Health concerns, quality criteria and cost pressure in comparison to proteins obtained using established technologies have prompted investigations into alternative sources. **Prof. Dr. Karin Kovar** outlines the OPTYTECH Project's innovative, forward-thinking response to this challenge

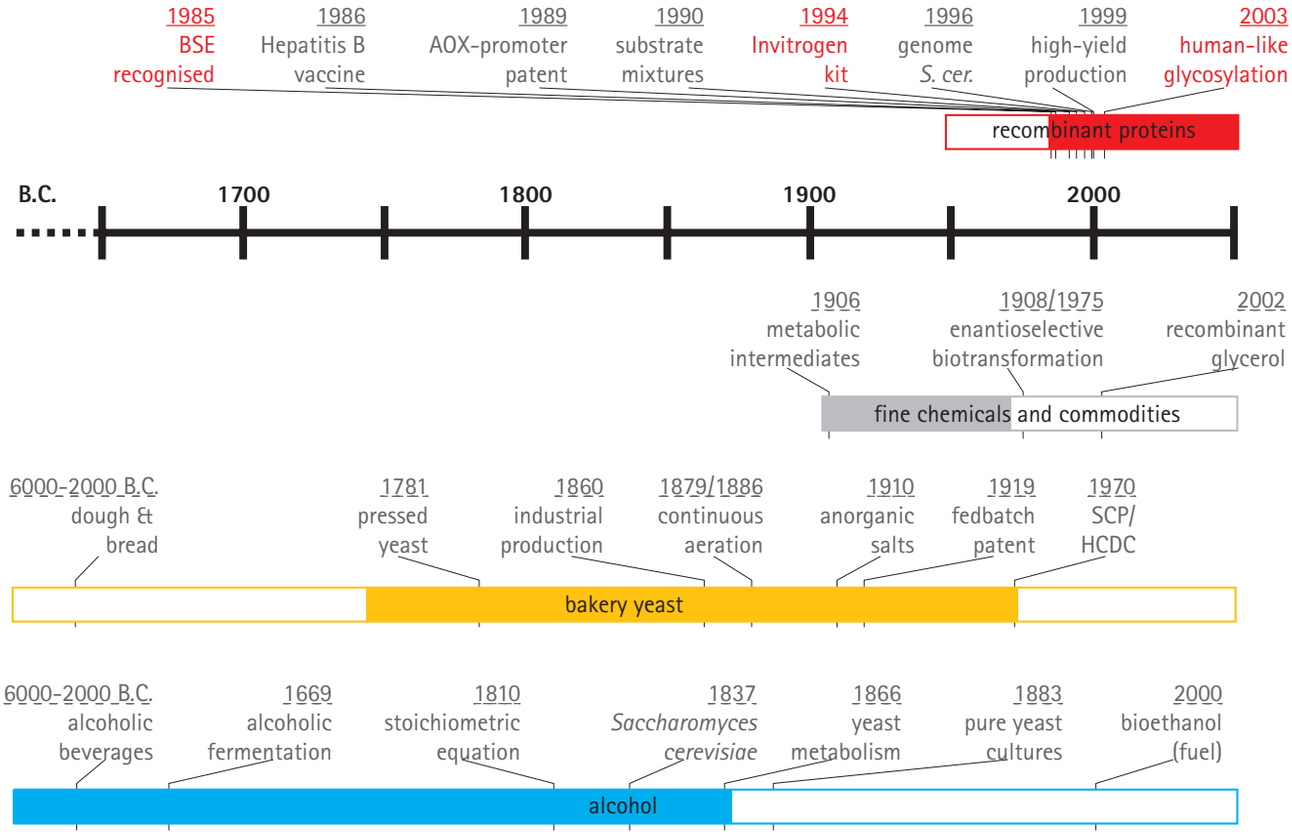
Biopharmaceuticals: why use engineered yeasts?

During close industry-university cooperation, cost-efficient and safe procedures to manufacture proteins for use in the pharmaceutical industry and in medicine have been developed. The activity initiated by the OPTYTECH Eureka-project has resulted in the establishment of a broader European research and training network, AIBY.

Why use yeasts to manufacture biopharmaceuticals?

Yeasts are a source of therapeutic and catalytic proteins (or biomass) and excreted metabolites, as well as whole cell catalysts for bioconversion reactions. This diversity, and the ease of handling when propagating yeast cultures on various scales (i.e. from the home to a large industrial bioreactor) has long attracted man's attention (Figure 1). Even the basic knowledge used when developing pharmaceutical production processes stems from a long tradition of food and feed production with yeasts.

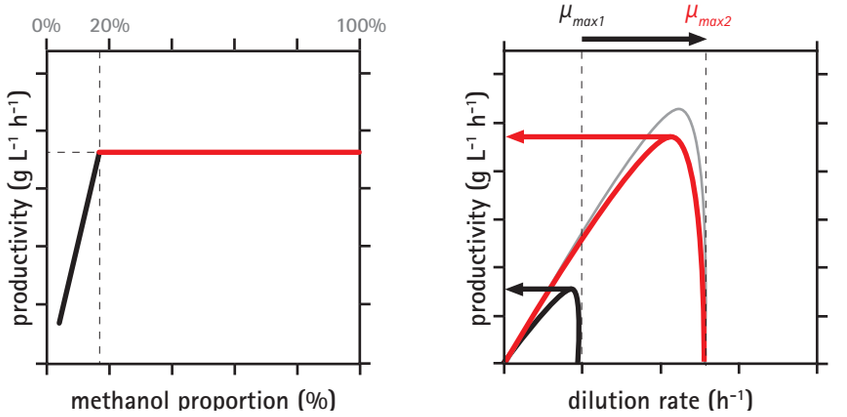
There are concerns about safety (e.g. potential contamination by transmittable animal diseases such as BSE or viruses in general) and homogenous quality when



Overview of the history of yeast use. Solid columns highlight the prosperous periods in the manufacture of a particular product

proteins for pharmaceutical applications are gained in the traditional way, from natural plant materials or slaughtered cattle, and by more elaborate means - e.g. mammalian cell culture systems. Yeasts potential as versatile, safe (i.e. GRAS affirmation, no endotoxins released), efficient expression hosts to meet market demands has been acknowledged. To exploit this, cost-effective procedures to produce properly processed, functional recombinant proteins of reproducible quality which don't cause an undesired immunogenic response are being sought.

Novel engineered yeast expression systems are suitable for secreted proteins, where typical eukaryotic posttranslational modifications like (humanised) glycosylation or disulfide bond formation are required. As, firstly, these products are currently produced using cost- and handling-intensive mammalian cell cultures and, secondly, insufficient manufacturing capacity in Europe is predicted, available manufacturing facilities for microbial (yeast and bacterial) systems could provide an alternative for some of the established and future products in the bio-pharmaceutical sector. Where process economy is a concern, yeasts thermotolerance and the ease of cultivation to (very) high cell densities with cheap mineral media are advantageous. "We recommend keeping an eye on developments in the next generation of yeast expression systems, which show great potential for further technological advancement and competitiveness in the European biotechnology sector," explains Dr. Hans-Peter Meyer of Lonza Ltd and consultant to the Swiss Commission for Technology and Innovation, CTI.



Rationale for enhanced product formation, effect of methanol to glucose ratio

Left-Effect of methanol to glucose ratio on the expression level of AOX; composition changing from 0 per cent methanol to 100 per cent (vice-versa for glucose). Right- Comparison of biomass productivity as a function of dilution rate (i.e specific growth rate) and substrate composition: 100 per cent glucose (hairline), 80 per cent: 20 per cent glucose to methanol mixture (red), 100 per cent methanol (black)

The next generation of yeast expression systems shows real potential for technological advancement in manufacturing biopharmaceuticals and making Europe more competitive

Productivity and quality enhancements

The most widespread manufacturing technology with *Pichia pastoris* is still the expression of a targeted heterologous gene under the tightly controlled *AOX1* promoter. "As the *AOX1* promoter is one of the strongest naturally occurring promoters and generally enables exceptionally high transcriptional levels to be achieved, *Pichia* production strains are much sought after in industrial manufacturing and are also an interesting target for further improvement at genetic level" (Dr. Thomas Purkarthofer, VTU Engineering GmbH).

Gene translation is induced by methanol, which is commonly applied as the only carbon substrate during production. The wide range of other substrates (e.g. glycerol, glucose, ethanol) *P. pastoris* is known to grow with are generally assumed to repress product formation when added with methanol, which triggers product formation when used alone. Although these conclusions on repressive effects have been drawn from batch cultivation data only, they are also supposed to be generally valid for other culturing modes such as the fedbatch mode frequently utilised in industry scale.

In contrast to batch (see above), in carbon-limited cultivation modes (i.e. chemostat or fedbatch cultures) these repressive carbon sources are used alongside methanol and do not negatively affect the expression of heterologous proteins, provided neither the carbon sources nor metabolites accumulate in the culture broth. For products whose formation is associated with biomass growth, improving growth performance leads to an increase in productivity, i.e. the grams of product which is formed per litre and hour. As glucose enables a specific growth rate (μ_{max}) more than twice as high as methanol and as product expression is not reduced (i.e. achieving the highest levels when the proportion of methanol in the carbon source supplied is > 20 per cent), culturing *Pichia* with a mixture of methanol and glucose may, in theory, also be favourable with respect to productivity (Figure 2). A procedure involving the carbon-limited addition of mixtures of methanol and glucose, which leads to a shortening of process duration due to higher productivities, has been developed and qualified with several heterologous enzymes, e.g. porcine and bovine trypsinogen/trypsin, horseradish peroxidise, beta-galactosidase, and selected therapeutic proteins. Further benefits over the traditional method, which involves the addition of pure methanol, are a reduction in evolved heat and enhanced process robustness, which gives some protection against disturbances in the control systems (PCS).

Depending on various factors (e.g. cultivation mode, extracellular environment, strain design), complex (or even glycosylated) products excreted by engineered yeasts may occur with different molecular variants. As such diversity can affect many of the later relevant therapeutic properties, homogenous (uniform) product quality is an increasing priority when optimising cultivation procedures. As the expressed product undergoes further degradation, not only is product composition no longer homogenous, but the formation rate of the molecular form targeted is also significantly reduced (Figure 3). A straightforward adaptation of the process control and feedstock composition has helped us avoid these undesirable product variations.

The crucial question for further research is to what extent is the physiology of (novel)

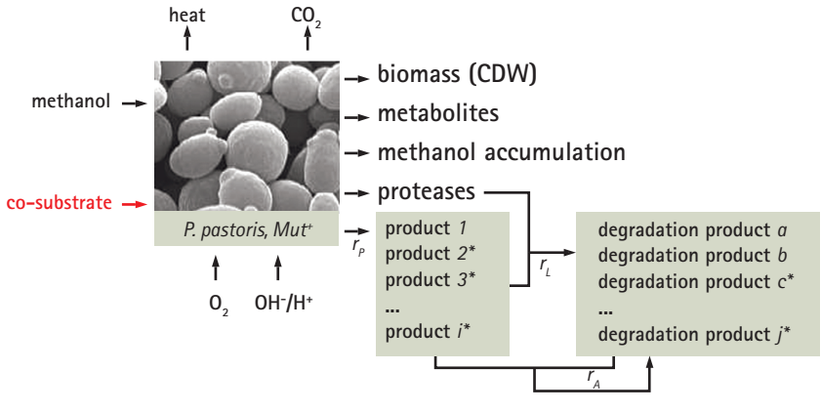
recombinant strains similar to that of wild-type strains, i.e. independent of the particular genetic modification introduced. A rapid development of biopharmaceutical processes, as required by current market conditions, could be achieved more easily if such generic knowledge were utilised directly in the development of new processes without being continually redeveloped for each newly cloned recombinant strain/product. "Rational design of high productivity processes might well be facilitated by an awareness of the gems of earlier basic research. Thus, the collateral rewards of our OPTYTECH activities are illustrated by high quality education and the sharing of teaching/learning resources in scope of the e-learning platform www.biotechLAB.net" (Prof. Ing. Karel Melzoch, Dean, Institute of Chemical Technology, Prague, Czech Republic).

Establishing the AIBY network

During the OPTYTECH (E! 3415) project partners from the Czech Republic and Switzerland jointly developed novel process strategies with recombinant *Pichia pastoris* strains for the manufacture of proteins for several applications, e.g. (1) reasonably priced therapeutic proteins, (2) enzymes to be used in the synthesis/modification of pharmaceuticals, (3) drug target proteins for structural analysis and high throughput screening, and (4) proteins for diagnostics. These strategies were implemented in industrial scale at the facilities of Lonza Ltd, a globally active Swiss company with its headquarters in Basel, Switzerland and an important microbial production site in the Czech Republic. The involvement of the universities in this endeavour was made possible by the support of the Czech Ministry of Education and the Swiss Commission for Technology and Innovation (KTI/CTI). As a complement to advanced molecular biology tools, the additional physiological studies on continuous cultures delivered valuable information for designing high-cell density manufacturing processes as well as feedback on possible directions for enhancing strain development.

The OPTYTECH project has reinforced knowledge transfer between the industry and universities, and exceeded the specific expertise, time and funds project partners could have individually contributed. "The financial support of OPTYTECH which has continued seamlessly over several years has allowed us to bring the *Pichia pastoris* system, previously established at our university for research purposes only, to the maturity required for its large industrial implementation" (Prof. Dr. Tobias Merseburger, Head of the Institute of Biotechnology, Zurich University of Applied Sciences, Wädenswil, Switzerland).

OPTYTECH also provided the impulse for a thematic symposium held in 2005, a follow-up being scheduled for May 2008 www.biotech2008.ch. At the 2005 event, not only were new partners from Austria and Belgium gained and the AIBY network (a cooperation initiative on Advances in Industrial Biotechnology of Yeasts) subsequently established in 2007, but the dissemination of knowledge to a wider audience was also made possible. ★



Main processes during the production phase

The product is formed while either methanol or a methanol and glucose mixture is continuously supplied in a carbon-limited mode. Most of the added carbon is converted into CO_2 (< 80 per cent) and biomass (> 20 per cent). The associated high oxygen demand is covered by an efficient air supply (3 vvm, pO_2 > 20 per cent) and the pH is controlled at given set points using ammonium hydroxide. High amounts of heat are transferred to maintain a constant cultivation temperature. The cells usually express the product targeted and several variants of its form (marked*), which undergo further possible degradation. The performance of these processes is described by rate of product formation (productivity, r_P), rate of proteolytic degradation (r_L) and rate of autocatalytic degradation (r_A)

At a glance

The founding partners of OPTYTECH, the initial project, are marked with an asterisk, all partners are given in alphabetical order

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Flanders Institute for Biotechnology, Ghent, B
***Institute of Biotechnology,** Zurich University of Applied Sciences, Switzerland
***Institute of Chemical Technology,** Prague, CZ
***Lonza Biotech s.r.o,** Kourim, CZ
***Lonza Ltd, Visp,** CH
Oxyrane UK Ltd, Manchester, U.K.
Research Centre Applied Biocatalysis, TU Graz, A
VTU Engineering GmbH, Grambach/Graz, A

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Prof. Dr. Karin Kovar is engaged in interdisciplinary applied research on process technology and fundamental physiological aspects of the development of biotechnological manufacturing processes.