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4th Japanese-Swiss Meeting on Bioprocess Development, Kyoto, 28–29 November 1994

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The Fourth Japanese-Swiss Joint Meeting on Bioprocess Development took place in Kyoto from 28 to 29 November 1994. It was organized by a Japanese Committee under the chairmanship of Prof. *Hideaki Yamada* (Toyama Prefectural University) and the honorary chairman, Prof. emeritus *Saburo Fukui* (chairman of the Japanese Bioindustry Association). Some twenty scientists from Switzerland and *ca.* 50 Japanese scientists participated in the meeting. Nine speakers from Switzerland were sponsored by the Swiss Priority Programme of Biotechnology.

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It was of interest to compare the progress made in bioprocess development over the last two years, after the third meeting in Montreux (1992), the second meeting (1990) in Kyoto, and the first meeting (1988) in Interlaken have set the quality brandmark in this conference series.

Successs in bioprocess development depends on several pillars, such as the knowledge about the involved microorganisms, their genetic variations and metabolisms, molecular biological properties and engineering designs of reactors, and last but not least the contributions of analytical techniques to characterize the microorganisms, and to control fermentation and downstream processing.

Where Do We Stand – An Introduction

After the opening addresses by Prof. *Hideaki Yamada* and Dr. *Hans Georg W. Leuenberger*, Prof. *Armin Fiechter* (ETH-Zürich), the initiator of this meeting series and organizer of Meeting 1 and 3, opened the first session with his view of the European biotechnology in the mid nineties. He showed with examples from his own working group, that substantial technical innovations have been made in Europe in the field of biotechnology, *e.g.* automated microbial and mammalian cell cultures, protein free media, automated on-line analysis for metabolites and enzyme activities, cell cycle synchronization, *etc.* Such technical innovations have been successfully commercialized by manufacturers of modern biotechnology equipment. However, the transfer of new innovations into marketable products in Europe did not keep up with the situation in USA or Japan. The low acceptance of biotechnology in Europe as well as the consecutive emigration of bioindustrial activities and related investments were given as reasons. Thus, Europe contributes only a minor share to the steadily growing market of biotechnologically manufactured products, particularly pharmaceuticals. On the other hand, Europe is in a leading position in environmental industries (reduction of energy demand, alternative energies, waste water and air pollution treatment, recycling of wastes). Prof. *Fiechter* discussed the prospects of European biotechnology also taking into consideration the opportunities which will be offered by exploiting the knowledge of genome sequences of man, animals, and industrially interesting organisms.

New Enzymes

Symbiotic microorganisms have been widely ignored due to the difficulty to obtain them as pure cultures. Prof. *Teruhiko Beppu* (Nihon University) investi-

CHIMIA 49 (1995) Nr. 7/8 (Juli/August)

gated a thermophile *Symbiobacterium thermophilum,* whose propagation is absolutely dependent from a concomitantly growing thermophilic bacillus strain. A glycoprotein excreted by the *Bacillus sp.* could be identified as the factor allowing growth of the *Symbiobacterium thermophilum.* Two genes of heat-stable tryptophanes and one gene of heat-stable β-tyrosinase have been cloned from *S. thermophilium* and expressed in *E. coli.* The sequence of all three enzymes exhibit high homology with their mesophilic counterparts. However, one of the tryptophanases from *S. thermophilium* showed a distinctly different temperature profile of its catalytic activity. This example demonstrates that symbiotic microorganisms represent a source of novel useful enzymes.

Biocatalysis

In a couple of presentations the stereospecificity or the regiospecificity of enzyme catalyzed reactions was used to perform reaction steps which are hardly accessible by chemical methods:

Dr. *Marcel Wubbolts* (ETH-Zürich) presented a broadly applicable method for the preparation of optically pure epoxides from inactivated vinylic bonds. Two monooxygenases from bacterial origin were used for this purpose: Alkane hydroxylase from *Pseudomonas oleovorans*, which is able to grow on alkanes, has been used for the synthesis of optically active epoxides from terminal alkenes, alkadienes, and allyl alcohols. The same enzyme mediated also the regiospecific introduction of oxygen in alkanes resulting in alkanols, alkanediols, and alkanoic acids. On the other hand, xylene oxydase from *Pseudomonas putida* growing on toluene, xylene, and other toluene derived compounds, accepts a wide variety of *meta-* and *para*-substituted toluene derivatives as substrates and converts them into the correspoding benzyl alcohols in addition to (substituted) styrenes, which are oxydized to optically active styrene epoxides. The application of the products for the synthesis of fragrances, polymers, pharmaceuticals and the textile dye indigo has been discussed. Recombinant strains producing alkane hydrolase and xylene oxydase have been generated and utilized in two-liquid phase fermentation systems. Such systems help to reduce the toxicity of the substrates and facilitate product recovery. The two enzymes share some interesting features: Both oxidize terminal methyl groups to alcohols and vinyl groups to optically active epoxides. Both depend on the same cofactors [NADH, FE(II)], both are membrane associated and exhibit a high homology at the amino acid sequence level.

A couple of recently developed enzyme catalyzed reactions have been presented by Dr. *Hans Georg W. Leuenberger* (*F. Hoffmann-La Roche AG*, Basel). All reactions have been applied to pharmaceutical chemistry on a larger scale with the aim to introduce chirality into the molecules. Commercially available hydrolases (Lipase P, Subtilisin *Carlsberg*, Lipase OF) afforded enantioselective and in two cases also regioselective hydrolysis of racemic esters. The resulting products have been obtained with excellent optical purity and served as precursors of different pharmaceutically active compounds (thrombin inhibitors, renin inhibitors, collagenase inhibitors, sleep inducer). In a last example, (*R*)-α-hydroxy-γ-amino-butyrate has been obtained in almost optically pure form by asymmetric reduction of the corresponding *N*-substituted α-keto ester with intact cells of *Candida parapsilosis* after removal of the protecting groups. All reactions have been optimized and scaled up so that 10–100 kg amounts of optically active drug precursors could be prepared.

A report on a successful collaboration between *Kaneka Corp.* and *F. Hoffmann-La Roche Ltd.* was given by Dr. *Junzo Hasegawa* (*Kaneka Corp.*, Hyogo). *Hoffmann-La Roche* had developed a method for the production of (4*R*,6*R*)-4-hydroxy-2,2,6-trimethylcyclohexanone, a useful building block for the synthesis of (3*R*,3'*R*)-zeaxanthin. Starting from 4-oxoisophorone, this building block was obtained after stereospecific C=C reduction with Baker's yeast, followed by a chemical reduction of the sterically less hindered 4-oxo group. *Kaneka* focused their work on the improvement of the microbial reduction step and on the separation of (4*R*,6*R*)-4-hydroxy-2,2,6-trimethylcyclohexanone from its (4*S*,6*R*)-diastereoisomer which occurs after chemical reduction. *Zygosaccharomyces rouxii* and *Debaryomyces hansenii* were identified as microorganisms able to catalyze the C=C reduction of 4-oxoisophorone more efficiently than Baker's yeast. On the other hand, the separation of the diastereoisomeric hydroxy ketones could be facilitated after stereoselective esterification of the undesired (4*S*,6*R*)-diasteroisomer with maleic acid anhydride. A technical production process can benefit from these results.

Dr. *Masao Motoki* (*Ajinomoto Co.*, Kawasaki) succeeded in isolating large amounts of microbial transglutaminase from the culture supernatant of *Streptoverticillium mobaraense*. This enzyme is able to catalyze the formation of intra- and intermolecular ε-(γ-glutamyl) lysyl crosslinks in several food proteins. Thus, physical properties of food proteins (gelation capability, viscosity, emulsion stability, thermostability, *etc.*) can be improved by means of this microbial transglutaminase. This enzyme is being commercially applied to the processing of meat and fish products.

Solvent resistant microorganisms have considerable application potential as catays-sts for lipophilic substrates. Prof. *Koki Horikoshi* (Tokyo University, Kawagoe) isolated a series of toluene resistant bacteria from soil samples. Investigations were focused on strain IH-2000, which grew well in media containing high concentrations of toluene, although it did not utilize toluene as a carbon source. It was identified as a *Pseudomonas putida* strain. This strain was also tolerant with regard to other toxic solvents, including aliphatic, alicyclic, and aromatic hydrocarbons, alcohols, and ethers. The stability of the solvent tolerance was strongly stimulated by Mg^{2+} and Ca^{2+} ions. Stable toluene resistant mutants could also be obtained from several conventional solvent-sensitive *Pseudomonas* strains (spontaneous mutation and selection in presence of toluene). Furthermore, a solvent tolerant strain has been isolated from *E. coli* K12 after NTG treatment and selection in solvent containing medium. Such solvent-tolerant microorganisms have been used in organic solvents for steroid biotransformations, hydrocarbon degradation as well as for the processing of fats and oils (per-solvent fermentation).

Production of Metabolites

C_{20} Polyunsaturated fatty acids (PUFA's) are of considerable practical interest due to their biological activities. Prof. *Sakayu Shimizu* (Kyoto University) has developed processes for the production of a variety of PUFA's using the fungus *Mortierella alpina* as a producer. A production process for arachidonic acid is already run on an industrial scale with a fermentation yield of *ca.* 4 g/l medium. Various mutants which are defective in the desaturation of fatty acids were obtained from *Mortierella alpina* after spore treatment with NTG. For example, a mutant defective in delta 5 desaturase (converts dihomo-γ-linolenic acid to arachidonic acid) produced 2.5 g/l of dihomo-γ-linolenic acid. A mutant defective in delta 12 desaturase produced three different n-

9 PUFA's including mead acid (1.9 g/l). The same mutant converted α-linolenic acid to eicosapentaenoic acid. Another mutant elongated linoleic acid to form 11,14-*cis*-eicosatrienoic acid which was further desaturated to a novel C_{20} fatty acid (5,11,14-*cis*-eicosatrienoic acid) by delta 5 desaturase.

Efficient mutants derived from *Bacillus subtilis* have been developed by Dr. *Saturo Asahi* (*Takeda Ltd.*, Osaka) for the industrial production of the pyrimidine nucleosides, uridine and cytidine, which are used as building blocks for pharmaceuticals. The following strategy has been applied. A *B. subtilis* strain (No. 122) has been selected, which accumulates large amounts of ortidine and orotic acid, both intermediates in the pyrimidine synthesis. In *B. subtilis*, the synthesis of UMP was found to be regulated by uridine nucleotides and the formation of CTP by cytidine nucleotides through feedback control. Uridine producers were selected as mutants, which are resistant to uracil analogues and deficient in uridine phosphorylase activity. Strain No. 556 produced 65 g/l of uridine in 75 h in a medium containing 20% glucose. The whole biosynthetic pathway was found to be free from feedback regulation by uridine nucleotides. Furthermore, the strain is deficient in homoserine dehydrogenase activity. This deficiency seems to play an important role in the uridine production. Cytidine producing mutants were selected on the basis of the following properties: Deficiency in cytidine deaminase activity, resistance to pyrimidine analogues and deficiency in homoserine dehydrogenase activity. Strain No. 615 was free from feedback regulation by pyrimidine nucleotides and produced 30 g/l of cytidine in a medium containing 20% of glucose.

Cyclodextrins (CDs) are useful in pharmaceutical and food industries. They are produced by the action of cylodextrin glucanotransferases (CGTase) on starch. The main products are α- or β-CD (six and seven glucose units, resp.). However, there has been no CGTase which produces substantial amounts of γ-CD (eight glucose units), although this product may also be useful for industrial applications. Prof. *Takeshi Uozumi* (University of Tokyo) selected *Bacillus ohbensis*, which produces 30% β-CD and 8% γ-CD from soluble starch, and cloned its CGTase in *Escherichia coli*. However, the productivity of this enzyme in *E. coli* was only 2% of that with *B. ohbensis*. Next, he constructed an expression plasmid useful in *B. subtilis* using the promoter of *B. subtilis* cellulase gene and pUB 110 as the vector. The CGTase gene was efficiently expressed and the CGTase was secreted through its own signal peptide. Productivity was triplicated by addition of the degQ gene and reached the same level as in *B. ohbensis*. Site directed mutagenesis was applied in order to improve the productivity of γ-CD. A mutant enzyme having Trp instead of Tyr at position 188 gave the best results with 15% of γ-CD and 13% of β-CD from soluble starch after a reaction time of 4 h at almost the same productivity as the wild type enzyme. Other substitutions of the amino acid 188, which is supposed to be near the catalytic center, showed modified product specificities but in no case a higher productivity of γ-CD. Studies by X-ray crystallography are expected to further elucidate the function of amino-acid residues at position 188 and its vicinity.

Public Perception – Strategies

Dr. *Oreste Ghisalba* (*Ciba*, Basel), who acts as director of the Swiss Priority Programme of Biotechnology (SPP Biotechnology), gave an overview on the activities of this programme: SPP Biotechnology was started in 1992 with a budget of *ca.* 50 Mio. CHF. Its aims are to support innovative application oriented projects, to link basic research, applied research and process development, to bring together researchers from universities and industry, to foster education in biotechnology, to set priorities and to assure the international competitiveness of Switzerland in biotechnology. For the first period (1992–1995) the following high priority research areas (modules) have been selected:

1) Processes for the production and purification of proteins for medical applications
2) Biotechnology: Bioengineering and biocatalysis
3) Neuro-informatics
4) Bioelectronics

5A) Biotechnology information and communication

5B) Biosafety research/development of biotechnology

6) Biotechnology of higher plants.

SPP Biotechnology also comprises an education programme for postgraduate studies, postdoctoral fellowships, junior group leaders, and sabbaticals. For the second programme phase (1996–1999), an additional module on 'Food Biotechnology/Utilization of Agricultural Raw Materials' is expected to be added. A more detailed overview on SPP Biotechnlogy has recently been published in this journal (*Chimia* **1994**, *48*, 93). Work presented by nine of the speakers at this meeting was sponsored by SPP Biotechnology.

Dr. *Richard Braun* (University of Bern) compared the public perception of biotechnology in Europe with the situation in Japan and USA. Actually, Europe and Japan compare well with respect to patents filed in this field, number of active companies and employees, whereas both together are found to be overwhelmed by USA. The major handicap of the German speaking countries in Europe, in which the strongest opposition towards gene technology prevails, possibly originating from the historic experience with the Nazi-regime, was identified in the missing information about and general discussion of benefits and potential risks. Interestingly, gene technology for medical applications has never been a target of criticism, and gene therapy is not prohibited. In the meantime, the European Federation of Biotechnology (EFB) and individuals in various countries have taken notice of this problem and targeted measures are being set to actively promote industrial biotechnology, *e.g.* by an EFB task group on public perception, the SAGB in Brussels, DFG in Germany, or the SPP in Switzerland. Emotional argumentation is one aspect, *e.g.* used by Gen Suisse; activities including posters, exhibitions, public meetings, brochure, debates or talks are other now. We need the science and technology to solve many of today's problems!

Dr. *Othmar Käppeli* (BATS, Basel) outlined the goals of the Agency for Biosafety Research and Assessment of Technology Impacts (BATS). This institution was opened in 1993 and devotes its efforts to the coordination of biosafety research in Switzerland. The activities include documentation and transfer of relevant knowledge concerning biosafety and technology impacts. In so doing, it acquires and communicates data on biotechnology benefits and impact. It establishes and maintains an information and documentation database, which covers international biosafety research projects and scientific literature, regulatory prededures, international meeting reports, and technology impact assessment studies. Furthermore, BATS makes available scientific information on biosafety and technology impacts, by elaborating, reviewing and summarizing scientific knowledge on particular topics, distributing the corresponding scientific background, teaching, and organizing meetings.

CHIMIA 49 (1995) Nr. 7/8 (Juli/August)

Applied Genetics

After characterization of the chloroplast NDA of a liverwort, *Marchantia*, Prof. *Kamji Ohyama* (Kyoto University) has now identified 32 introns in the mitochondrial DNA of this organism. Several introns interrupt the gene coding for a cytochrome oxidase subunit at sites identical to fungal mitochondrial introns. Type I and type II introns can encode proteins, *e.g.* RNA maturase. Nearly 50% of the type II introns can encode sequences highly related to maturase. Between 40 and 50% similarity was determined with maturases from *Neurospora* or *Podospora*. These results strongly suggest that the maturase genes have been horizontally transferred between plants and fungi, and are not necessarily vertically inherited from a common ancestor only.

Genome Analysis Programme

The co-ordinator, responsible for chromosome XIV of the yeast genome analysis program of Europe, Japan, USA, and Canada, Dr. *Peter Philippsen* (Biocenter, University of Basel) reported very positively on the progress, and sketched the further work to be done. He expects the 1.4×10^7 base pair long genomic sequence containing probably 6000 to 7000 protein coding genes to be finished by late 1996, since the present speed is approximately 10^4 base pairs per day. Chromosomes I to III, VI, VIII, and XI are already sequenced, and almost 50% of the genes are completely new, only some 20% display motifs indicating certain functions. This poses a major challenge on this programme and the problems are thought to be solved by exploiting bioinformatics, sound human sense (*e.g.* visual inspection of sequences), and a lot of experimental work in the lab, such as analyzing gene disruptions, construction of synthetic lethalities or 2-hybrid systems. Moreover, one tries to define families of putative genes and to group interacting proteins. However, it must be expected that the functional analysis will be significantly more expensive than the sequencing programme. But this is the pressing issue: To know more about and to be able to tune a host organism.

Host Organisms

Prof. *Mitsuyoshi Ueda* (Kyoto University) tries to improve the host organism *Saccharomyces* by exploiting new functional promoters. Unfortunately, the several promoters reported as practical in this yeast are derived from genes involved in glycolysis; they required relatively expensive inducers. Genes encoding peroxisomal enzymes in *Candida tropicalis* were higly expressed when cloned in *S. cerevisiae*, accounting for up to 30% of extractable protein. This means that new powerful promoters useful in *S. cerevisiae* have been found, which can be induced by cheap sources such as acetate. For instance, under the promoter of isocitrate lyase from *C. tropicalis*, β-galactosidase from *E. coli*, catalase from *C. tropicalis*, or glutamic acid decarboxylase from rat brain have been successfully expressed in *S. cerevisiae*; further modifications like omission of the terminator resulted in up to 100-fold higher activity when compared with the parent organisms. This leads to the expectation that Baker's yeast will rapidly develop as a top performance host organism.

Enzymes, Proteins, and Cells

The stereochemistry of reactions catalyzed by D-amino-acid transferases was in the focus of the presentation of Prof. *Kenji Soda* (Kyoto University). He and his group found a thermo-stable enzyme, abundantly produced by a newly isolated thermophilic *Bacillus*. This enzyme and BCAT from *E. coli*, are so far unique with respect to their primary and 3-D structure, as well as their stereospecificity for a *re*-face C(4') proton transfer. This possibly reflects the molecular evolution of aminotransferases. In this new enzyme, a large *N*-terminal domain is linked to a smaller *C*-terminal one with a hingy peptide. There are strong indications that the *C*-terminal works just as a chaperon.

An interesting approach to ameliorate α-glucan-phosphorylases from various plant tissues (potato leafs and tubers) for an industrial conversion of glycans like amylose, amylopectin, maltodextrins, or glycogen was reported by Prof. *Toshio Fukui* (Osaka University). The problem is that the nature enzymes' affinities, *e.g.* for glycogen, vary by more than three orders of magnitude. A chimerical construct of the type-I isozyme carrying an equivalent insert from a type-H isozyme from potato (close to the active site of the enzyme) showed a significantly improved affinity for glucans. Another chimerical construct with an insert from rabbit muscle enzyme showed even higher affinity to a wide variety of glucans, thus broadening the substrate spectrum considerably. These constructions rendered the enzymes' affinity flexible. These enzymes are stable over years and can be used in immobilized form for direct production of glucose-1-phosphate; unreacted starting materials are recycled and the product is concomitantly purified simply by ultrafiltration and electrodialysis.

Monclonal secretory IgA antibodies for use as a passive vaccine for the protection of mucosal surfaces have been discussed by two researchers from Lausanne: Dr. *Lorenz Rindlisbacher* (University of Lausanne) reported on the characterization and improvement of the antibodies, whereas the production has been discussed by Dr. *Ian Marison* (EPF-Lausanne). The production is tricky because these antibodies are of dimeric nature, linked together by the so-called J chain, and the secretory protein component which derives from polymeric immunoglobulin receptor upon cleavage. The molecule is highly glycosylated. An application of the mixture of the individual components has not been successful. In the meantime, several different chimerical sIGAs have been constructed, and are expressed either in insect or mammalian cell lines (*baculo-* or *vaccinia* virus recombinants). Among several reactor types tested (mice *in vivo*, T-flasks, CSTRs in batch and fed-batch mode), the hollow fiber reactors outperformed all others by virtue of achievable IgA concentration and volumetric productivity; however, the active fraction of product was as low as 34%. It was found important to monitor and control the concentrations of glucose, several amino acids and ammonium ions. The on-line measurements have been accomplished by using FIA and HPLC. Ammonium ions have been effectively removed by applying a pH gradient across a hydrophobic membrane *in situ*, while the same membrane could be exploited for bubble free oxygen supply to the culture. Such a membrane can be reportedly applied either in an external loop, as a flat (reactor bottom) membrane, or in tubular form. Satisfactory purification is performed using Ni-chelat and Concanavalin A affinity chromatography.

CHO cells cultivated in serum- and protein-free medium are exploited as research object to study the effects of various growth factors by Prof. *Hans M. Eppenberger* (ETH-Zürich). Only such a defined culture system allows causal-analytical interpretation of results. Insulin or basic fibroblast growth factor stimulate cultures and induce a morphological change. Expression levels of cyclin E are significantly elevated in such cultures. (Wild type) CHO cells, when transfected to express cyclin E constitutively, reached

a specific growth rate of 0.8 d^{-1} in protein free basal medium. These cells show the same qualitative behavior as bFGF stimulated cells, but cyclin E was found to be twice as effective. Further research will be directed towards deliberately switching cyclin E on and off by controllable additions to the medium, *e.g.* tetracycline. This may open the way to control the proliferation of cells reproducibly and predictably.

Dr. *Toshio Kokubo* (IRL, Takarazuka) presented some work on an *in vitro* amidation process for the preparation of α-amidated peptides. Many biologically active peptides have the α-amide structure at their *C*-terminal. These cannot be directly produced with microorganisms by means of recombinant DNA. It is known that an endopeptidase specific for a proline residue, prolyl endopeptidase (PEP), is a very useful catalyst for the enzymatic coupling to prepare α-amidated peptides. Since PEP cleaves a peptide specifically at the *C*-terminal side of a proline residue, it can catalyze condensation in the reverse manner of the hydrolysis. However, to have industrial significance, its thermostability had to be improved. A gene of PEP was cloned from *Flavobacterium meningosepticum* and expressed in *E. coli* under the control of a strong promoter, *tac*. The expression level reached 110 mg/l protein by induction with IPTG. The engineering of PEP was achieved by random mutagenesis with chemical mutagens and following screening for a thermostable mutant of PEP. After conversion to the duplex form, the mutated gene was subcloned back into the original plasmid to give a library of PEP mutants, which were screened for residual activities after heat treatment. The gene isolated from the clone was found to have base substitution at two positions. This thermostable mutant of PEP was employed to catalyze the *in vitro* amidation of LH-RH and oxytocin precursors.

Prof. *Hidehiko Kumagai* (Kyoto University) described a procedure to produce L-DOPA by tyrosine phenol lyase. Tyrosine phenol lyase (TPL) is a pyridoxal phosphate(PLP)-dependent enzyme and catalyzes α-, β-elimination, β-replacement, reverse of α-, β-elimiation and racemization reactions of tyrosine and its derivative amino acids. TPLs were purified from cells of *Escherichia intermedia* and *Erwinia herbicola*. TPLs can catalyze the synthesis of L-DOPA from pyrocatechol, pyruvate and ammonia, whereas L-DOPA is a useful drug for the treatment of *Parkinson*isms. The author reported on work to clone the gene that codes for TPL of *E. herbicola* and the determination of its nucleotide sequence. The deduced protein product was 456 residues long with a molecular weight of 51364 Da.

Prof. *Horst Vogel* (EPF-Lausanne) reported on the controlled incorporation of polypetides and proteins into lipid mono- and bilayers on solid support, providing important understanding of the structural organization of membrane components. This information can be used to design biosensors and molecular electronics. Specifically, the author described the formation of two-dimensional microscale structures on solid surfaces. A new class of synthetic lipids is used, so that the lipids covalently bind to a gold surface *via* their disulfide bridge. The structures are built by spreading a mixture of conventional and sulfur-bearing lipids on the water surface of a *Langmuir* trough. The monolayer phase separates to give domains of conventional lipids. The size of the domain depends only on the self-organization properties of the mixture and its manipulation on the water surface. The film is subsequently transferred to a gold substrate, where the thiolipids bind covalently to the support. The conventional lipid can be washed away to leave domains of bare gold. In these regions polypetides and proteins can be addressed through a subsequent self-assembly step while completing the lipid monolayer to a bilayer. Two examples were reported, one involving antigenic peptides by terminal gold-attached cysteins, demonstrating the patterned antibody binding to surfaces, and the other involved natural neuroreceptors forming a functional active channel protein on solid supports.

Polysaccharides

Dr. *Kazuya Otsuji* (*Kao Corp.*, Ibaraki) presented work on polysaccharide production by cultured cells of *Polianthes tuberosa*. Only a few industrial production procedures are known to produce useful metabolites by plant cell cultures. One such process has been described by the author, involving the mass production of polysaccharides by liquid cultures of *Polianthes tuberosa* cells. The report was focused on the effects of plant growth regulators and viscosity of the culture medium on the production of polysaccharides, the chemical structure of the polysaccharides, and their use in a cosmetic product.

Application of Proteins

Prof. *Masashige Shinkai* (Nagoya University) presented a promising approach for production and medical application of submicroscopic antibody-conjugated magnetic particles. 5–35 nm large magnetites were coated and the tumor specific antibodies (colonic cancer or human glioma) were attached *via* their carbohydrate chains. Such magnetoliposomes can be stably stored when cooled. When applied to tumor cells, they are incorporated in 3–12 times greater amounts than by non-targeted control cells. Upon exposure of such loaded cells to a magnetic field, the larger magnetites were the better heaters and evolved > 100 W g^{-1} in a 240 kHz field at > 20 kA m^{-1}, or raised the temperature of the unperfused tissue to 56° within 10 min. There is at least two-fold application of the targeted particles in diagnosis as well as in therapy: Localization of the target, *e.g.* by magnetic resonance imaging (MRI), and direct hyperthermal treatment of targeted tumor tissues; in the latter case, the attainable temperature depends greatly on the blood flow rate.

Clinical Applications

Dr. *Kazuo Aisaka* (*Kyowa Hakko Kogyo Co., Ltd.*, Tokyo) outlined the importance of enzymatic analysis in clinical chemistry. Based on the enzymatic oxidation of cholesterol with cholesterol oxidase and the possibility to quantitate hydrogen peroxide with high sensitivity, *Kyowa Hakko Kogyo Co.* commercialized a kit for the determination of serum cholesterol. In order to improve the productivity of cholesterol oxidase, genetic engineering techniques were applied, and the company succeeded in the hyperproduction of cholesterol oxidase using *Streptomyces lividans* host-vector systems. The CHOD-encoding gene from *B. sterolicum* was cloned by colony hybridization in *E. coli*. The CHOD gene was then expressed in *Streptomyces lividans*, resulting in a 20-fold secretion of the enzyme as compared with *B. sterolicum*. In the course of the studies, another type of cholesterol oxidase (CHOD-II) was found. The expression cloning was carried out by searching halo-forming clones on cholesterol plates using a *E. coli* host-vector system. The amino-acid sequence of CHOD-II was completely different from that of CHOD-I. The author described tests to differentiate between CHOD-I and CHOD-II, and it was found that CHOD-II was more stable than CHOD-I. A new kit is now available,

with a number of performance improvements.

Engineering

Oxygen supply is often the limiting factor in industrial fermentations. One approach to overcome this problem is to improve the bioreactor engineering and the aeration systems. Dr. *Pauli T. Kallio* (ETH-Zürich) reported on another successful approach: Improvement of the oxygen utilization efficiency by genetic manipulation of the host organism. Increased bioprocess productivities have been obtained by expressing a gene encoding a hemoprotein (VHb) from *Vitreoscilla sp.* in various host organisms. VHb binds the available oxygen and makes it available for biosynthetic tasks. VHb-expressing *E. coli* cells reached under microaerobic conditions approximately 40% higher cell density than the wild type strain and enhanced production of recombinant proteins. Intracellular expression of VHb in *Streptomyces coelicolor* increased the production of actinorhodin 10-fold and cephalosporin C production was increased 4-fold when VHb was expressed in *Acremonium chrysogenum*. VHb expression in *Corynebacterium glutamicum* enhanced the yield of L-lysine by *ca.* 30%. Furthermore, VHb was also expressed in higher organisms as *Saccharomyces cerevisiae* (30% increase in ethanol production) and recombinant Chinese hamster ovary (CHO) cells (40–100% increase in tPA production). Possible mechanisms and models of VHb action have been discussed.

A new bioreactor with **re**movable, **s**terilizable **rot**ating **d**iscs (RESTROD) for the investigation and exploitation of microbial biofilms has been promoted by Dr. *Paul Péringer* (EPF-Lausanne). A major application field is the degradation of xenobiotics under monoseptic conditions. He described the degradation of *p*-toluenesulfonate as recalcitrant model compound with a *Comamonas testosteroni* strain. Fixed biomass processes are strong with respect to high biomass concentration, high efficiency and stability, resistance to toxicity and compactness of the process. The new reactor allows complete bulk mixing with controlled, uniform shear stress, cultivation with constant specific growth rate, and easy sampling of the biofilm. The three reactors, operated in parallel, were fed at a dilution rate of 0.5 h^{-1} in order to minimize growth of suspended cells; the maximum specific growth rate of the organism was 0.37 h^{-1} at the conditions chosen to mimic real contaminated effluents, and the thickness of the biofilm reached 200–250 µm after some 25 d, while the liquid phase composition stabilized already 7 d earlier. The reactor proved useful for studying augmentation of biofilms, resident and invading biofilm microbes and population distribution.

An alternative scale-up criterion, namely constant fluid velocity distribution patterns, was reported by Dr. *Hideo Tsujimura* (*Suntory Ltd.*, Osaka) to be more powerful than constant k_La or P/V for aerobic, shear sensitive mycelial microbes. Two successful examples were the peroxidase and arachidonic acid production with *Arthromyces* and *Mortierella*, respectively. The new method consists of simulating local velocity distribution profiles, classifying the spaces according to velocities (in four classes) and calculating the respective mass-, motion-, and energy balances for reactors at different scale. In both paradigmatic cases, the productivities at the 10 m^3 scale were equal or greater than at the 10 l lab-scale. It could be shown by reference experiments that this concept was superior to the classical criteria of either constant impeller tip speed or of constant k_La.

Rapid dynamics of substrate consumption of microbes such as *Saccharomyces* and *Escherichia* have been reported and interpreted in terms of a dynamic kinetic model by Dr. *Bernhard Sonnleitner* (ETH-Zürich). The experimental evidence for short termed substrate accumulation in the supernatant, overflow metabolite excretion and re-consumption based on the development of automated, rapid, high-resolution on-line analytical tools and high performance bioreactor equipment. A dynamic extension of the *Monod*-type substrate consumption kinetics reflected an inherent economical strategy of microbes to save energy: Only when required by an increased extracellular substrate supply, cells start to express the necessary surplus of enzymes to process the extra substrate. This delay results in an overshoot of substrate concentration, whereas decrease of substrate supply ends in immediate extracellular limitation. One conclusion is that macroscopic steady states can exist unless cells are in physiological steady state; this has, of course, significant influence on the design of monitoring and control tools. Inconstant operation of bioprocesses is expected to allow more productive and stable cultures than constant operation, because cells are forced to express higher levels of enzymatic activities.

An elegant solution to a general problem of process development, namely limitation in time, was provided by Dr. *Claude A. Chassin* (*Lonza Inc.*, Basel). Two examples of enzymatic and whole cell catalyzed processes, originally not considered to be economically feasible, were given to describe the successful strategy: Modeling is considered a much more powerful tool than purely empirical procedures to design a process whenever only few quantitative data characterizing the reaction kinetics are available along with engineering constraints. The modeling circle used was quite simple: Verbal and mathematical formulation of the relevant states and effectors, model simulation, parameter sensitivity testing and experimental verification of predictions are straightforward, and drastically reduce both, development time and costs of process design. Understanding the process is forced by this approach and this pays back on the production scale, since it is crucial for quality maintenance.

The isolation of high value biopharmaceutical products from fermentation broths usually requires several time-consuming separation steps. Prof. *Heinz B. Winzeler* (Polytechnic School, Winterthur) described a special membrane separation system, offering new opportunities to simplify downstream processing. The most prominent problem in membrane filtration is the accumulation of retained material, forming a secondary membrane with subsequent fouling, which leads to a decline in flux with time. Mass transfer through membranes with fluid mechanics is a relatively new field of theoretical and experimental exploration to improve the mass transfer. A spiral channel configuration induces *Dean* vortices to control concentration polarization and fouling on the membrane surface, and results in a constant mixing throughout the flow path. A single channel module had been developed in cooperation with *Bioengineering Ltd.* Scale-up is simple and requires stacking of seveal spiral plates. One of the many benefits of the system is that it has no moving parts or seals.

Analytical Methods Used in Biotechnology

Several analytical techniques and their biotechnological applications were presented at this meeting.

Dr. *Andreas Engel* (Biocenter, Basel) explained the need for alternative analytical tools to study membrane protein structures. Scanning probe microscopy (SPM)

CHIMIA 49 (1995) Nr. 7/8 (Juli/August)

is a relatively new method to study membrane proteins in their native environment, *i.e.*, the lipid layer and buffer solution. Atomic force microscopy (AFM) is used to measure the force between the scanning tip of the instrument and the sample surface, and exploits this signal to trace the surface with a servo system. Atomic-scale resolution, *i.e.*, 1 nm lateral and 0.1 nm vertical resolution, allows to visualize structural changes of proteins which can be correlated with their biological activity. Examples from the literature were cited. In one case, cholera toxin bound to ganglioside integrated in a bilayer, as well as crystalline membranes reconstituted from *E.coli* OmpF porin and phospholipids were adsorbed to freshly cleaved mica and imaged in solution by AFM. In particular, the extracellular and the periplasmic side of the porin trimers could be identified.

Prof. *Isao Karube* (University of Tokyo) reported on studies to develop biosensors for process and environmental monitoring. He described a fish freshness sensor, the results of which favorably compared with HPLC investigations. Novel biosensors, based on amylase hydrolysis and maltose sensors, combined with the appropriate fluid handling system, were used for the measurement of damaged starch in flour. *Karube* also described a biochemical oxygen demand (BOD) sensor, incorporating an electron mediator without an oxygen electrode. Furthermore, three new sensors were discussed to measure very small amounts of cyanide in river water. The first type uses a two-step enzyme reaction (rhodanase, sulfite oxydase) and a luminol reaction; the second type is based on the fact, that respiration activity of *Saccharomyces cerevisiae* is inhibited by cyanide ions; and the third type uses the inhibition of luminol chemiluminescence by cyanide ions.

Dr. *Ursula E. Spichiger* (Technopark Zürich) outlined the newest trends in electrochemical sensor development. The coupling of redox enzymes to amperometric electrodes is a well-established technique for the preparation of biosensors. This approach combines the simplicity of amperometric designs with the substrate selectivity or specificity of an enzyme, resulting in cost-effective systems. Amperometric electrodes are especially suited to design miniaturized sensors. Some examples have been discussed, among them xanthine oxidase (XOD), peroxidase (POD), lipophilized with caprylic aldehyde, and sulfite oxidase (SO) based sensors. The detection limit for hypoxanthine is at 10 nM, whereas the response time (t_{95}) is *ca.* 10 s. The sensor discriminates ascorbate and therefore is recommended for food investigations. The mediator is certified as being safe.

Ursula Spichiger proposed the development of optical biosensors, so-called biooptodes.

Prof. *H. Michael Widmer* (*Ciba Ltd.*, Basel) reported on analytical developments to characterize proteins, agglomerates and cells by field-flow fractionation (FFF). FFF is a relatively new pseudochromatographic method, capable of separating and analyzing a broad spectrum of samples, ranging from *ca.* 5 kDa to more than 10^{15} kDa. The main advantage of FFF is its characteristics to separate analytes without the need for a stationary phase, thereby avoiding surface exposure. He described a fully automated asymmetrical flow FFF system, built from standard HPLC equipment. This system allows to study the formation of protein agglomerates during production and storage of pharmaceuticals. In a cultivation processes of hybridoma cells, producing immunoglobuline G (IgG), the production of IgG was monitored. The results compare favorably with the results obtained from ELISA experiments. Finally, the degree of chemical conjugation of a toxin to an antibody as a function of cross-linkers attached to the toxin was determined by asymmetrical flow FFF, and the molar ratio of toxin and antibody was derived from these measurements.

The meeting in Kyoto was of high quality, both parties could benefit from the mutual exchange of experience in the field of bioprocess development. In summary, it may be stated that the Japanese researchers are very strong in the practical application of bioprocesses and they enjoy a larger variety of commercialized products than the Swiss counterparts. However, there are areas, where the Swiss still contribute significantly to bioprocess development, particularly in the field of engineering devices and the analytical aspects of biotechnology.