Institution: Newcastle University

Unit of Assessment: UoA5

Title of case study: Increasing the yield of important enzymes for industry

1. Summary of the impact

Bacillus species constitute an industrially-important group of bacteria that are used worldwide to produce carbohydrate and protein-digesting enzymes on a large scale. While the bacteria secrete native enzymes at an economically viable rate, generating strains of bacteria that could do the same for non-native enzymes has been an industry challenge. Researchers at Newcastle University have collaborated with industry since the early 1990s to study the mechanism of protein secretion in *Bacillus*. They discovered bottlenecks in the protein secretion pathway and used that knowledge to engineer more productive strains of bacteria. Since 2008, companies, including Novozymes (the world's largest manufacturer of industrial enzymes), have developed strains of bacteria, based on the Newcastle findings, for use in their manufacturing processes improving yields by more than four orders of magnitude in some cases.

2. Underpinning research

Key Newcastle University researchers

(Where people left/joined the University in the period 1993-2013, years are given in brackets)

- Professor Colin Harwood led the work at Newcastle; he was a Senior Lecturer in the Department of Microbiology and Immunology (1976-1999), a Reader in Molecular Microbiology (1999-2001), then Professor in Molecular Microbiology.
- Professor Peter Emmerson collaborated with Harwood on one of the studies; he was a Professor in the School of Biochemistry and Genetics (1983-2002).

Background

Many species of bacteria secrete biological molecules into the extracellular space to assist with the uptake of nutrients, or otherwise modify their environment so that it is more favourable for growth. This phenomenon has been understood and exploited by industry for some time, particularly for the production and harvesting of enzymes – biological catalysts – which have many commercial applications.

Proteins destined to be secreted by bacteria are initially produced inside the cell, where they are prevented from adopting their final conformation (shape) by interaction with proteins called chaperones. The secretory proteins are then moved across the cell membrane through specialised transporters, and once outside they fold into their final conformation in a process often facilitated by a further set of proteins.

Research

In 1998 Harwood's group, in collaboration with researchers at the Institut Jacques Monod in France, published the results of study on the mechanism of secretion of the enzyme alpha-amylase by the commercially important bacterium *Bacillus subtilis*. The researchers showed that when *B. subtilis* was engineered to secrete alpha-amylase originating from another species of bacterium, the yield of non-native enzyme was much lower than the yield of native enzyme. Experiments suggested that the cause of this was proteolysis of the non-native enzyme during, or at the end of, transport across the cell membrane, and *in vitro* experiments showed that when the concentration of Ca^{2+} was increased in bacterial cultures (thereby stabilising protein folding) the yield of alpha-amylase increased. The results implied the presence of a quality-control system in the secretory pathway of *B. subtilis* that is activated by mis-folding of non-native proteins during transport and which degrades them before they can pass beyond the cell wall into the extracellular medium (R1).

In the same year, Harwood and colleagues published a paper in which they identified a candidate extracytoplasmic protease in the secretion-pathway quality-control system, namely wall protease A (WprA). A strain of *B. subtilis* in which WprA was not expressed was capable of secreting

significantly higher levels of intact and active non-native alpha-amylase (R2). This work was extended to show the influences of protein charge, rate of folding and protein engineering changes on the yield of secretory proteins (R3).

Using the model protein recombinant *Bacillus anthracis* protective antigen (rPA) expressed in *B. subtilis* as an experimental system, Harwood and colleagues explored the role of other parts of the secretory pathway (such as enzymes that modify the composition of the cell wall, R4) in determining the stability of secreted proteins, and which ultimately determine the yield of protein recovered. They also showed that while certain individual modifications to the secretory pathway improved yield, combinations of them often did not.

Harwood, in collaboration with Dr Rocky Cranenburgh at Cobra Biologics, recently published (delayed in order to protect IP, EV a) a proteomic analysis of *B. subtilis* strains in which various combinations of the genes for ten extracytoplasmic proteases had been deleted (R5). Those genes included *wprA* (encoding the aforementioned quality-control protease), *htrA* and *htrB* (encoding two other quality-control proteases also active at the cell wall or wall/membrane interface), and *nprE* and *aprE* (encoding extracellular proteases which Harwood had previously shown to have an important role in maintaining cell integrity once culture growth has plateaued (R6)). The researchers demonstrated that quality-control protease deletion mutants were able to secrete significantly higher levels of the rPA than unmodified strains both in the laboratory and at an industrial scale in fermenters.

3. References to the research

(Newcastle researchers in bold. Citation count from Scopus, July 2013)

R1. **Stephenson K, Carter NM, Harwood CR**, Petit-Glatron MF and Chambert R (1998) The influence of protein folding on late stages of the secretion of alpha-amylases from *Bacillus subtilis. FEBS Lett*ers 430(3):385-9. DOI: 10.1016/S0014-5793(98)00698-X. **29 citations.**

R2. **Stephenson K** and **Harwood CR** (1998) Influence of a cell-wall-associated protease on production of alpha-amylase by *Bacillus subtilis*. *Applied & Environmental Microbiology* 64(8):2875-81.DOI: not available. **51 citations.**

R3. Jensen CL, **Stephenson K**, Jørgensen ST and **Harwood CR** (2000) Cell-associated degradation affects yield of secreted engineered and heterologous proteins in the *Bacillus subtilis* expression system. *Microbiology* 146:2583-94. DOI: not available. **18 citations.**

R4. **Thwaite JE**, Baillie LWJ, **Carter NM, Stephenson K, Rees M, Harwood CR** and **Emmerson PT** (2002) Optimization of the cell wall microenvironment allows increased production of recombinant *Bacillus anthracis* protective antigen from *Bacillus subtilis*. *Applied & Environmental Microbiology* 68(1):227-34. DOI: 10.1128/AEM.68.1.227-234.2002. **34 citations.**

R5. **Pohl S,** Bhavsar G, Hulme J, Bloor AE, **Misirli G,** Leckenby MW, Radford DS, **Smith W**, **Wipat A**, Williamson ED, **Harwood CR** and Cranenburgh RM (2013) Proteomic analysis of *Bacillus subtilis* strains engineered for improved production of heterologous proteins. *Proteomics* 13(22). DOI: 10.1002/pmic.201300183. **No citations yet; published online Aug 2013.** ***Harwood was involved in the conception and design of the study and the preparation of the manuscript.***

R6. **Stephenson K**, Bron S and **Harwood CR** (1999) Cellular lysis in Bacillus subtilis; the affect of multiple extracellular protease deficiencies. *Letters in Applied Microbiology* 29(2):141-5. DOI: 10.1046/j.1472-765X.1999.00592.x. **12 citations.**

Selected funding awards

- European Commission. 2000-2. £173,000. *Engineering the cellular quality control systems of Bacillus subtilis for the production of high value-added proteins.*
- European Commission, FP6 grant. 2004-8. £201,000 to Newcastle of total grant value of EUR 2 million. *BACELL Health: Bacterial stress management relevant to infectious disease and biopharmaceuticals.*
- DTI/BBSRC Link grant. 2003-6. £209,000. *The Bacillus Cell Factory: A New Tool for the Biomanufacturing Industry.*

4. Details of the impact

Background

Worldwide, *Bacillus* species are by far the most important producers of industrial enzymes, accounting for about half of all production. They therefore significantly underpin the global market for industrial enzymes that was worth around $4bn in the year 2012. The applications for industrial enzymes are diverse: for example, they are used during the production and quality-control of food and drink, they are an important component of non-food products (such as clothes washing powders), and they can be used to produce bio-energy sources (such as ethanol).

Industry has wanted to expand the range of different proteins that *Bacillus* could produce (for example, to include therapeutic proteins), because the bacteria can be grown so efficiently on a large-scale in fermenters, from which protein can be easily harvested. However, while *Bacillus* secrete native protein- and carbohydrate-digesting enzymes at high levels (of the order of grams per litre) into the culture medium, they do not secrete non-native proteins at an economically viable rate. Yields from strains engineered to produce non-native proteins were up to ten thousand-times lower than yields of native proteins.

Knowledge transfer to industry

Harwood conducted research with industrial scientists that led to a greater understanding of the mechanism of protein secretion in *Bacillus*. Insights from that programme of work formed the basis for innovations within the manufacturing industry. Of particular importance is Harwood's discovery that quality-control proteases (such as WprA) and other proteases, active at the bacterial cell membrane and in the culture medium, were degrading much of the non-native protein synthesised by *Bacillus*. Those research insights fed directly into the development by commercial companies of strains of bacteria in which those particular aspects of the protein secretion pathway were altered so that the bacteria secreted much higher amounts of intact non-native proteins.

Two biomanufacturing companies, Cobra Biologics and Novozymes, have confirmed the significant and material role of Harwood's research in informing their development of novel producer strains of bacteria.

Cobra Biologics is a wholly owned subsidiary of ML Laboratories plc. Cobra is a contract manufacturing organisation with three facilities, two in Sweden and one in the UK, where recombinant proteins, DNA and viruses (many of which are for therapeutic uses) are produced for other companies in the life sciences sector:

"Based on previous knowledge developed by the Harwood group, a series of strains [of Bacillus subtilis] *with combination* [deletion/downregulation] *of the secreted feeding and quality control proteases were systematically constructed. Specific strains showed very substantial improvements in rPA* [recombinant protective antigen, a model protein] *production from concentrations in the µg per litre range to approximately 1 g per litre in industrial fermentations."* (EV a)

Novozymes is the world's largest producer of industrial enzymes, with a 47% share of the global market in 2012 and total sales of about $2bn:

"There is no doubt the Harwood group over the past 20 years has provided and continues to provide very valuable data and has helped us make informed choices in relation to our strain development program aimed at enhancing the yield of industrial enzymes."

"… during the late 1990s/early2000s, Novozymes and the Harwood group jointly generated a series of hybrid alpha-amylases that helped to elucidate the fate of secretory proteins following their release from the translocase … The discovery that amylases are degraded significantly in the vicinity of the cell membrane/wall turned out to be crucial in our understanding and use of the extracytoplasmic chaperone, PrsA, and the role of membrane- and wall-associated proteases like WprA. The data on cell-wall proteases and their role in the degradation of industrial proteins and the lysis of cells was also important in cell refactoring". (EV b)

Commercial impact

Both Cobra Biologics and Novozymes have incorporated new strains of bacteria, underpinned by this research, into their manufacturing processes after 2008. The strains are used by Cobra Biologics in fee-for-service contracts with major biotechnology and pharmaceutical companies, whereas Novozymes manufactures enzymes and then sells them directly to industry.

Cobra Biologics:
"… the resulting strains [of Bacillus, with downregulated or inactivated quality control or feeding proteases] *have been used by Cobra Biologics in two fee-for-service contracts in the period 2008-2013, one client being one of the largest international pharmaceutical companies with a multi-billion dollar turnover. One of the products was an industrial enzyme (in 2011) and the other a therapeutic protein (in 2012). Both clients continued to use the expression systems after they were transferred from Cobra once the contracts were completed."*(EV a)

Novozymes:
"Since 2008 we have incorporated protease-deficient production hosts, and are continuously evaluating the use of protease-deficient host strains as a valuable option in our enzyme production processes. WprA is also in this family of proteases which can be beneficial to remove from hosts. Several enzymes would have been very difficult to bring to the market without this knowledge about proteases. The actual yield-improvements resulting from the collaboration [with Harwood at Newcastle University] *and the resulting business impact must remain trade secrets, but the protease-deficient strains have made significant and valuable contributions to yield improvement."* (EV b).

5. Sources to corroborate the impact

EV a. Statement from the Head of Molecular Biology, Cobra Biologics Ltd.

EV b. Statement from a Senior Manager (department for bioengineering of bacterial production strains), Novozymes A/S.