E. coli Expression System Efficiently Secretes Recombinant Proteins into Culture Broth

Susanne Leonhartsberger

The bacterium *Escherichia coli* is one of the most popular hosts for producing recombinant proteins. In addition to its simplicity, safety, and known genetic properties, a major asset is the fact that transformation of *E. coli* with foreign DNA is easy with well-established genetic manipulation methods. So generation of stable cell lines is a speedy process. The major advantage of *E. coli,* however, is its ability to produce proteins in large quantities and to grow very quickly compared with mammalian cells, which enables excellent space/time yields. Still, the use of *E. coli* for production of complex molecules like heterodimers, those containing complex disulfide bonds, or glycosylated proteins remains a challenge.

Overexpression of recombinant genes often results in formation of inactive protein aggregates (inclusion bodies) from which biologically active proteins can be recovered only by complicated and costly denaturation–refolding processes. Another often-cited drawback to *E. coli* is difficulty in recovering substantial yields of correctly folded proteins.

Product Focus: Proteins, peptides, enzymes, and antibody fragments

Process Focus: Production

Who Should Read: Product and process development scientists, project managers, manufacturing

Keywords: *E. coli*, secretory production, process optimization, microbial expression

Level: Intermediate

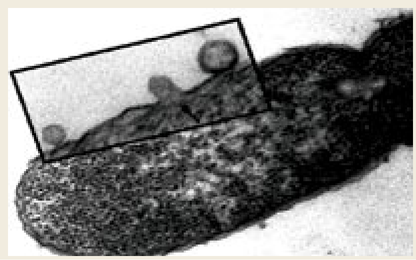
One ultimate goal of all production processes is to achieve correct folding of the protein of interest. Cellular cytoplasm, the major location where proteins are usually synthesized, maintains reducing conditions that impede formation of disulfide bonds. So one approach to recovering high yields of correctly folded recombinant proteins is to have them secreted into the periplasmic space because its oxidative environment favors formation of disulfide bonds, and certain disulfide isomerases catalyze the process. The secretory production of recombinant proteins provides several additional advantages over cytosolic production. The N-terminal amino acid residue of a secreted product is identical to a natural gene product after cleavage of its signal sequence. And there appears to be much less protease activity in the periplasmatic space than in the cytoplasm of *E. coli.*

Advantages of Protein Secretion

Extracellular production of recombinant proteins is even more advantageous than periplasmatic production. Proteolysis of a recombinant protein by periplasmatic proteases is prevented, and most important, secretion facilitates downstream purification efforts substantially by eliminating the need to disrupt cells for protein recovery. Thus purification begins with less inhomogeneous material and requires fewer processing steps to yield a pure product. Moreover, extracellular production can allow higher recombinant protein expression titers because product accumulation is not restricted by the limited volume of the periplasmatic compartment.

Finding the right host/vector system constitutes one of the first steps on the way to an efficient manufacturing process.
WACKER BIOTECH GMBH (WWW.WACKER.COM/BIOLOGICS)

Photo 1: Secretion strain WCM105 (electron micrograph of a single cell)



To take advantage of the above benefits, various strategies have been developed for extracellular production of recombinant proteins in *E. coli* (**1**). They can be excreted into their culture medium after treatment of cells with various agents: glycine, chloroform, lysozyme, and Triton X-100 surfactant from Dow Chemical Co (www.dow.com). L-form cells (cells without cell walls) present an alternative, but they are sensitive to lysis.

It has been shown that recombinant proteins fused to outer-membrane protein F (*ompF*) or to the C-terminal hemolysin secretion signal can be directly excreted into supernatant. Also, proteins secreted into the periplasmic space can be released into the culture medium by coexpression of certain genes: *kil, out, tolAIII,* or the bacteriocin release protein (BRP) gene. None of these strategies has so far proven to be suited for large-scale protein production.

Superior Secretion

Wacker Biotech has developed a system based on a proprietary *E. coli* strain (WCM105), which is designed to secrete recombinant proteins at high yields and purity into culture broth. By contrast with known commercial-scale *E. coli* expression systems, this allows the secretion of target proteins into supernatant in their native conformation. Therefore, the system simplifies primary recovery and purification processes — thus minimizing downstream costs.

The Wacker secretion system uses the Sec pathway for transport of the recombinant product into the periplasmatic space. Sec translocase provides a major pathway of protein translocation from the cytosol across the cytoplasmic membrane in bacteria (**2**). SecA ATPase interacts dynamically with SecYEG integral membrane components to drive transmembrane movement of newly synthesized preproteins. The premature proteins contain short signal sequences that allow them to be transported outside the cytoplasm. We have used a number of such sequences (*pelB, ompA,* and *phoA*) for efficient secretory production of recombinant proteins in *E. coli*. In the Wacker system, optimized and proprietary signal sequences and expression plasmids facilitate this step (see below). In the periplasm, the signal sequence is cleaved off by signal peptidase to yield a mature protein product with its natural N-terminus.

In a second step of synthesis, correctly folded proteins are released from bacterial periplasmatic spaces into the culture broth. This step is mediated by a unique feature of the proprietary WCM105 strain, which has a modified outer membrane for efficient secretion of recombinant proteins (Photo 1). By contrast with other "leaky" *E. coli* strains described in literature, WCM105 is robust for large-scale fermentation. Figure 1 shows data from a 4500-L scale producing a therapeutic protein (hirudin). Because of structural modifications in the outer membrane of WCM105, its secretion efficiency is superior to that of other secretory strains described in literature (Figure 2).

The combination of WCM105 with an optimized expression plasmid allows production of proteins at high yields. By a simple cell-separation step, target products can be isolated in their soluble, native, and active form from culture broth. They are present at high purity, and yields as high as 7 g/L have been obtained (Figures 1 and 3). Because the system is based on the genetically well-characterized *E. coli* K12, it is suitable for production of therapeutic proteins in compliance with CGMP standards (**3**).

Various Proteins/Peptides

Several proteins of prokaryotic and eukaryotic origin have already been produced in high yields by the Wacker system, including various enzymes as well as antibody fragments and peptides. No restrictions with protein properties have been observed so far. Proteins with wide-ranging molecular weights (7–120 kDa) and pI values can be efficiently produced, and the system will produce molecules with amino acids that differ from methionine at position 1. Furthermore, WCM105 efficiently produces proteins with correctly formed disulfide bridges. Examples include hirudin, which is a 7-kDa protein containing three disulfide bridges, or antibody fragments with four intramolecular disulfide bridges.

A major challenge is the biological production of peptides, which is of interest because chemical synthesis of larger ones involves many steps, which can translate to high costs. For example, synthesis of a 36–amino-acid peptide can require more than 100 steps. But biological production in *E. coli* is difficult because of proteolytic degradation. So derivatives of WCM105 have deletions in genes that code for proteases. These strains efficiently produce peptides by peptide carrier fusion and subsequent cleavage (Figure 4).

Figure 1: Growth curve and production of hirudin (a 6.9-kDa protein with three disulfide bridges) on a 4.500-L fermentation scale

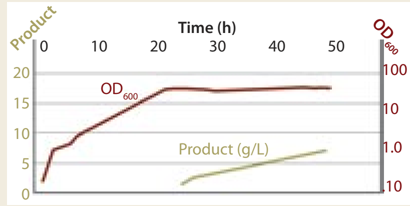
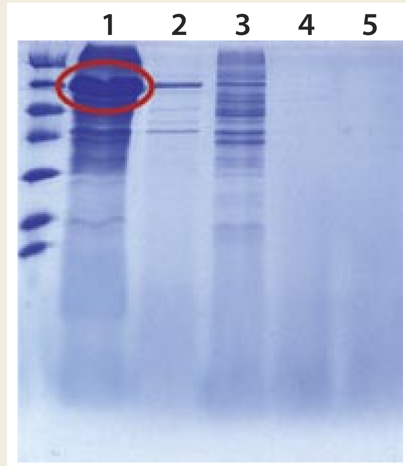
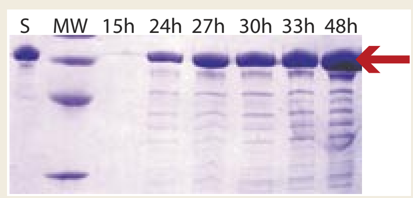


Figure 2: Secretion efficiency of different *E. coli* strains (SDS-PAGE, 15-µL culture supernatant of different strains after fermentation in rich medium); strains harbored identical protein expression plasmids, and the protein of interest is highlighted.



[1] WCM105 from Wacker Biotech
[2] BLR from Novagene (Ref #6)
[3] RV308 from Genentech (ATCC31608; EP0677109 Esperion/Pfizer)
[4] K802 (ATCC33526; Ref #7)
[5] MM28 (CGSC#6785; Ref #8)

Figure 3: Production of cyclodextrin glycosyltransferase EC 2.4.1.19 (a 70-kDa prokaryotic protein) by the Wacker secretion system; 2 µL of culture supernatant taken during fermentation at different points in time were applied to an SDS-PAGE (arrow highlights the protein of interest).



Comprehensive Toolbox for Optimizing Secretion

Production of hydrophobic, toxic, or complex recombinant proteins in *E. coli* periplasm can be limited by low secretion levels and folding problems, which lead to inclusion body formation. In such cases, the yield of native protein will be low.

Figure 4: Protease deletion strain of WCM105 Δ3 proteases allows biotechnological production at 50 mg/L (in shake flasks) of a 39-aa peptide.

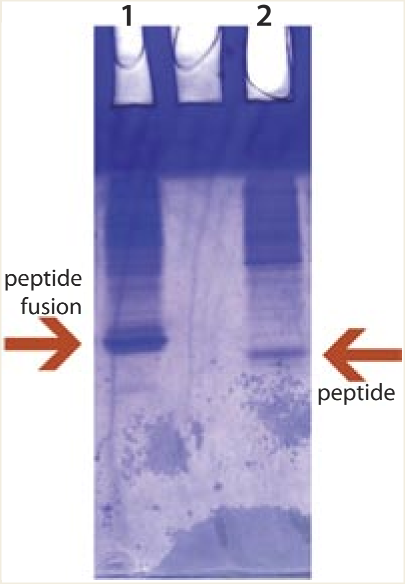
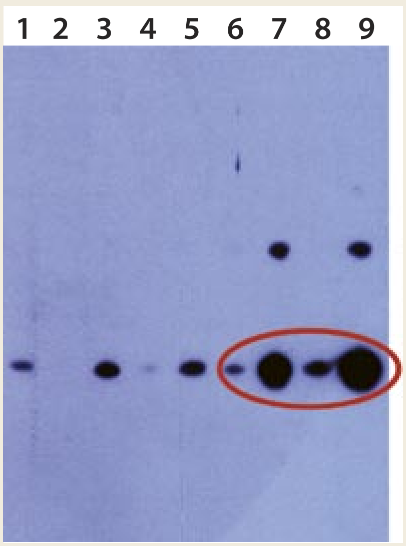


Figure 5: Secretion of interferon α2b is improved by optimized signal sequences. Interferon α2b was produced by strain WCM105 and secreted into the supernatant (in shake flasks with rich medium). Expression plasmids contained the gene encoding interferon α2b fused to different signal sequences. For each lane, after 48 h (lane 2, 4, 6, and 8) and 72 h (lane 3, 5, 7, and 9) incubation times, 1 µL supernatant was applied to an SDS-PAGE and developed in a Western blot using anti-interferon antibodies.



[1] Interferon α2b standard
[2,3] *phoA* signal peptide
[4,5] *ompA* signal peptide
[6,7] WACKER signal peptide 1
[8,9] WACKER signal peptide 2

To overcome those limitations and enhance protein production, Wacker developed proprietary signal peptides to optimize secretion of proteins into the periplasm. The efficiency of these peptides compared with standard signal peptides is shown in Figure 5, with interferon α2b as a model protein. Alternatively, the Wacker system allows use of standard signal sequences such as *phoA, ompA,* and *pelB*.

Various expression plasmids with different origins of replication for different copy numbers of recombinant genes can aid in fine-tuning and optimization of expression levels. A typical expression plasmid is pEX, which contains the tac promoter and the lacIq repressor, but other expression plasmids may be used as well.

The coexpression of periplasmatic chaperones or disulfide-bond (Dsb) family proteins can improve the efficiencies of secretory production and protein folding (**4**). Cytoplasmic and periplasmatic chaperones assist in correct folding of secreted proteins and prevent formation of inclusion bodies. Several helper elements can optimize expression, solubility, or secretion of recombinant products. Such elements can be introduced into the system either as additional genes on the expression plasmid or encoded on helper plasmids added along with it. Helper elements include cytoplasmatic chaperones, components of the secretion apparatus, periplasmatic chaperones and disulfide bridge formation factors.

Sometimes, efficient protein secretion is hindered by degradation of target proteins attributable to cell-envelope proteases such as DegP, OmpT, protease III, and Tsp. Using protease-negative mutant strains helps minimize proteolysis, as shown in a published case of peptide production (**5**). Genetic variants of WCM105 (such as protease deletion mutants) can overcome such degradation of target proteins.

Downstream costs are a major factor in biologics production. The Wacker secretion system is applicable for a broad range of proteins including antibody fragments and peptides. It offers efficient and reliable secretion of recombinant products into culture medium. So it simplifies both primary recovery and purification processes to facilitate cost-efficient manufacturing.

Production of biologics in the fermentors of Wacker Biotech. WACKER BIOTECH GMBH (WWW.WACKER.COM/BIOLOGICS)

References

1 Choi JH, Lee SY. Secretory and Extracellular Production of Recombinant Proteins Using *Escherichia coli*. *Appl. Microbiol. Biotechnol.* 64(5) 2004: 625–635.

2 Pugsley AP. The Complete General Secretory Pathway in Gram-Negative Bacteria. *Microbiol. Rev.* 57(1) 1993: 50–108.

3 Contact info.biologics@wacker.com and ask for the expert report evaluating the Wacker Secretion System.

4 Arié et al. Chaperone Function of FkpA, a Heat Shock Prolyl Isomerase, in the Periplasm of *Escherichia coli*. *Mol. Microbiol.* 39(1) 2001: 199–210.

5 Park SJ, Georgiou G, Lee SY. Secretory Production of Recombinant Protein By a High-Cell Density Culture of a Protease Negative Mutant *Escherichia coli*. *Biotechnol. Prog.* 15(2) 1999: 164–167.

6 Ray et al. Production of Salmon Calcitonin By Direct Expression of a Glycine-Extended Precuror in *Escherichia coli*. *Prot. Expr. Pur.* 26, 2002: 249–259.

7 Yang et al. One Hundred Seventy Fold Increase in Excretion of an Fv Fragment Tumor Necrosis Factor Alpha Fusion Protein (sFv/TNF-α) from *Escherichia coli* Caused by the Synergistic Effects of Glycine and Triton X-100. *Appl. Environ. Microbiol.* 64, 1998: 2869–2874.

8 Nagahari et al. Secretion into the Culture Medium of a Foreign Gene Product from *Escherichia coli:* Use of the *ompF* Gene for Secretion of Human β-Endorphin. *EMBO* 4, 1985: 3589–3592.

***Susanne Leonhartsberger**, PhD, is senior R&D manager at Wacker Biotech GmbH, Hans-Knöll-Straße 307745 Jena, Germany, 49-3641-579-920, fax 49-3641-579-921; Susanne.Leonhartsberger@wacker.com; www.wacker.com/biologics.*