INDUSTRIAL ECOLOGY

Biotechnology, bioremediation, and blue genes

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Until now, the majority of biotechnologists have come from faculties of biology rather than chemistry, and the economic and ideological imperatives of biotechnology have come more from the pharmaceutical than the chemical industries. But this situation has recently shown signs of change. Consider the remarks of Ronald Breslow, president of the American Chemical Society, in the August 26, 1996 issue of *Chemical and Engineering News*: "...The chemical community, including our major chemical companies, has decided that we can and must be environmentally benign. The biblical injunction, 'Hurt not the earth, neither the sea, nor the trees,' is guiding a lot of modern chemical effort..."

Certainly the economic incentives have always been present. If these are now to be joined by a real commitment on the part of the chemical industries to make "green chemistry" the norm rather than the exception, biotechnology will be presented with major new opportunities to take advantage of maturing expertise at manipulating microbial chemical factories. The fate of Genencor International's (GCI, South San Francisco, CA) biological process for the production of indigo dye may serve as one test of these ideas.*

The basis of this process derives from the pioneering work of David Gibson's laboratory, where it was first shown that the naphthalene dioxygenase (NDO) of *Pseudomonas* could convert indole (the final intermediate in tryptophan biosynthesis) to indoxyl, a reactive compound that undergoes oxidative dimerization to form indigo (Ensley, B.D., Gibson, D.T., and Laborde, A.L. *J. Bacteriol.* 149:948-954, 1982; Ensley, B.D. et al. *Science* 222:167-169, 1983). How these experimental demonstrations were translated into a complete industrial process that incorporates the solutions to a variety of scientific challenges, as well as the realities of commodity chemical use and commerce, is an instructive and fascinating story.

The first biotechnology company to realize the potential of using a cloned NDO in a commercial setting was Amgen (Thousand Oaks, CA), which acquired the intellectual property and successfully constructed the first metabolic operons in *Escherichia coli* capable of directing the de novo synthesis of indigo from glucose (Murdock, D. et al. *Bio/Technology* 11:381-385, 1993). For reasons that may have to do with the spectacular success at producing erythropoietin, corporate commitment to commodity chemical R&D was not equal to the complexities of the indigo problem, until the program was wholly acquired by GCI and continued in 1989 to the present.

Genencor's approach to resurrecting commercial biological indigo involved a comprehensive series of genetically engineered metabolic transformations. First, it was necessary to coax *E. coli* to commit sufficient glucose carbon to the production of chorismic acid, the key common precursor of the aromatic amino acid synthetic pathways. The approach was to amplify the genes of the common pathway, overexpress each enzyme, combine them, and monitor production of a chorismate intermediate. Overexpression of a feedback-resistant *aroG* product, that directs phosphoenol pyruvate toward chorismate, turned out to be sufficient.

The next problem, diverting the excess chorismate to tryptophan synthesis (and not tyrosine or phenylalanine) was solved by amplifying the *trp* operon. This led to near-theoretical yields of tryptophan over two-thirds of the production phase. The solution to the final metabolic engineering problem, getting *E. coli* to accumulate indole instead of condensing it with serine, was provided by a mutant tryptophan synthase that could no longer tunnel indole between the α and β subunits, and therefore releases free indole.

Combining these alterations with the engineered NDO operon, was (or so they thought) the last step in the biological end of developing a competitive commercial process. And on this basis, Genencor scientists went on to perfect a production scheme using *lac* control elements and coordinated derepression during glucose limitation of the numerous engineered constructions. With this in-house, the company proceeded with the required Environmental Protection Agency (Washington, DC) filings, process-product validation, and scaleup to multiple 30,000 liter runs, the product from which was used to dye 150,000 yards of denim. The result? The blue jeans were too red! The reason? Too much indirubin in the indigo.

Solving the indirubin dilemma meant first understanding the source of the unwanted by-product and then inventing a means to control its production. The chemical culprit turned out to be isatin, an oxidative derivative of indoxyl that accumulates to varying degrees during neutral pH fermentation (but is essentially absent from the von Baeyer industrial synthesis) and reacts with indoxyl to form the red-hued indirubin. The amount of isatin can be controlled in part by adjusting the dissolved oxygen during fermentation. Additional, and critical, control was provided by the cloning of an isatin hydrolase activity. This enzyme, which converts isatin to isatic acid, a colorless, nontoxic compound, was found in an indole-degrading natural soil isolate of *Pseudomonas*, from

whence it was activity-cloned and optimized for expression in *E. coli*. The overall effect of these labors was to reduce the indirubin content of the indigo dye pastes to levels comparable to those found in synthetic material.

Based on this, a new production strain, which now contained over 18 kb of stable, coordinately regulated, additional, genetic information in 15 open reading frames was developed; the attendant EPA documentation refiled; a new production process validated at the 14, 300, 3000, and 30,000 liter scales, and finally, new textile dye trials, involving two independent mills and over 200,000 linear yards of denim, were performed. This time the judgment by the mills and jeans manufacturers was quite different. The Genencor indigo was indistinguishable from synthetic material in handling, dye-mix preparation, and most importantly, performance in-process.

How do the two versions of indigo compare on other criteria? Synthetic indigo production involves eight unit operations—using and producing highly toxic chemicals—and involves messy, potentially hazardous, processes, including cyanylation, saponification, and sodium-ammonia reactions that require special conditions to protect workers and the environment. Recombinant indigo requires three unit operations—fermentation, cell separation, and product formulation—uses water instead of organic solvents, corn syrup as a carbon source, and produces coproducts (biomass and CO₂) instead of waste products.

The economics of the biological process are also appealing. Based on an input of raw glucose at 15 cents per pound, the GCI process produces 90 cents worth of indigo (\$.75/lb, 12% yield based on carbon), one cent of biomass (\$.04/lb, 28% yield), and three cents of carbon dioxide (\$.05/lb, 60% yield). The overall economic picture is such that the recombinant indigo can be offered to textile manufacturers at a cost equal to the synthetic material. Whether they, and the chemical companies that now produce indigo, will have the foresight, courage, and commitment to environmental stewardship, so well expressed by Ronald Breslow, to introduce this striking example of the use of genetic technologies for industrial bioremediation, remains to be seen. But it seems, to this observer at least, that there is an entire generation of ecologically conscious consumers who would be happy to know that green chemistry and blue genes made their jeans blue. ///

Process description based on a talk by Scott Power (of Genencor) at the Danvers Symposium on "Recombinant DNA biotechnology: Focus on metabolic engineering" (Oct. 6-11, Danvers, MA).