Pichia Power: India's Biotech Industry Puts Unconventional Yeast to Work

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As a platform for making recombinant proteins, *E. coli* is hard to beat. This universal gut bacterium grows quickly and eagerly, and—with simple genetic tinkering—will make virtually any foreign protein. However, *E. coli*, a prokaryote, can't handle posttranslational modifications; the proteins it makes are often misfolded and insoluble, requiring expensive processing steps to make them usable. Mammalian cells, in contrast, fold and modify proteins with ease but are much harder to culture. One organism that potentially combines the advantages of bacterial and mammalian expression systems is *Pichia pastoris*, a harmless species of yeast that feeds on methanol. Being a single-celled organism, it is easy to grow and manipulate, while as a eukaryote, it can perform complex manipulations on proteins. These attributes have made it a mainstay of the recombinant protein industry in India. "*Pichia* gives high yields of well-expressed proteins," says Harish Iyer, head of research and development at Biocon, the first company to market recombinant human insulin made using *Pichia*. "It is a wonderful workhorse for us."

Pichia's role in biotechnology dates back to 1969 when researchers first learned about the organism's remarkable ability to feed on methanol. Since at that time methanol could be made cheaply from waste natural gas, oil company researchers were intrigued by the idea of using this yeast to make protein-rich animal feed. They developed the technology to grow the organism to cell densities as high as 130 g/l. However, the oil crisis of the 1970s, coupled with a fall in price of soybeans—a competing source of animal feed protein—killed this effort.

Researchers then turned their attention to using this prolific yeast as an expression system for foreign genes. In the early 1980s, molecular biologists at the Salk Institute Biotechnology/Industrial Associate, Inc., (SIBIA), in La Jolla, CA, isolated the principal gene for the yeast's alcohol oxidase promoter, which is highly effective at controlling the expression of foreign genes. The team went on to develop vectors, strains, and tools for genetic manipulation of the organism. The combination of a strong promoter and very high cell densities "resulted in strikingly high levels of foreign proteins," recalls James Cregg, who led the SIBIA team. One of the team's first achievements was cloning the surface antigen of the hepatitis B virus (HBsAg), a process that was later repeated by several Indian companies with great commercial success. In contrast, when HBsAg is expressed in *E.coli*, "you just get 'rocks' or inclusion bodies of misfolded protein that are apparently not protective against the virus," says Cregg.

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Since then, researchers worldwide have used *Pichia* to make recombinant versions of several dozen proteins—bacterial, fungal, viral, plant, and human—with expression levels comparable to *E.coli* and higher than tissue cultures. "*Pichia* doesn't have toxins like *E.coli* does, or viruses like tissue cultures do," says Cregg. "It is an excellent expression system, perhaps one of the best."

Despite its advantages, *Pichia* has not been widely used in the United States and Europe to make human therapeutics. (One of the very few *Pichia*-derived injectable biologics approved for use in the U.S. is a recombinant human serum albumin made by Japan-based Mitsubishi Pharma Corporation.) This is partly because glycosylation patterns in yeast cells tend to differ from their mammalian counterparts; studies suggest that glycoproteins made using fungal systems might trigger adverse immune responses when injected into mammals. More importantly, many elements of the *Pichia* expression system are protected by patents by Tucson, AZ, based Research Corporation Technologies. "Because of patent concerns, only a handful of U.S. companies have worked with *Pichia*," says Cregg. In contrast, several Indian biotechnology companies have embraced this yeast as a platform for making recombinant products, encouraged by intellectual property regulations that until recently were less stringent than those in the U.S. and Europe.

In 1997, Hyderabad-based Shantha Biotech announced India's first *Pichia*-derived product, a hepatitis B vaccine based on a recombinant form of the HBsAg antigen. Earlier work by Cregg and other researchers provided guidelines for doing this, but transitioning the technology from the lab to the factory required a major effort. "We initially struggled to get good levels of expression," recalls Revathi Chaganti, then the sole molecular biologist at the company. The protein tended to associate with the membrane, and it was tricky to extract it without disturbing its structure. "Even now we recover only about 30% of it," she says. Most of the published work dealt with shake-flask cultures; scaling the system to industrial-size fermentors was a challenge. "We started with a 10 L fermentor and increased it by stages to 750 L," says Chaganti. From such humble beginnings, the company now makes more than 100 million units of the vaccine each year. It sells the product in more than 50 countries and provides 40% of UNICEF's supply. Prior to Shantha's effort, the multinational GlaxoSmithKline was the sole provider of this vaccine in India, charging more than $10 per dose; Shanta's entry into the market caused its price to plunge, and it now sells at 15 cents a dose.

During the past few years, the market for the hepatitis B vaccine in India has grown fiercely competitive, as several other companies have entered the fray. One of the contenders is Indian Immunologicals, a veterinary biologics manufacturer for whom this vaccine was the first foray into recombinant DNA technology as well as the *Pichia* expression system. ''We had 25 years of experience with mammalian cells,'' says V. Srinivasan, director of research and development at the Hyderabad-based company. ''Compared to that, *Pichia* was a much easier system to work with.'' Downstream processing, however, required more effort; it was particularly challenging to attain purity levels of 97% and above, according to Srinivasan. ''It is not the expression system alone that matters–you have to couple it with powerful downstream processing,'' agrees Krishna Ella, chairman and managing director of another hepatitis B vaccine manufacturer, Bharat Biotech, who switched to *Pichia* after initially working with a *Saccharomyces* platform. Yet another platform, *Hansenula polymorpha*, a methylotrophic yeast similar to *P. pastoris*, is used by Mumbai-based Wockhardt for making its version of the vaccine.

In 2000, Biocon, India's leading biotechnology company, unleashed *Pichia* on insulin, another hot item in the Indian biologics market. ''It was a singularly challenging project,'' recalls Iyer, who led the company's effort. ''Here we were, a band of 10–20 people, competing with multinationals like Eli Lilly and Novo Nordisk, with their hundreds of researchers.'' As with HBsAg, expressing the protein was the easy part. ''There wasn't much difficulty in transforming the microbe or getting high yield,'' says Iyer. The challenge came from the whole series of activities required to commercialize the product: extraction, purification, clinical studies, regulatory approval, etc. It took the better part of four years to complete these stages. ''And then one day we had our first formulation, a vial with a label that had Biocon's name on it,'' says Iyer. ''It was a tremendously rewarding moment.'' The product, which was released in 2004, is now registered in around 40 countries and sells millions of units each year.

Indian manufacturers cite several reasons for choosing *Pichia* over other platforms. For instance, Chaganti observes that *Pichia*-derived proteins, in contrast to those made using *E.coli*, have intact disulfide bonds and don't need expensive refolding stages. ''All you need to do is to break open the association with the membrane, put it on a column, and purify it,'' she says. However, Chaganti and others also noted some shortcomings with *Pichia*. She points out that some proteins give poor yields unless their gene is optimized to eliminate rare codons. Further, during secretion, glycoproteins such as granulocyte-macrophage colony stimulating factor (GM-CSF) get over-glycosylated and may get N-terminal amino acids cleaved off. ''Because of this we could not use *Pichia* for producing GM-CSF, though the expression levels were very good,'' says Chaganti. *Pichia*'s ability to feed on cheap methanol is a major selling point; however, even here there is room for improvement, says Iyer. He points out that much of the methanol consumed by the organism is burnt to produce heat instead of the ''useful work'' of making protein. ''How to shunt the process away from heat into work is a major unsolved problem with *Pichia*,'' says Iyer. ''We need to do some fundamental work here.''

Until recently, Indian companies were more interested in reproducing and adapting results from the literature than in performing fundamental research. However, with recent legislation that brings Indian patent laws in compliance with World Trade Organization (WTO) and Trade-Related Aspects of Intellectual Property Rights (TRIPS) requirements, the situation is changing. Shantha Biotech, for instance, is working for the development of a novel *Pichia*-based expression system in collaboration with the Centre for Cellular and Molecular Biology, a research institute in Hyderabad. Other companies and research institutes are engaged in similar efforts. ''Our initial effort was one of indigenization rather than innovation,'' says biochemist P.N. Rangarajan of the Indian Institute of Science in Bangalore, who developed the *Pichia* strain used by Indian Immunologicals for their hepatitis B vaccine. ''But now we are doing more basic research and are hoping to come up with innovative things in *Pichia*.'' He points out that despite *Pichia*'s wide use in biotechnology, many aspects of the organism—such as how it attains such high cell densities or how methanol turns on the expression of its alcohol oxidase promoter—remain a mystery. To investigate these issues further, Rangarajan has launched a new research program at the Institute. ''We are getting very exciting results,'' he says.

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