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 1 strain M B101.

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.

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 1 M B101 and self-replicating 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 lac 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
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. 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. 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 1.6 times more rHGH per gram of dry biomass than E. coli.

Refolded rHGH isolated from P. fluorescens extracts was as active as commercially available standard in a viral inhibition assay. Although a greater percentage of the active cytokine (gal1 3) was soluble in E. coli prima- rily as soluble protein (96%), whereas only 48% of the gal1 3 was soluble in E. coli. Protein purified from both expression systems by Ni+ affinity chromatography was as active as in an ELISA assay, as has been shown for E. coli-derived material.

The pBl expression signal and the E. coli expres- sion/secretion system or the pbb secretion signal for the P. fluorescens expression/secretion system were fused to the N-termi- nus of the gal2 open reading frame to test for Gal2 secretion to the periplasm. The pbb-gal2 fusion expressed in E. coli resulted in 1.6 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 pbb-gal2 fusion expressed in P. fluorescens resulted in 10 g/litre (at the 20 litre scale) of fully processed protein. The major- ity (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-galactosidase ELISA. Although a greater per- centage of the total protein was found in the soluble frac- tion for the E. coli construct as compared to the P. fluorescens pbb-gal2 construct, the overall yield in P. fluorescens of processed protein was sig- nificantly 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 3.13.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% of total cell protein in high cell density cultures at pilot scales.

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. A 20 litre fermentation of P. fluorescens strain DC240 over 24 hours yielded a final O.D 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 pro-tein (Figure 2). The nitrilase holoenzyme is a dode-camer of 400,000 Daltons M.W. and all of this pro-