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Session 2

Microbial Catalysis and Metabolic Engineering

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The engineering of microbial cell factories for production of fine or bulk chemicals is a multidisciplinary effort that involves genetic engineering (overexpression, deletion or introduction of genes), physiological engineering (cultivation and adaptation of the catalyst to the appropriate process conditions) and biochemical engineering (process configuration, protocols for down-stream processing etc.). Any application oriented project in the field of industrial biotechnology must involve an "omics" analysis. This does not necessarily mean the application of transcriptomics, proteomics, metabolomics, etc. but especially, and invariably, an economics analysis. As pointed out by Cameron and Lievense (Proceedings 25th Symposium p 805) any application oriented project should:

- be business driven,
- leverage existing infrastructure,
- provide for integration along the value chain,

The goals for metabolic engineering of microbial biocatalysts for industrial application are:

- *faster* (reduction of process time),
- *more* (higher final concentrations for a beneficial down-stream processing),
- *cheaper* (scale-up to appropriate size of equipment, reduce feedstock costs, reduce power input etc.).
- *more efficient* (higher yield of product on substrate and thus less by-products),
- *cleaner* (less pollution by the overall process).

All of these goals are interconnected and, when reached, lead to conditions of extreme stress for the biocatalyst: Product concentrations of over 100g.l^{-1} are by no means exceptional (production of ethanol, acetic acid, amino acids) and may even exceed 200 g.l^{-1} (production of citric acid by

Aspergillus niger ; production of gluconate salts by Gluconobacter spp; and calcium lactate by lactic acid bacteria).

Frequently the biocatalyst also has to cope with toxic substrates such as lignocellulose hydrolysates, aromatic substrates, for example benzaldehyde in the production of L-phenylacetylcarbinol (an intermediate in ephedrine synthesis) by yeast and the synthesis of β-lactam antibiotics from toxic side chain precursors by fungi and streptomycetes. Last but not least, every fermentation process finally results in limitations for one or more nutrients. It is therefore crucial to make adequate choices with respect to the wild type organism that is subjected to metabolic engineering attempts: In many cases it is much simpler to engineer pathways than to increase the resistance to stress as the latter parameter is often ill-understood. Thus, when considering biocatalysis at elevated temperatures (that often proceed at faster rates) thermophilic microorganisms should be engineered in their metabolic pathways as for example practiced by Lynd and co-workers with *Clostridia* for simultaneous saccharification and fermentation of lignocellulosics. For production of alcohol at low pH yeasts and moulds have an advantage as these organisms generally have a higher acid resistance than (sugar-consuming) bacteria. Production of lactic acid, rather than lactate salts, is more easily achieved by genetic engineering of (acid-resistant) yeasts than by increasing the acid tolerance of lactic acid bacteria, etc. This philosophy is also fundamental in attempts to develop solvent-tolerant *Pseudomonas putida* strains for the production of toxic aromatic chemicals. With the rapid growing amount of microbial genomes that are sequenced and the increasing number of microbes that become genetically accessible, it can be expected that biodiversity will deliver us with a variety of biocatalysts that exhibit a natural resistance towards the particular stress that is prevailing in a certain process. However, other considerations also apply, such as the "GMO" issue that is particularly relevant in cases of metabolic engineering of "non-conventional micro organisms" (i.e. other than *S. cerevisiae, E. coli, A. niger, T.reesei, B. subtilis*, etc.).

A powerful method in the engineering of biocatalysts for the production of chemicals is to apply selective pressure. This has been termed evolutionary engineering, despite the fact that the nature of the mutations that are enriched is nearly always unpredictable. The method has been successfully applied to genetically engineered *Saccharmoyces cerevisiae* strains for enhancing the rate of anaerobic xylose utilization. In this way (xylose isomerase-containing) yeast strains can be selected that exhibit anaerobic growth on xylose in mineral media with a growth rate (μ max) that exceeds $0.1h^{-1}$ and with high alcohol yields (i.e. without xylitol production). In this and other cases the selection of improved mutants cannot be directly connected to a particular event in transcription since the expression of several hundred genes may to be changed as a result of the selection procedure. This calls for prudence with respect to the guidance that "omics" techniques may provide in the optimization of biocatalysts for

production processes. This especially holds for the improvement of traditional biotech processes such as alcohol, citric acid and penicillin production.

For example, the first biotechnological process patented (end of 19th century) was that of citric acid production by *Aspergillus niger.* However, nowadays it is still unknown what makes a particular *A.niger* strain a good-producing biocatalyst for the conversion of glucose to citric acid. Similarly, minor but significant, improvements in the production of pencillins by *Pencillium chrysogenum* are still being made by classical mutagenesis of high-producing (industrial) strains. Presently it must be concluded that the introduction of a new pathway in a manipulatable host, complicated as it may be, seems to be easier than the optimization of an existing production strain towards the theoretical maximum product yield.

Progress in the implementation of lignocellulosic biomass as a feedstock for the production of fuels and chemicals is slow but definitely progressing towards a full-scale industrial process. Presently several pilot plants are in operation, notably the Iogen facility in Ottawa, and a Swedish plant that has been inaugurated in May 2004. No doubt ethanol will be the first product from lignocellulosic biomass that will reach the market. This relates to the fact that the down-stream processing is relatively simple: pure product can be obtained by distillation. Whether and to what extent the dirty, toxic lignocellulose hydrolysates can also be applied for the production of other (non-volatile) chemicals is still under study. In our opinion this cheap but undefined feedstock primarily offers a realistic option in case of volatile chemicals. Not only in the USA, Canada and Brazil, but also on the European continent and Japan, much emphasis is given to the addition of ethanol to petrol. The European Commission has "ordered" the addition of 2% ethanol by January 2005 and a yearly increase by 0.75% to 5.75% in 2010. In 2003, the Japanese government "permitted" the blending of ethanol with gasoline. It is therefore to be expected that international symposia on the manufacturing of fuels and chemicals from plant biomass will continue into the next decade, as will sessions on microbial catalysis and metabolic engineering, which form an integral part of such symposia.