

Alternatives to Mammalian Expression

Bacterial, Yeast, and Cell-Free Systems Are Among Options Attempting to Replace Perennial Favorite

K. John Morrow Jr., Ph.D.

As the market for recombinant proteins continues to skyrocket, biotech is exploiting a range of options to deal with multiple possibilities. At last month's CHI "Peptalk" conference, participants explored advances in a number of different expression systems, including bacteria, yeast, mammalian cells, and even cell-free systems.

A widely used protein-expression system is *Pichia pastoris*. A number of companies that provide molecular reagents also make plasmids and vectors for manipulating *Pichia*.

The focus of VTU Technology (www.vtu.com), however, is contract development using the *Pichia* platform, according to Thomas Purkarthofer, Ph.D., head of the company's R&D biotechnology division. "Our capabilities are based on molecular technologies available in the public domain, combined with a proprietary set of synthetic variants of the *Pichia pastoris* AOX1 promoter."

The AOX1 promoter, inducible with methanol, is known for its power. In order to simplify patent concerns, VTU uses its own optimized plasmids not available publicly, but with no particular IP except for the promoter variants.

With this approach, the company has developed customized high-performance strains for the production of secreted proteins for use in the pharma, chemical, diagnostics, and agricultural industries. The VTU promoter library of synthetic variants of AOX1 spans a range of activities and expression characteristics in order to match promoter properties and specific expression requirements to a given target protein, as well as efficient expression strains for coexpression of auxiliary proteins required by a particular protocol.

These strains were isolated and modified individually in order to provide the highest possible level of function. "Our key to success is a combinatorial strategy, matching promoter variants with target and auxiliary genes to maximize protein output," Dr. Perkarthofer explained.

The company is in a rather unique situation, in that the technology division was recently formed as a unit of the main company, VTU Holding, which includes engineering and energy-production divisions. This means that there are ample opportunities for collaborative efforts in developing upstream and downstream processes with respect to questions of energy consumption, reactor design, and efficient management of a scale-up program.

Nothing succeeds like overexpression, as the mammalian-cell protein-expression platforms has so dramatically proven. As

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scientists, program managers, and regulators have become increasingly more comfortable with mammalian-cell expression programs over the years, it has become virtually impossible to dislodge them from their pinnacle. Nonetheless, alternatives keep appearing, and many have received widespread endorsement.

Recombinant Protein Production

An interesting bacterial alternative is under development at NIZO Food Research (www.nizo.nl), according to Igor Mierau, Ph.D., project manager for gene expression and fermentation. The company is investi-

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According to Delphi Genetics, its technology has a number of appealing properties, but perhaps the most important is the scalability of the system.

News Bioprocessing Highlights

Tekmira Secures Manufacturing and Development Commitment from Alnylam

Tekmira Pharmaceuticals (www.tekmirapharm.com) expects to receive a minimum of \$11.2 million over the next three years from Alnylam Pharmaceuticals (www.alnylam.com) for process development and manufacturing services. This commitment is part of Tekmira's manufacturing relationship with Alnylam through Phase II clinical development for RNAi therapeutics that utilize Tekmira's SNALP technology, including Alnylam's clinical-stage product candidate ALN-VSP.

The manufacturing and development funding is in addition to ongoing research activities between the two companies and in addition to any milestone payments that may be received from Alnylam.

Under the Tekmira-Alnylam partnership, Tekmira is eligible to receive up to \$16 million in milestones on each and every RNAi therapeutic advanced by Alnylam or its partners that utilizes Tekmira's technology, as well as royalties on product sales.

Genencor and BRAIN to Produce Biobased Chemicals from Renewable Feedstock

Genencor (www.genencor.com) and BRAIN (www.brain-biotech.de) will collaborate in the field of the biobased fermentative production of industrially relevant biochemicals from renewable raw materials.

Genencor will contribute its capabilities in metabolic pathway engineering and biomanufacturing of industrial bio-products and BRAIN will be tapped for its expertise in the fields of metagenomics and screening technologies, to pursue product targets for the chemical industry.

BRAIN will provide Genencor access to these technologies and especially to its metagenome resources of some 150 million genes of yet uncultured microorganisms. Enzymes and biosynthetic pathways of interest will then be genetically engineered in microbial production strains for the production of important biochemicals.

This new collaboration expands upon a successful partnership established in 2004, where the two companies joined forces to develop a new enzyme product platform.

ProFibrix Obtains Access to Crucell's PER.C6 Technology

ProFibrix (www.profibrix.com) inked a commercial license agreement with Crucell (www.crucell.com) for PER.C6®. ProFibrix will utilize PER.C6 to manufacture recombinant human fibrinogen at levels that support the development and commercial roll-out of new products. Fibrinogen is at the heart of all ProFibrix products and is an essential part of nature's own injury-repair mechanism.

The company's lead product Fibrocaps® is based on fibrinogen derived from human blood plasma and is a dry powder, topical hemostat that stops acute and

severe bleeding during surgery or after trauma injury, according to the company. Initially recombinant fibrinogen will be developed for systemic applications in hemostasis and later on for the development of tissue-repair products.

Novartis Scores \$486M Eight-Year Flu Vaccine Contract

The U.S. Department of Human and Health Services (HHS) Biomedical Advanced Research and Development Authority awarded Novartis (www.novartis.com) a contract totaling \$486 million over eight years to design, construct, validate, and license U.S. cell-based influenza vaccine manufacturing facilities in Holly Springs, NC.

The facilities will provide a pre-pandemic supply of vaccines and the capacity to manufacture 150 million doses within six months of declaration of a pandemic. Commercial production of pre-pandemic and seasonal flu vaccines is planned after completion, which is expected in 2012.

Under the contract, Novartis is responsible for preconstruction document development, land use and zoning, construction, commissioning, validation and licensing of the facilities. The contract also requires Novartis to provide two commercial-scale annual lots of pre-pandemic vaccine for a minimum of three years. In addition, HHS has the right to exercise options to purchase additional influenza vaccines over 17 years.

Protein Expression

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gating *Lactococcus lactis*, a traditional cheese and butter-producing microorganism.

The bacteria has a number of advantageous properties that lends it to protein-expression platforms. These include absence of endotoxin, inclusion bodies, and sporulation, as well as a range of gene-expression alternatives, within and outside the cell membrane. Moreover, there are a variety of options for control of gene expression, such as nisin, Zn²⁺, and lactic acid-based regulation. To complement the regulatory options, numerous promoters have been described in peer-reviewed literature.

Dr. Mierau and his colleagues have paid special attention to the nisin system. This 34-amino acid peptide is a bacteriocin, a proteinaceous antibiotic that inhibits the growth of similar or closely related bacterial strains. The nisin-controlled gene expression system (the NICE system) is based on the autoregulation of nisin biosynthesis and drives regulated overproduction of a range of proteins by *L. lactis* and its cousins.

According to Dr. Mierau, there is a wealth of literature describing control of gene expression through the NICE system, including overexpression of folate production, expression of a variety of pro- and eukaryotic membrane proteins, bacterial surface antigens, and bioactive peptides.

Using the plasmid pNZ8150, a wide range of proteins have been expressed in *L.*

lactis, e.g., the antibacterial protein Lysostaphin, with yields in the 300 mg/L range. An easy purification scheme produced 90% pure material.

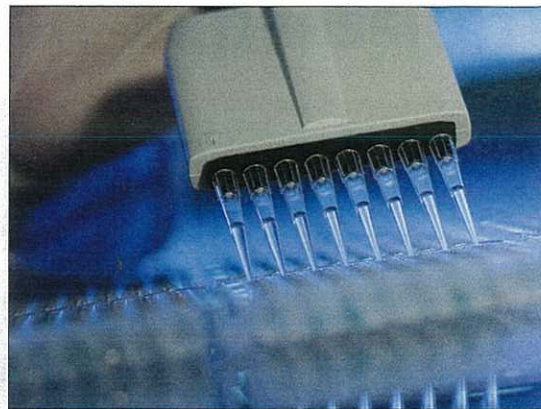
Finally, the Mierau group, in collaboration with Edmund Kunji of the Medical Research Council in Cambridge, U.K., has generated eukaryotic membrane proteins, including the yeast mitochondrial carrier protein and the KDEL-receptor 7-helix protein. So while the system may lack some of the panache ascribed to mammalian protein-expression systems, it appears to have promise for specialized projects for which *E. coli* and other expression systems may not be suitable.

Elastin-Like Biopolymers

There is an insatiable demand for new protein-purification platforms. A notable contribution to this gaggle of approaches is based on the use of elastin-like biopolymers, as described by Ariel Boutaud, Ph.D., director of research at Phase Bioscience (www.phasebio.com).

Much of the protein-purification technology within the biotech industry is based on the use of affinity chromatography, in which an expressed recombinant protein is fused to a peptide having high affinity for a particular ligand. A resin column is constructed with the ligand coupled to it, and the crude protein soup is passed through the column,

VTU Technology offers protein-expression services with the methylotrophic yeast *Pichia pastoris*. Its capabilities are based on molecular tools and a proprietary set of synthetic variants of the *Pichia pastoris* AOX1 promoter.



leaving the target protein bound to the column. Subsequently the peptide tail can be removed, leaving the protein target.

While this approach is ubiquitous throughout the industry, it has notable shortcomings, including high cost and the need for specialized equipment and expensive resins. Scale-up to industrial levels is a hit-or-miss proposition, frequently frustrating and expensive, and yields can be paltry with significant loss.

The deltaPhase recombinant expression/purification system is based on the transition properties of elastin-like polymers and their ability to retain this inverse temperature-phase transition when conjugated to other molecules. The phase transition offers a new method of purification for therapeutic proteins and peptides. The process consists of fusing an ELP sequence to the N or C terminus of the polypeptide of interest by recombinant DNA techniques. The DNA coding for the polypeptide or protein tagged with an ELP is introduced into an expression system (e.g., *E. coli* or mammalian cells) for production.

Once the protein ELP fusion is produced, the cells are lysed in the case of an *E. coli*

expression system or the culture supernatant is collected for a mammalian-expression platform. The ELP fusion protein is then phase transitioned to form insoluble aggregates, which are isolated by centrifugation or filtration. After isolating the aggregated ELP protein, it is resolubilized by decreasing the temperature. The ELP may then be cleaved enzymatically from the fusion protein. Another cycle of phase transition purification will separate any uncleaved product from the ELP, which remains in the insoluble fraction and leaves behind the purified product in the soluble fraction.

"We believe that the deltaPhase technology offers an innovative method for protein/polypeptide purification and avoids costly chromatography," Dr. Boutaud stated. "We would argue that PhaseBio's deltaPhase technology is the first new production and purification method developed in the last 20 years."

Freedom from Antibiotics

E. coli is recognized as the workhorse of recombinant DNA manipulation. A longstanding feature of this technology is the requirement for antibiotics during the clonal

Novozymes Exploits a Venerable Bioproduction Tool for Contract Manufacturing Operations

Novozymes (www.novozymes.com), formerly part of NovoNordisk, bases much of its contract manufacturing capability on yeast-based expression, which it believes to be the gold standard in microbial protein expression, according to Dermot Pearson, marketing director. Novozymes' experiences in biomanufacturing have enabled Novozymes to move rapidly into the biomedical arena.

Yeast sports many positive features for biomanufacturing. "We can push CHO mammalian cells to produce more than a gram per liter of protein a day, but this requires long growth cycles," Pearson continues, "whereas with yeast you can achieve five grams per liter per day using inexpensive media."

Other advantages include the fact that yeast secretes protein into the medium and users do not have to deal with inclusion bodies or endotoxins. "For proteins that don't require glycosylation, yeast is ideal," Pearson adds, "but we have also engineered yeast strains so they are deficient in both n-linked and o-linked glycosylation."

Novozyme has aggressively developed albumin fusion technology, joining albumin to various therapeutic molecules to improve their delivery. A prime example is Albuferon, constructed by linking albumin to interferon alpha. "With our technology we get exceptional expression," Pearson states.



Novozymes' Lund facility is a cGMP plant for development and production of the company's biopharmaceutical product range. It also offers contract process development and manufacturing services.

Because yeast has been employed for industrial purposes for so long in the manufacture of food and alcoholic beverages, there is a great repository of engineering knowledge available on mega-large-scale manufacturing. While thousands of liters are routinely handled in the biotechnology industry, winemaking dwarfs these figures, with behemoth fermentors in the range of hundreds of thousands of liters. This level of upscaling could provide massive yields of product if such feats of production are required.

Promega's Protein Purification Project

Promega (www.promega.com) recently introduced a couple of new tools to make life easier for recombinant protein aficionados. HaloTag, despite a name that conjures up cherubim and seraphim, is a solid, down-to-earth product. According to Kate Qin Zhao, Ph.D., a senior research scientist in proteomics R&D, the HaloTag concept was originally based on a bacterial dehalogenase, which will covalently attach to a set of chloroalkane ligands with different functional groups, such as fluorescent dyes, biotin, and solid surfaces. By forming a fusion protein with a gene product of interest, it can perform as a powerful tag and purification device. According to Dr. Zhao, it has been successfully applied to a variety of biotech tasks, such as live-cell imaging, protein interactions, and protein immobilization.

Now a next-generation HaloTag 7 has been configured in order to increase its structural compatibility with its numerous fusion protein partners. The newly redesigned model boasts a

number of desirable features, including superior performance compared to GST, MBP and His-tags with better protein recovery, a lower nonspecific contaminants.

Fusion tags are frequently introduced to facilitate protein purification of recombinant proteins. Removal of the tag after purification is usually done by engineering a cleavage site between the tag and the encoded protein recognizable by a site-specific protease, such as one from tobacco etch virus (TEV). Promega scientists have introduced a TEV linker that allows the protein of interest to be efficiently cleaved from the HaloTag 7 protein, greatly improving the quality of purification. So the system produces proteins with superior yield, purity, and specific activity by providing a simple method for fusion protein detection and quantification. The HaloTag protein purification system will be released this spring.

Dr. Zhao also discussed two cell-free protein expression systems, the S30 T7 H

selection and protein-expression phases, in order to stabilize the plasmids that carry the essential genetic information. This need for antibiotics represents a substantial impediment to the straightforward manipulation of bacterial strains and vectors, according to Philippe Gabant, Ph.D., CEO and founder at Delphi Genetics (www.delphigenetics.com). Moreover, regulatory questions make the use of antibiotic-resistance factors undesirable and under the best of circumstances, plasmid instability can frequently lead to low yields and introduce unwanted variability during manufacturing.

To confront this problem the company took advantage of its expertise in bacterial poison antidote genes. This is a recently developed selection and stabilization tool, based on naturally occurring poison-antidote systems. As Dr. Gabant explains, the selection gene targets DNA replication, an essential bacterial process, specifically the enzyme DNA gyrase. If the selection gene is unleashed, it shuts off DNA gyrase and the cell is doomed. The antidote protein interacts with the selection protein and blocks its lethal activity.

"This constitutes a powerful alternative to antibiotics for clone selection and plasmid stabilization," Dr. Gabant stated.

In designing the production strains, the selection gene is integrated into the bacterial chromosome, but it fails to kill the cell as long as it is repressed by the antidote gene, which is engineered into the plasmid. So without the use of antibiotics the strain is stabilized for a variety of different applications. The plasmid, pStaby1.2, is available with or without antibiotic-resistance genes. It performs admirably with protein-production levels and plasmid DNA levels 3 to 5 times higher than in the case of strains lacking the bacterial selection system, Dr. Gabant added.

The Delphi technology has a number of appealing properties, but perhaps the most important is the scalability of the system. Any *E. coli*-based protein-production system

can be upgraded to industrial levels in which the necessity of antibiotics to keep the genes in place is no longer a consideration. Not only does this constitute a major technical simplification, but the cost savings involved in the omission of antibiotics from thousands of liters of medium are substantial.

Isotopic Enrichment

"Bacteria can be coaxed into producing just one protein," says Masayori Inouye, Ph.D., professor at the Robert Wood Johnson Medical School (rwjms.umdnj.edu). For a number of years, Dr. Inouye and his colleagues have been studying a bacterial toxin that can be used to turn *E. coli* into a factory producing a single protein of interest.

The Inouye group has evaluated MazF, an endoribonuclease that degrades only messenger RNA by cleaving it at a specific nucleotide site. The researchers designed an *E. coli* strain that carried this gene, they then

followed up by adding a protein-producing gene of interest, which lacked the specific cleavage site. When the MazF gene is turned on, all other protein synthesis grinds to a halt, since all the other messengers are rapidly degraded. The overwhelming amount of protein produced is now the target protein, which can be specifically labeled by adding ^{13}C and ^{15}N to the culture medium.

Dr. Inouye envisions the technique as a means of producing pure protein easily and rapidly for structural and functional studies of proteins in intact, living cells using nuclear magnetic resonance.

The Inouye platform, referred to as SPP (single protein production), can be adapted to maximize production of virtually any protein, either membrane bound or cytoplasmic, according to the group. This approach* to dealing with protein purification allows the investigator to forego the use of affinity columns or other costly materials

and hardware. Moreover, it allows for structural studies to be conducted on proteins that are toxic to other systems.

The genes that constitute the platform can be transferred to eukaryotic systems, including yeast and mammalian cells. Given the need for industrial-scale protein-purification technology that can escape the burden of complex resins, columns, and ligands, the technology has great intrinsic appeal.

In the bioprocessing field, all the low-hanging fruit was carted away long ago, so it is a tribute to the biotechnologists profiled in these pages that they were able to address longstanding problems in a fresh and creative fashion. If these technologies prove to be acceptable on a large scale, they could make major inroads into areas of production and purification that have so far resisted easy solutions, and have relied upon time-tested but laborious and expensive technologies. GEN

Breakthroughs by lunchtime.



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Luminex

Yield Protein Expression System and the TNT® T7 Insect Cell Extract Protein Expression System. She claims they offer a number of important advantages. The prokaryotic S30 System is a native coupled-transcription/translation device that features high protein productivity per DNA input, with yields as high as 500 µg/mL/hr at 37°C. Because of its ease of manipulation, it can be used as an efficient screening tool. It is scalable and as little as 5 µL reaction mixtures can be driven with 100 ng plasmid DNA. And the system has the flexibility to permit use of multiple detection methods, including Coomassie, FluoroTect™, Transcend™, HaloTag® Protein purification and His/HQ tag/HaloTag.

Finally, the TNT T7 Insect Cell Extract Protein Expression System, designed as a eukaryotic system, uses a DNA template with a T7 promoter for protein yields up to 75 µg/mL. "We believe it offers a higher probability of obtaining proteins with high specific activity," states Dr. Zhao. ■