ESETEC®: EFFICIENT MANUFACTURING OF A PEGYLATED ANTICALIN®

For the Pieris AG, WACKER's secretion technology ESETEC® was used to successfully secrete up to 5 g/l of an Anticalin® into the culture medium. An efficient downstream process was developed for purifying an Anticalin® from the culture supernatant and for manufacturing pegylated Anticalin®. The GMP-compliant manufacturing is based on the simplified downstream process made possible by ESETEC®.

Anticalins®

Anticalins® are engineered proteins that specifically bind targets, such as small molecules, peptides and proteins, respectively. Anticalin® structures derive from protein scaffolds of natural lipocalins. Like antibodies, Anticalins® have structurally conserved (constant) and variable regions that are responsible for target binding. Systematic changes to the structure and conformation of the variable loops have yielded Anticalins® with diverse target binding specificities. Anticalins® therefore represent a platform technology for the development of new therapeutics. Anticalins® are an interesting alternative to antibodies, because they penetrate tissue more efficiently, can be easily produced by microbial expression due to their simplier biochemical characteristics, and are generally well tolerated by the human immune system (please visit www.pieris-ag.com for further information).

How Does ESETEC® Work?

ESETEC® has been developed to secrete recombinant products into the culture broth during fermentation in order to enable very high product yields. The system works by a two-step mechanism:

- In the first step, the target product is transported across the cytoplasmic membrane into the periplasm via the Sec pathway. While crossing this membrane, the signal peptide is cleaved off which releases the native product.
- In the second step, the correctly folded product is uniquely secreted from the periplasm into the fermentation broth across the outer membrane.

The ESETEC® strain derived from *E. coli* K 12 is stable during fermentations in volumes of up to 4,500 l.

Figure 1

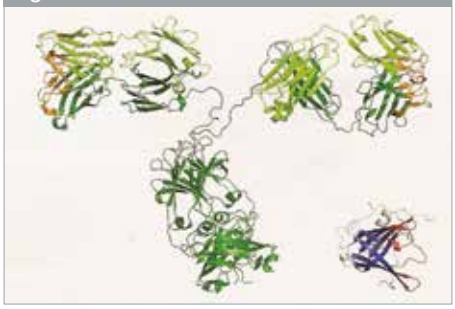

Figure 1: Antibodies (left) are much larger than lipocalins (right). The hypervariable loops of the antibody are shown in orange, the hypervariable loops of the lipocalin are in red, and the conserved ß-barrel is in blue. This figure was kindly provided by Pieris AG.

ESETEC® Successfully Produces High Yields of Anticalins®

Lipocalin scaffold proteins are mostly soluble, secretory proteins that often have one or two disulfide bridges. Thus, until now Anticalins® have been microbially expressed by secretion into the periplasm. Due to limited yields in the periplasm we explored the possibility of using ESETEC® to produce Anticalin® in higher yield through secretion into the culture medium. Fig. 3 (see next page) shows an example of an Anticalin® that was successfully produced by ESETEC®. In this case ESETEC® increased the product yield 40–50 fold when compared to microbial expression followed by secretion into the periplasm. Moreover, ESETEC® succeeded in producing another Anticalin with yields of 5 g/l active protein/culture medium (see Fig. 2). Thus, ESETEC® is the system of choice not only for producing antibody fragments in high yield but also for alternatives to antibodies that are based on engineered protein scaffolds like Anticalins®.

Figure 2

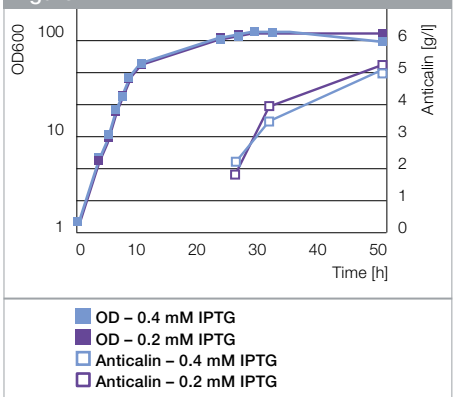

Figure 2: *E. coli* cell growth and product formation of an Anticalin® as a function of fermentation time. Growth was stable and yielded up to 5 g/l of product.

Efficient GMP-Compliant DSP of an Anticalin®

For the Anticalin® that was secreted into the culture medium by ESETEC®, an efficient downstream process to produce pegylated Anticalin® was developed. The process required just two chromatography steps to yield highly purified Anticalin® and, after pegylation, just one further chromatography step for polishing (see Fig. 3). Product yield after DSP is generally higher when the starting material has been produced by ESETEC® because, unlike ESETEC®, other expressions require extra purification steps, such as homogenization, periplasm preparation, and solubilization/refolding.

Furthermore, as the culture supernatant does not contain as many contaminants as compared to homogenization supernatants, DSP requires fewer steps than are needed for purification of proteins that accumulate inside the cytosol or the periplasm of *E. coli*. Thus, our process for producing pegylated Anticalin® demonstrates all the time and cost saving benefits which ESETEC® offers by enabling high product yields and by implicating more efficient, straightforward downstream processing. The whole process is GMP-compliant and was implemented at WACKER, including the pegylation and all the analytical methods for in process control and product quality control.

Figure 3

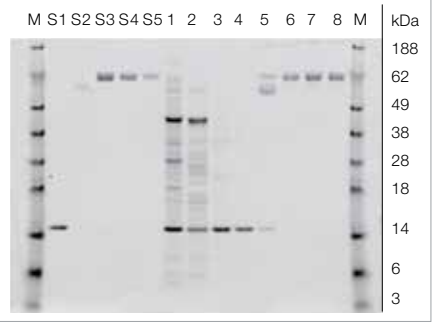

Figure 3: SDS-PAGE image of the purification of pegylated Anticalin®: M - Protein molecular weight marker, S1-S5 standard positive controls: S1 - free Anticalin®, S2 - free PEG, S3-S5 - pegylated Anticalin® in different concentrations. Lanes: 1 - after clarification, 2 - after filtration, 3 - main fraction from capture chromatography, 4 - main fraction from intermediate chromatography, 5 - after pegylation, 6 - main fraction from polishing chromatography, 7 and 8 - final product

Figure 4

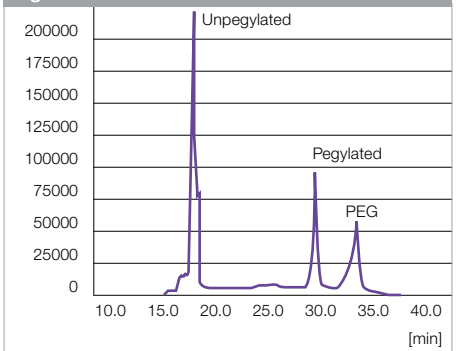

Figure 4: Quality control (in process control) of a pegylation reaction mixture by HPLC. Successful separation of unpegylated protein, PEG and pegylated protein allows their quality and quantity to be assessed.

Protein Pegylation at WACKER

As illustrated with the Anticalin®, WACKER has acquired the expertise needed for protein pegylation. WACKER can develop a GMP-compliant process for pegylated protein products including:

- Sourcing of activated PEG for our customers from qualified suppliers
- Analysis of activated PEG for release
- Creating optimized conditions for the pegylation reaction
- Analytical characterization of unpegylated protein, PEG, and pegylated product (in process control); see Fig. 4
- Testing the pegylated protein product for release (quality control)
- Scale-up of the pegylation reaction to manufacturing scale according to cGMP.

Additional Information

For details on antibody fragment (Fab) production by ESETEC®, please refer to the info sheet "Case Study - ESETEC®: High-Yield Production of an Active Antibody Fragment" and to the info sheet "ESETEC® : Unique Protein Production using *E. coli*".

Availability

Wacker Biotech's ESETEC® technology is available for cGMP-compliant manufacturing of clients' products.
Please contact us for more information.

ESETEC®

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