CHIMIA 50 (1996) Nr. 9 (September)

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Lung material was spiked with high infectious titers of the rodent adapted scrapie ME 7 as model for BSE and processed through the scaled down version of the manufacturing steps. The samples were then tested in C57BL mice carrying the Sinc gene. An 18 log reduction in titer of infectious agent was observed. These results in addition to all other quality control measures and the fact that the bovine lungs are only obtained from countries which are considered to be BSE-free indicate an extremely high BSE safety factor in *Trasylol®* production.

Recombinant Factor VIII – a Glycoprotein from Perfusion Cultures of Mammalian Cells

Coagulation Factor VIII deficency is an inherited disease resulting in uncontrolled bleeding episodes in Hemophilia A patients. Treatment of Hemophilia A patients is done by applying external Factor VIII formerly obtained from human plasma and now available as recombinant Factor VIII (rFVIII). *Kogenate®* which is the tradename for rFVIII from *Bayer* represents the first worldwide licensed recombinant glycoprotein manufactured from continuous perfusion culture of genetically engineered mammalian cells. In addition, rFVIII is the first worldwide approved recombinant protein for chronic life-time treatment.

For *Kogenate®* production [2] baby-hamster-kidney 21 cells transfected with the human Factor VIII gene are used. These cells are cultured in a specifically designed serum-free medium that allows high yield expression of this complex, highly glycosilated protein. Large-scale cultivation is performed in deep-tank fermenters using a continuous, high cell density cultivation process. The suspension cultures are operated in a perfusion mode for up to six months under steady state conditions, allowing a high degree of culture control. High cell density is achieved by in house developed cell retention systems yielding up to 30-fold increased densities compared to batch or fed batch cultures. Thus, a 500 l perfusion culture produces as much rFVIII as a 15000 l batch fermenter.

The rFVIII containing harvests from fermentation are processed batch-wise through a multistep purification system including ion exchange, gelfiltration and affinity chromatography. The key step is immunadsorption chromatography using monoclonal antibodies against Factor VIII.

The antibodies are also produced in a continuous perfusion process from hybridoma cells, purified and coupled to a glass beads matrix. The rFVIII purification process is very powerful in removing host cell and other impurities from the product. The final impurity levels are in the picogram range per clinical dose for DNA and nanogram range per dose for protein impurities.

In addition the purification process has the capability to remove viruses which is achieved by a combination of virus inactivation and virus clearance of the chromatography steps. A validation study showed that the overall titer reduction was 6–12 logs depending on the model virus.

The worldwide approval of *Kogenate®* indicate that the production concept of continuous perfusion fermentation coupled with batchwise purification is an efficient alternative to batch cultures for the production of pharmaceutical proteins from mammalian cells.

[1] C.F. Gölker, M.D. Whiteman, K.-H. Gugel, R. Gilles, P. Stadler, R.M. Kovatch, D. Lister, M.H. Wisher, C. Calcagni, G.E. Hübner, *Biologicals*, in press.

[2] B.G.D. Bödeker, *Transfusion Med. Rev.* **1992**, VI, 256.

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Industrial Bioprocesses for the Production of Substituted Aromatic Heterocycles

Rainer Glöckler* and **Jean-Paul Roduit**

1. Product Range

The chemical oxidation of alkylated pyridines, pyrazines, and pyridine- and pyrazinecarboxylic acids and the ring hydroxylation of *N*-heteroaromatic carboxylic acids often leads to formation of by-products caused by non-specificity of the chemical reaction. We have developed several biotransformation reactions for the production of specifically functionalized aromatic *N*-heterocycles. For example, the regioselective enzymatic oxidation of methyl groups has been extensively studied.

1.1. *Enzymatic Oxidation of Methyl Groups*

Pseudomonas putida ATCC 33015 grown on xylene as sole carbon and energy source, oxidizes many methylated heteroaromatic five- and six-membered rings to the corresponding monocarboxylic acids (*Scheme 1*). The oxidation of 2,5-dimethylpyrazine to 5-methylpyrazine-2-carboxylic acid (MPCA) was studied in more detail resulting in a 15-m^3-scale production process reaching concentrations of 24 g MPCA/l with an analytical yield of >95% [1].

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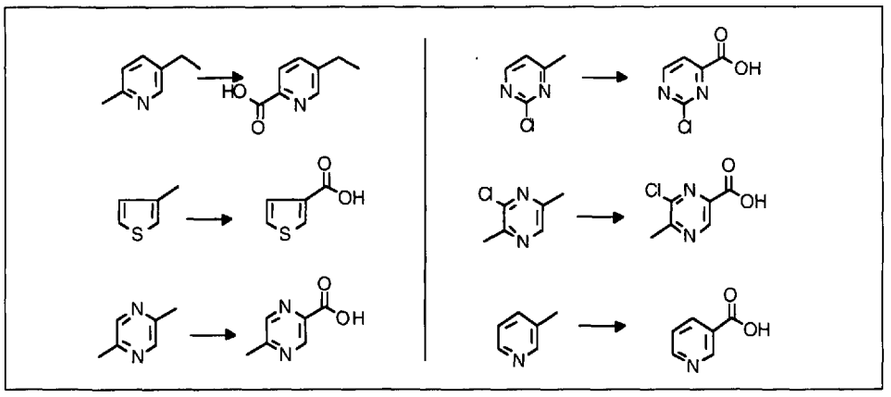
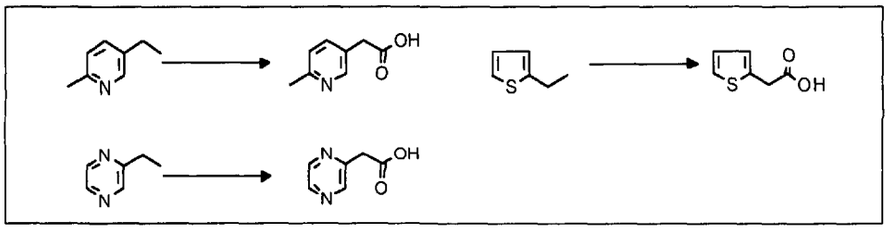
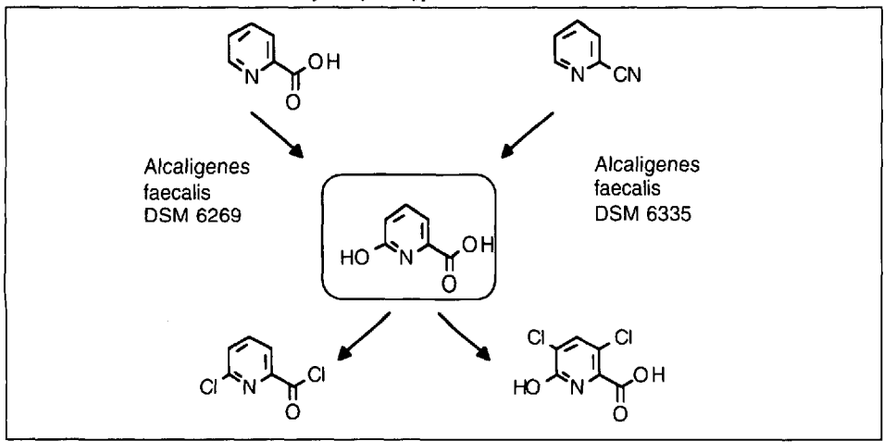
Scheme 1. *Oxidation of Heterocycles Bearing Methyl Groups to the Corresponding Heteroaromatic Carboxylic Acids with Cells of* Pseudomonas putida *ATCC 33015 Grown on Xylene*



Scheme 2. *Terminal Oxidation of Ethyl Groups at Aromatic* N*-Heterocycles with Whole Cells of* Pseudomonas oleovorans *ATCC 29347 Grown on Octane as Carbon Source*



Scheme 3. *Microbial Production of 6-Hydroxypicolinic Acid*



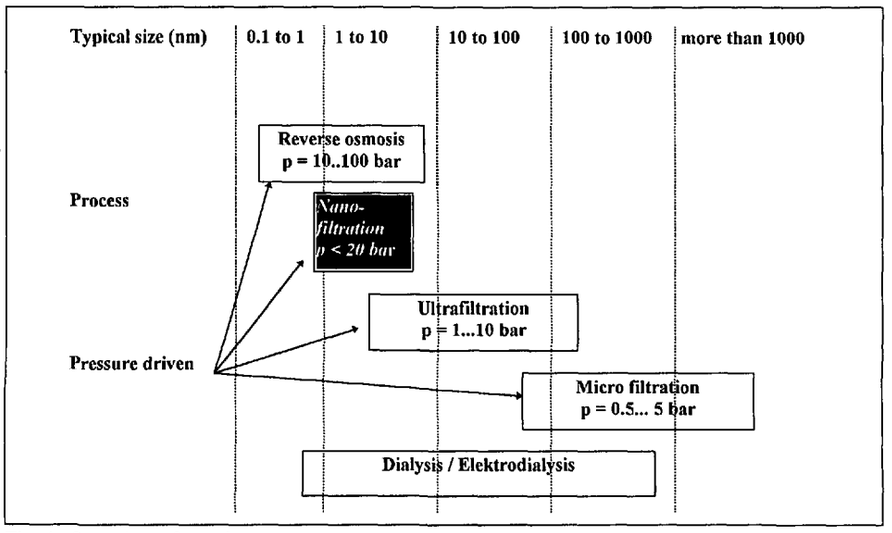


Fig. 1. *Overview of membrane processes*

1.2. *Enzymatic Oxidation of Ethyl Groups*

It has been shown that another *Pseudomonas* strain (*Ps. oleovorans* ATCC 29347), grown on octane as the sole carbon and energy source, catalyzes the specific oxidation of ethyl groups at heterocycles to the corresponding acetic-acid derivatives (*Scheme 2*). The oxidation of 5-ethyl-2-methylpyridine to (5-methyl-3-pyridyl)acetic acid demonstrates the selectivity of this type of reaction. No form of by-product with an oxidized methyl group could be detected [2].

1.3. *Selective Ring Hydroxylations*

Lonza has developed several processes for selective hydroxylation of *N*-heteroaromatic carboxylic acids (*Scheme 3*). The first example is the production of 6-hydroxypicolinic acid with *Alcaligenes faecalis* DSM 6269 starting from picolinic acid. There, we were able to produce > 98g/l in 42 h [3]. It is also possible to start from 2-cyanopyridine using *Alcaligenes faecalis* DSM 6335 as the biocatalyst [4–10]. Analogous to this process, the production of 6-hydroxynicotinic acid starting from nicotinic acid has been achieved. With *Achromobacter xylosoxydans* as biocatalyst we produced > 100 g/l of 6-hydroxynicotinic acid within 12 h [11]. We were also able to produce 6-hydroxynicotinic acid using *Agrobacterium sp* DSM 6336 grown on 3-cyanopyridine.

2. Product Inhibition

Most of the processes above described have a common problem, namely substrate or product inhibition or both. The problem of substrate inhibition can be solved with a computer-controlled feed strategy based on the on-line measurement of the starting material by FIA, HPLC, or GC [12]. This method is widely used by *Lonza* [13].

To solve the problem of product inhibition, it is necessary to remove the inhibiting product from the fermentation broth and the biocatalyst. This could be done with several methods. Here three methods are described in more detail:

- nanofiltration
- ion exchange
- reactive extraction

2.1. *Nanofiltration*

Today, membrane separation methods are becoming more and more important for the down-stream processing (DSP) of biomolecules. Dialysis is well-known in medicine in the form of haemodialysis. In industry micro- and ultrafiltration for cell

separation, electrodialysis for the separation of charged molecules, and reversed osmosis for concentration are well-established methods. The difference between the methods is the driving force. For dialysis diffusion caused by the gradient of concentration over the membrane is the driving force. Micro- and ultrafiltration are pressure-driven processes. In electrodialysis, an electrical field causes separation of charged molecules.

A few years ago, a new membrane process appeared on the marked for DSP, namely nanofiltration. In terms of the size of molecule that it can separate nanofiltration is located between reversed osmosis and ultrafiltration. The cutoff is between 200 and 2000 Da and it is also a pressure-driven process (*Fig. 1*). Separation is caused by not only size- but also by charge-exclusion because membranes can be functionalized with negative or positive charges (*Fig. 2*). Therefore, it is possible to separate inorganic salts, colored molecules and other charged substances from the non-ionic product by nanofiltration. But the pH must be adjusted so that the product will be in the isoionic form. We have developed down-stream processes based on nanofiltration for our production processes. To solve the problem of 'product inhibition' it is necessary to adapt the technology to be used in-line during fermentation.

2.2. *Ion Exchange*

An ion-exchange resin consists of an insoluble matrix, to which charged groups have been covalently attached. The bound counterions are replaced by product ions having the same charge and are retained in the column. Neutral molecules and molecules having the same charge as the functional groups of the resin are eluted from the column. Another ion is used to displace and elute the ionically attached product.

To remove the inhibiting product, it is necessary that it is bound to the ion exchanger. This was tested in *Lonza* as an ISPR method to separate 3-pyridylacetic acid from the fermentation process, resulting in a 2.5-fold longer biotransformation phase and a 20% higher productivity rate over the whole process [14] (*Fig. 3*).

2.3. *Reactive Extraction*

Reactive extraction is being studied in a cooperation between the Swiss Federal Institute of Technology in Lausanne and *Lonza* in Visp, using 3-pyridylacetic acid and other products as model substances. The result of this work is summarised in another abstract in this issue of *Chimia* [15].

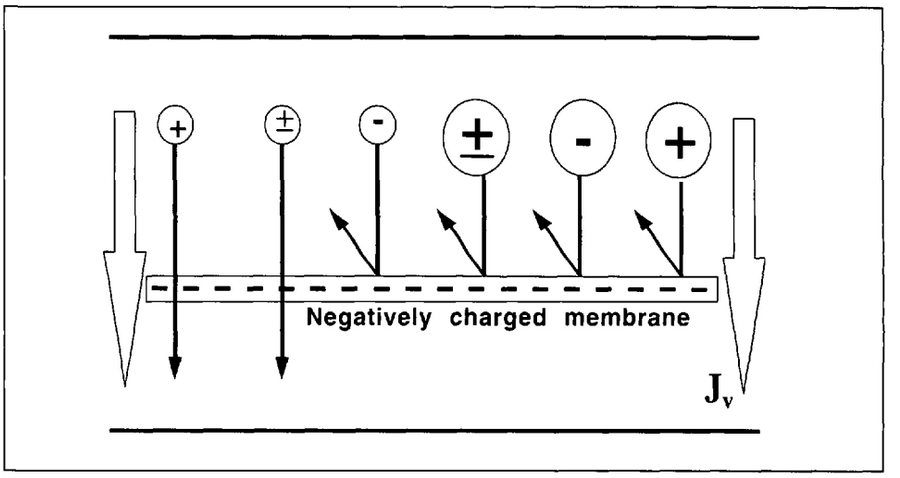


Fig. 2. *The principal of separation with charged nanofiltration membranes*

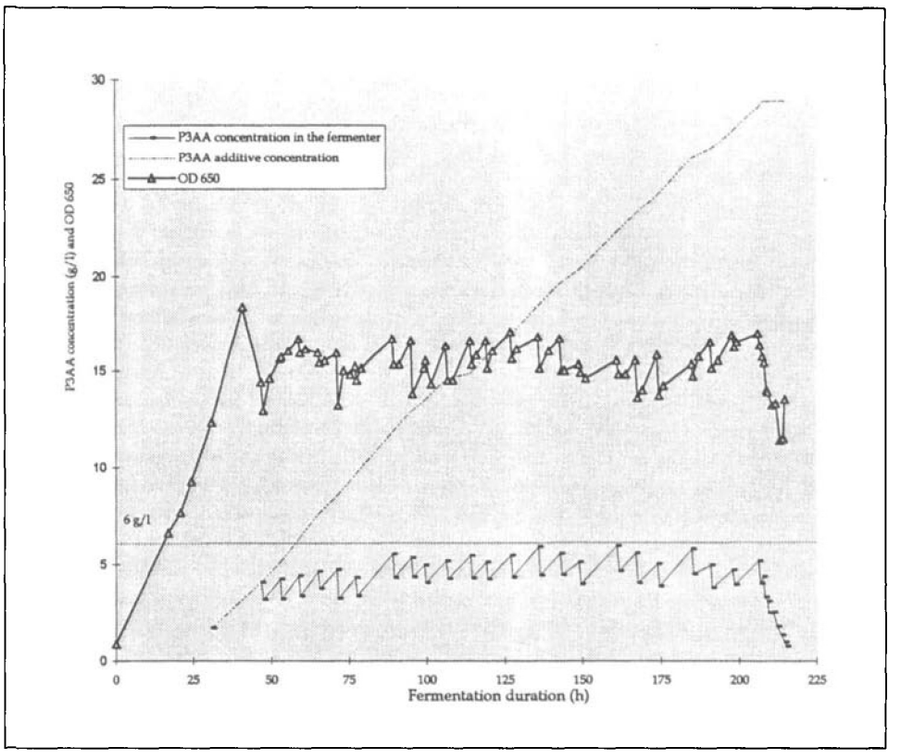


Fig. 3. *Biomass (OD650) and 3-pyridylacetic-acid (P3AA) concentration measured by HPLC in the fermenter during an ISPR experiment.* Each decrease of product concentration is due to an Amberlite IRA 416 run.

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