with conventional and improved strains.