Pseudomonas fluorescens - A robust manufacturing platform

Charles Squires and **Henry Talbot** of **Dowpharma** introduce a new microbial expression platform

Efficient expression of the target protein in a cell line or other host organism can be a bottleneck to protein pharmaceutical production. Dowpharma, a contract manufacturing services unit of Dow Chemical, has developed a manufacturing platform for high-yield production of non-glycosylated protein pharmaceuticals. This platform is derived from *Pseudomonas fluorescens* biovar I strain MB101.

The system's performance is based on a combination of a robust host strain and the availability of extensive molecular biology and bioinformatics tools. These include a range of stable plasmid vectors of various copy numbers, non-antibiotic-dependent plasmid maintenance, engineered host strains for stringent control of gene expression, the ability to export proteins to the cell's periplasmic space and a functional genomics capability. The system also features optimised high cell density fermentation.

Soluble and active protein yields in excess of 25 g/litre have been achieved with a variety of protein types ranging from enzymes for industrial use to proteins for pharmaceutical applications. These are routinely optimised in 20 litre fermentors and have been successfully scaled for commercial production of a number of products.

Biology of *P. fluorescens*

P. fluorescens is an abundant and natural component of the microbial flora of soil, water and plants. The American Type Culture Collection has designated strains of *P. fluorescens* under Biosafety Level 1, which defines them as "...having no known potential to cause disease in humans or animals".

In recent years, *P. fluorescens* has been used in many applications, including ice nucleation (for snow making and minimisation of frost damage to plants), producing insecticidal proteins and the control of plant diseases. Its ability to catabolise a wide variety of natural and synthetic compounds, such as chlorinated aliphatic hydrocarbons, and produce a variety of enzymes has led to wide use in bioremediation and biocatalysis.[1]

The genome sequence of this *P. fluorescens* strain was determined as part of the development of the organism into a production platform strain for the manufacture of proteins, peptides and metabolites. Knowledge of the entire genome allows directed changes to be performed quickly to individual genes or groups of genes, thus facilitating sophisticated pathway engineering and enhancement of specific gene expression in *P. fluorescens* (see below). *P. fluorescens* is also easy to manipulate genetically, enhancing this capability.

Core expression platform

The current platform for recombinant protein production in *P. fluorescens* comprises a family of host strains derived from *P. fluorescens* biovar I MB101 and self-replicable but non-conjugative expression plasmids of varying copy number. The host strains are stable, amenable to genetic or molecular manipulations and can be cultivated to high cell densities in fully defined mineral salts media in standard fermentors without oxygen enrichment, even at very high cell densities.

A variety of self-replicating plasmid vectors are available, onto which target genes of interest may be cloned and expressed. These plasmids are compatible and, if needed, can be stably maintained in the same cell. Expression of heterologous genes is driven by transcription promoters of varying strengths, such as the *ben*, *ant*, *tac* and *lacUV5* promoters.

Optimal translation initiation signals and strong transcription terminators are also available. Derivatives of the *lac* (*tac* and *lacUV5*) promoter can be regulated by introduction of a *lacI* gene into the host strain, permitting induction by isopropyl-thiogalactopyranoside. Induction by lactose is possible in *P. fluorescens* strains bearing the *lac* operon from *E. coli*.

Prokaryotic microorganisms such as *P. fluorescens* and *Escherichia Coli* can secrete proteins to the extracellular medium or to the periplasmic space, an intracellular compartment that lies between the inner and outer membranes. Such secretion can be of value in decreasing the costs of protein recovery. The biochemical apparatus to form protein disulphide bonds in prokaryotes lies within this periplasmic space, because the cytoplasm, where protein synthesis takes place, is too reduced to allow these bonds to form chemically. All disulphide-bonded proteins in prokaryotes are found in this space or are secreted entirely outside the cell.

Many mammalian-derived proteins of therapeutic interest, including monoclonal antibodies, and many types of engineered antibody fragments contain disulphide bonds and therefore should be secreted at least to the periplasmic space if they are to be formed *de novo* in an active state. Secretion is typically mediated by a short, 20-40 amino acid leader sequence on the amino terminus of secreted proteins. This sequence is called a secretion leader and is specifically removed in the periplasmic space by proteolytic action.

Secretion leader sequences capable of efficiently directing the secretion of heterologous proteins to the periplasm of *P. fluorescens* have been found by a combination of genomic and functional means.

Of several signal sequences identified in this way, the signal from phosphate binding protein (*pbp*), in particular, has been found to be effective at efficiently transporting single chain antibodies and other mammalian-derived proteins. In addition, quantitative removal of the secretion leader has been demonstrated with *P. fluorescens* and secretion has been shown to result in the recovery of disulphide-bonded proteins in their native, active conformations.

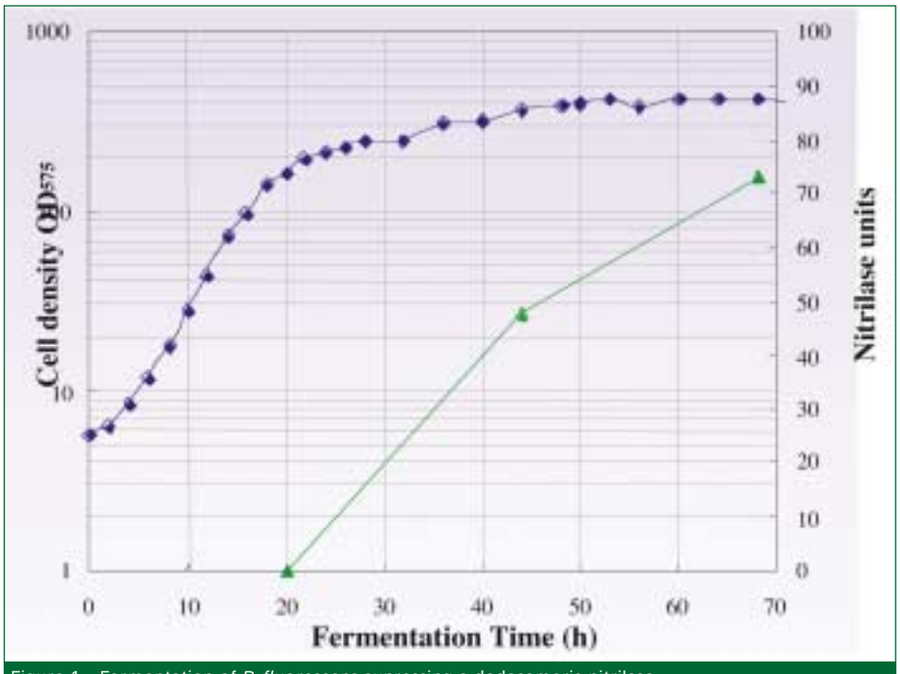

Figure 1 - Fermentation of *P. fluorescens* expressing a dodecameric nitrilase

Reprinted from **July/August 2004 Speciality Chemicals Magazine**

Production of proteins

The expression of several therapeutic proteins in the *P. fluorescens* system has been compared side-by-side with expression in *E. coli* using the T7 expression system.[2] In each case the results obtained using *P. fluorescens* and the *tac* promoter were equivalent to, or had some advantages over, the commonly used T7 promoter system in *E. coli*.

Commercial recombinant human growth hormone (rhGH) is currently produced in *E. coli* cytoplasmically as inclusion bodies that can be readily refolded to an active form.[3] For this study, the rhGH gene was cloned and expressed cytoplasmically in both the *P. fluorescens tac* expression and the *E. coli* T7 expression system; then rhGH production was evaluated at the 20 litre fermentation scale. *P. fluorescens* produced 16 times more rhGH per gram of dry biomass than *E. coli*.

Refolded rhGH isolated from *P. fluorescens* extracts was as active as commercially available rhGH, as determined by an *in vitro* cell proliferation assay. Also, secretion of rhGH using the *pbp* secretion leader sequence has been demonstrated with *P. fluorescens*. In this case rhGH was found in the soluble protein fraction, at more than 2 g/litre, and the secretion leader was shown to be correctly and completely removed.

Human gamma interferon (γ-IFN) is another example of a therapeutic protein currently produced in *E. coli* as inclusion bodies. Studies have found that γ-IFN is difficult to refold.[4] The comparative expression of γ-IFN in *P. fluorescens* versus *E. coli* revealed a significant advantage for recovery of the active cytokine in the *P. fluorescens* expression system.

Figure 2 - Coomassie-stained reducing SDS-PAGE showing expression level of nitrilase

The *E. coli* construct produced approximately 2 g/litre of insoluble γ-IFN protein at the 20 litre scale. The *P. fluorescens* construct produced approximately 4 g/litre of the cytokine during a typical 20 litre fermentation. SDS-PAGE analysis of soluble and insoluble fractions showed that the majority of the protein (95%) was present in the soluble fraction when produced in *P. fluorescens*. This soluble protein was readily purified in a single ion-exchange chromatographic step to greater than 95% purity. Purified *P. fluorescens*-expressed γ-IFN was found to be as active as the commercially available standard in a viral inhibition assay. These two case studies clearly demonstrate that *P. fluorescens* can produce more and better quality protein that the traditional *E. coli* system.

Figure 3 - Light micrographs of *P. fluorescens* cells before and after induction of nitrilase

Monoclonal antibodies and engineered antibody fragments represent a large percentage of therapeutic and diagnostic proteins currently in the development pipeline.[5] Two anti-β-galactosidase single chain antibodies, gal13 and gal2, were produced in the cytoplasm or secreted to the periplasm of *P. fluorescens*.[6,7]

The gal13 antibody, produced cytoplasmically, demonstrated an eightfold increase (0.5 to 4 g/litre) in volumetric yield at the 20 litre scale when produced in *P. fluorescens* as compared to expression in *E. coli*. Gal13 was produced in *P. fluorescens* primarily as soluble protein (96%), whereas only 48% of the gal13 was soluble in *E. coli*. Protein purified from both expression systems by Ni+ affinity chromatography was found to be active in an ELISA assay, as has been shown for *E. coli*-derived material.[7]

The *pelB* secretion signal[8] for the *E. coli* expression/secretion system or the *pbp* secretion signal for the *P. fluorescens* expression/secretion system were fused to the N-terminus of the *gal2* open reading frame to test for Gal2 secretion to the periplasm. The *pelB-gal2* fusion expressed in *E. coli* resulted in 16 g/litre (at the 20 litre scale) of protein, of which about 54% was processed by the cell, resulting in the removal of the secretion signal and indicating that the protein was secreted to the periplasm. Of that 54%, only 11% was found in the soluble fraction.

The *pbp-gal2* fusion expressed in *P. fluorescens* resulted in 10g/litre (at the 20 litre scale) of fully processed protein. The majority (96%) of the Gal2 protein produced in *P. fluorescens* was found in the insoluble fraction. Protein purified from both expression systems was found to be active in an anti-β-galactosidase ELISA. Although a greater percentage of the total protein was found in the soluble fraction for the *E. coli* construct as compared to the *P. fluorescens pbp:gal2* construct, the overall yield in *P. fluorescens* of processed protein was significantly higher.

A wide range of industrial enzymes belonging to the glycosidase (EC 3.2.1.x), nitrilase (EC 3.5.5.x), and phosphatase (EC 3.1.3.x) families, both of mesophilic and hyperthermophilic origins, have been produced in *P. fluorescens* at high levels. Several native and laboratory evolved β-amylases derived from environmental *Thermococcus* strains accumulated to amounts of >25% total cell protein in high cell density cultures at pilot scales.[9]

Similarly, native and optimised nitrilase genes have been expressed leading to very high yields of protein in a soluble and active form in *P. fluorescens*.[10] A 20 litre fermentation of *P. fluorescens* strain DC240 over 20 hours yielded a final OD and dry cell weight of over 400 and 100 g/litre respectively (Figure 1). Enzyme yield is more than 25 g/litre in the fermentor, or over 50% total cell protein (Figure 2). The nitrilase holoenzyme is a dodecamer of 400,000 Daltons MW and all of this protein is expressed in a soluble and active form. Figure 3 shows the appearance of the cells before and after nitrilase expression, which is clearly consistent with extremely high protein expression.

Conclusions

The *P. fluorescens* strain described here has many favourable properties in terms of the rapid, efficient expression of therapeutics that is increasingly required for higher volume products. The organism is grown in a completely defined mineral salts medium with no added animal components, antibiotics or organic nitrogen of any kind.

The fed-batch fermentation process is well characterised and scale up is predictable and rapid. Cell densities of >100 g/litre dry cell weight are routinely obtained in standard fermentation vessels, without oxygen supplementation. The organism is unusually well suited to high-level expression and will tolerate a wide range of conditions. Recovery and downstream purification procedures are standard and consistent with those employed with *E. coli*.

Knowledge of the genome sequence facilitates rapid strain engineering to improve gene expression in *P. fluorescens* as well. The established protein expression techniques employed during fermentation runs can be monitored by functional genomic tools to gauge the metabolic state of host cells and provide temporal profiles of desired gene transcript and protein production. These data can then be used to improve strain performance further.

Extensive pathogenicity and toxicology studies have shown the organism to be safe therapeutics duction. Work is continuing, in order to add data relevant for regulatory submissions. The combination of high volumetric and specific expression of a broad range of therapeutic proteins, coupled with the potential for soluble, active, and secreted products, makes *P. fluorescens* a compelling alternative for the microbial expression of biologicals for human health.

For more information, please contact:
Charles H. Squires/Henry W. Talbot
Dowpharma
5501 Oberlin Drive
San Diego
CA 92121
USA
Tel: +1 858 352 4398/4434
E-mail: csquires@dow.com or hwtalbot@dow.com

References:

1. M.G. Wubbolts & B. Witholt. in T.C. Montie (ed.), Pseudomonas, vol. 10. pages 271-329 Plenum Press, New York, **1998**
2. F.W. Studier, *J. Mol. Biol.* **1991**, *219*, 37-44
3. A.K. Patra *et al., Protein Expr. Purif.* **2000**, *18*, 182-92
4. J. Haelewyn & M. De Ley, *Biochem. Mol. Biol. Int*, **1995**, *37*, 1163-71
5. G. Walsh, *Nature Biotechnology* **2003**, *21*, 865-870
6. P. Martineau & J. M. Betton, *J. Mol. Biol.* **1999**, *29*, 921-9
7. P. Martineau, P. Jones & G. Winter, *J. Mol. Biol.* **1998**, *280*, 117-27
8. P.A. Nolan *et al., Gene* **1994**, *134*, 223-7
9. T.H. Richardson *et al., J. Biol. Chem*. **2002**, *277*, 26501-7
10. G. DeSantis *et al., J. Am. Chem. Soc*. **2003**, *125*, 11476-7