Biotechnology for fats & oils

The World Conference on Biotechnology for the Fats and Oils Industry will get under way Sunday, Sept. 27, 1987, in Hamburg, West Germany, at the Congress Centrum. The conference will run through Friday, Oct. 2, 1987.

The conference is designed to provide discussion of current and potential applications of biotechnology to the fats and oils industries. AOCS and the German Society for Fat Research (DGF) are primary organizers, with additional support provided by the Japan Oil Chemists' Society, Institute des Corps Gras and Stazione Sperimentale degli Oli e dei Grassi. The DGF will hold its annual meeting Wednesday, Sept. 30, and Thursday, Oct. 1, in conjunction with the world conference.

The prestigious Normann Medal will be awarded during a joint ceremony of the world conference and the DGF meeting on Sept. 30. World conference registrants will be able to attend DGF technical sessions; however, German will be used almost exclusively at the DGF meeting. English is the official language for the world conference.

Speakers at the opening ceremonies Monday morning, Sept. 28, will include Klaus von Dohnanyi, First Mayor of Hamburg, and Ministerial Director Kurt Eisenkramer, representing the Minister of Agriculture. Keynote talks opening the plenary program Monday will be given by P.K. Stumpf of the University of California at Davis, USA; Colin Ratledge, University of Hull, England, and Tsuneo Yamane of Nagoya University, Japan. The program will include six technical sessions extending through Friday.

The conference also will include an international exposition by companies that provide services, equipment and supplies to the industry. The exhibition will open Sunday, Sept. 27, and continue through Thursday. A series of coffee breaks and social events will provide opportunity for informal conversation among registrants and speakers. Spouses and other guests may register for a program that includes the all-conference social events and other activities.

Abstracts for the conference are published in this issue of *JAOCS*, beginning on page 1242.

Organizing committees

The following persons have served on the committee that developed the program for the world conference: T.H. Applewhite; A.R. Baldwin; R.K. Downey, Agri Canada Research Station; K.F. Gander, Unilever Research; A.M. Gavin, EMI EX/IM Inc.; I. Hara, Toyo Soda Mfg. Co.; S. Hayano, Japan Oil Chemists' Society; A.G. Hildebrandt, Bundesgesundheitsamt; Y. Karube, Tokyo Institute of Technology; W.W. Klassen, BARC/ARS, U.S. Department of Agriculture (USDA); J. Lyon, AOCS; A. Macrae, Unilever Research; T.L. Mounts USDA's Northern Regional Research Center (NRRC); S.L. Neidleman, Cetus Corp.; J. Ohlrogge, USDA's NRRC; L.H. Princen, USDA's NRRC; C. Ratledge, University of Hull; J. Rattray, University of Guelph; H.J. Rehm, Universität Münster; G. Robbelen, Georg-August University; R.D. Schmid, Henkel KGaA; K. Smith, American Soybean Association; C. Wandrey, Institute Biotechnology, Kernforschungsanlage; B. Werdelmann, Henkel KGaA; T. Yamane, Nagoya University, and T. Yoshida, Tokyo University of Fisheries.

Serving on the executive committee for the conference are T.H. Applewhite, chairman; Karl F. Gander, co-chairman; H. Bruning, DGF executive secretary, and Jim Lyon, AOCS executive director.

Social events

World conference registrants may take part in a number of social events. The first event will be an opening reception Sunday evening, Sept. 27, from 5:30 to 7 p.m., in the exhibit area at the Congress Centrum Hamburg.

On Monday, the city of Hamburg will host a reception in the Town Hall from 6 to 7:30 p.m., open to all meeting registrants. On Tuesday evening, an organ concert, open to everyone, will begin at 7 p.m. in the Petri Church. Thow who purchase tickets can then attend a German evening with dinner, drinks and entertainment, at the Fleetenkieker.

For those purchasing tickets, a more formal dinner-dance will be held Wednesday evening at the famed Atlantic Hotel. The event will start at 7 p.m. On Thursday, there will be an optional evening boat trip. The boat will leave at 5:30 p.m. for a trip on the Elbe River; a buffet dinner will be provided on board.

Business attire will be proper for the Sunday, Monday and Wednesday evening events; more casual attire is acceptable for Tuesday and Thursday evening events.

Spouses' program

Registrants for the spouses' program are invited to participate in the all-conference social events as well as the Spouses' Program, and may purchase tickets for any of the optional social events.

On Monday, Sept. 28, there will be a city tour during the morning and a visit to the antique center. The afternoon will be free for shopping.

Tuesday morning, participants will board a boat for a trip on Alster Lake and surrounding waterways. The day's tour will include the old warehouse district and a visit to the open-air museum in Kiekeberg. Lunch will be included. The return will be via the free port.

Wednesday will be an open day, while Thursday will include a visit to one of the most ancient areas in West Germany, the city of Luneberg, where participants will tour the cloisters and the Kronen Brewery museum. Lunch is included.

Program at a Glance

Sunday, September 27, 1987

14.00–18.00	Registration–Congress Centrum Hamburg
17.30–19.00	All-Conference Opening Mixer–Congress Centrum Hamburg. Exhibits will be open.

Monday, September 28, 1987

08.00–17.00	Registration–Congress Centrum Hamburg
08.45–09.40	Opening Ceremony
09.40–10.00	Break
10.00–17.30	Exhibits open
10.00–11.45	Keynote Addresses
11.45–13.30	Lunch
13.30–15.10	Session I
15.10–15.30	Break
15.30–16.45	Session I continued
16.45–	Discussion Session
18.00–19.30	Reception sponsored by the City of Hamburg

Tuesday, September 29, 1987

08.00–17.00	Registration
08.00–09.40	Session I continued
09.40–10.00	Break
10.00–17.30	Exhibits open
10.00–12.05	Session II
12.05–13.30	Lunch/Poster Session I
13.30–14.20	Session II continued
14.20–16.25	Session III
16.25–16.40	Break
16.40–	Discussion Session
19.00–19.50	Organ Concert at Petri Church. Open to all participants and spouses.
20.00	Optional Event: German Evening at Fleetenkieker.

Wednesday, September 30, 1987

08.00–17.00	Registration
08.00–09.35	Session III continued
09.35–10.00	Break
10.00–17.30	Exhibits open
10.00–12.00	Normann Award Ceremonies
12.00–13.30	Lunch/Poster Session II
13.30–	Discussion Session
19.00–24.00	Optional Event: Dinner-Dance at the Atlantic Hotel.

Thursday, October 1, 1987

08.00–17.00	Registration
08.25–09.40	Session IV
09.40–10.00	Break
10.00–17.30	Exhibits open
10.00–12.30	Session IV continued
12.20–13.55	Lunch/Poster Session III
13.55–14.20	Discussion Session
14.20–16.00	Session V
17.30–21.00	Optional Event: Boat Trip and Buffet on the Elbe River

Friday, October 2, 1987

08.00–16.00	Registration
08.00–09.15	Session V continued
09.15–09.40	Break
09.40–10.50	Discussion Session
10.50–12.05	Session VI
12.05–13.30	Lunch
13.30–16.00	Session VI continued
16.00–16.15	Closing remarks
16.15–	Discussion Session

Exhibitors

Atlas Industries A/S Baltorpvej 160, DK-2750 Ballerup (Copenhagen), Denmark. Atlas will exhibit its "Dry Condensing System," a vacuum system for the oleochemical industry. The dry condensing (DC) system is used for creating vacuum in edible oil deodorization, fatty acid fractionation and other processes taking place at pressures from 1 to 10 torrs. An alternative to steam ejector systems, the DC system is designed to reduce energy and water consumption. Fat and other materials captured in the process are not mixed with large quantities of water and motive steam. They, therefore, can be easily separated and recovered. Atlas Industries' representatives at the conference will be Finn Hansted and Henrik Hoeg-Petersen.

ATT-Verfahrenstechnik GmbH, Ludgeristr. 9, 4400 Münster, West Germany. ATT-Verfahrenstechnik offers design, planning, construction and commissioning of processing plants for reaction and separation. Services include countercurrent film processes for deacidification and deodorization of edible oils and fats, and distillation and fractionation plants for raw fatty acids. These processes are designed for higher exchange efficiency, lower pressure drop, lower thermal load, lower residence time, lower operating costs and lower environmental load.

Bruker Analytische Messtechnik GmbH, Silberstreifen, D-7512 Rheinstetten 4, West Germany. Bruker Analytische Messtechnik will display the NMR spectrometer "minispec" pc120 for determination of: solid fat content in fat compositions; oil content of oil-containing seeds; oil content of powdered milk products; droplet size in emulsions; and oil and/or moisture in processed or unprocessed oil-containing products.

Caffaro S.p.A., Via Privata Vasto, 1, 20121 Milano, Italy. Caffaro will feature various types of "PROLIT" bleaching earths, including PROLIT PN, PROLIT RF, PROLIT S and PROLIT AE. These are used in the process of refining fatty substances of any origin and for a variety of industrial and commercial uses.

Coesfeld GmbH Mess-Technik, Iggelhorst 19, D-4600 Dortmund 1, West Germany. Coesfeld will be exhibiting testing equipment for investigation of the crystallization behavior of fats, rotating viscometers and penetrometers.

Extraktionstechnik GmbH, Humboldtstrasse 56, 2000 Hamburg 76, West Germany. Extraktionstechnik will provide an overview of its activities and available equipment for the processing of all vegetable oil-bearing materials and vegetable oils and fats. The exhibit will feature the company's latest improvements in extraction, refinery and pollution control technology, including the SLF physical refiner/deodorizer, low temperature pollution-free condensation and wastewater-free extraction plants. Engineers and corporate staff will be on hand with photo display.

Franz Kirchfeld GmbH, Königsalle 17, D-4000 Düsseldorf 1, West Germany. Kirchfeld will feature its conventional and non-caustic refining processes, with capabilities up to 600 tons per day. Included are its low-energy systems for deodorization, hydrogenation and winterization.

Heinz Schumacher V.D.I., Schmuckshöhe 8a, 2000 Hamburg 63, West Germany. Heinz Schumacher, a consulting engineer, will be available to discuss desolventizing (DTDC), expanders, and fullfat-soymeal of different qualities for special applications.

Körting Hannover AG, Badenstedter Str. 56, D-3000 Hannover 91, West Germany. Körting Hannover will exhibit its multi-stage steam jet ejector units for processing plants in the edible oil industry, for flash cooling and crystallization.

Krupp Industrietechnik GmbH, Werk Harburg, P.O. Box 900880, 2100 Hamburg 90, West Germany. Pictures and information on the Krupp Food Technology program, including turn-key plants and single machines/apparatus for edible oil production, animal offals processing (rendering) and the detoxification of food and animal feed, will be displayed.

Novo Industri A/S, Novo Alle, DK-2880 Bagsvaerd, Denmark. Novo Industri will display a new enzyme technology for modification of fats and oils synthesis of fatty esters and modification of lecithin. Also, enzyme technology in general will be presented, including a comprehensive range of enzymes for detergents.

Süd-Chemie AG, Postfach 20 22 40, D-8000 München 2, West Germany. Information will be available on Süd-Chemie's highly active TONSIL bleaching earths used for the adsorptive decolorization and purification of oils, fats and waxes, as well as the advanced Girdler and NF-20/FS-40 nickel catalysts used in hardening oils such as fish, rapeseed and canola, and various fatty acids.

Tintometer GmbH, Westfalendamm 73, D-4600 Dortmund 1, West Germany. Tintometer will have on display instruments for color measurement and color grading according to Lovibond and other international specifications.

S.A. Fractionnement Tirtiaux, 601, Chaussee de Charleroi, Fleurus B-6220, Belgium.

Westfalia Separator AG, P.O. Box 37 20, 4740 Oelde, West Germany. Westfalia Separator will feature the refining separator-type RSA 100-01-777, a low noise version with self-cleaning bowl for degumming, neutralizing and winterization of oils and fats. The unit has capacity up to 300 tons/day.

Keynote Session

K-1 Lipid Biotechnology: Through The Looking Glass

P.K. Stumpf
University of California, Davis, California, USA

The biosynthesis of economically important fatty acids has been resolved in recent years. The individual enzymes have been isolated, purified and characterized; the flow of precursors required to supply the substrates for fatty acid synthesis has been defined. With these data at hand, a new thrust has been brought forth, namely the input of the concepts of molecular biology to the solution of a number of problems that until now have not been resolved. If and when these problems are elucidated, application of these new solutions will have a profound effect on the agro-economics involved in the annual production and consumption of over 60 million metric tons of vegetable oil throughout the world. Discussion will focus on some of the problems facing plant scientists in their attempts to understand and control plant lipid biosynthesis, some possible solutions will be suggested, and the impact of these solutions on the economy of nations will be examined.

K-2 Lipid Biotechnology: A Wonderland for the Microbial Physiologist

Colin Ratledge
University of Hull, Department of Biochemistry, Hull HU6 7RX, United Kingdom

Although the oils and fats industry relies and is based upon animal and plant lipids, the impact of microorganisms through their contribution to biotechnology is now beginning to be felt in many commercial sectors. Microorganisms have the advantages over plants and animals of a prodigious growth rate, wide choice of growth substrate, wide range of product diversity and, usually, considerable ease of mutation to give greatly enhanced yields and/or productivities of the desired materials. Their main disadvantage, however, is one of cost, it being much more expensive to produce one ton of yeast or bacteria than one ton of soybeans. Nevertheless, although microorganisms cannot compete in price against the bulk oil and fat commodities, they can serve as potential sources of some higher priced materials—γ-linolenic acid and perhaps cocoa butter—as well as producing novel lipids such as biosurfactants and biopolymers like poly-β-hydroxybutyrate. Microorganisms and their enzymes are also the means of accomplishing many transformation reactions, as discussed in the keynote presentation of Dr. Yamane. Microorganisms also provide a sophisticated waste disposal system for handling any unwanted materials arising from fat processing by upgrading them to improved oils and fats or even to produce animal feed protein. Microorganisms, because they are so easily handled, have proved to be excellent model systems for studying the basic biochemistry of lipid accumulation (where else but microorganisms could you find cells with more than 80% lipid contents?), fatty acid biosynthesis and regulation, as well as providing the basis to understand and accomplish genetic changes in higher organisms. For the success of these biotechnological activities, the combined inputs of the biochemist, engineer, geneticist and microbiologist have been required but because microbial lipids has been one of the less well researched areas, the field has become a wonderland for the innocent physiologist to enter and to make simple but interesting discoveries—perhaps the last area of biochemistry where it may be possible to do so.

K-3 Enzyme Technology for the Lipids Industry: An Overview by a Biochemical Engineer

Tsuneo Yamane
Department of Food Science and Technology, Faculty of Agriculture, Nagoya University, Furo-cho, Chigusa-ku, Nagoya 464, Japan

First, enzymes useful in the lipids industry, i.e., lipase, phospholipases (phospholipase A_2 and phospholipase D) and lipoxygenase, are summarized and classified from the viewpoints of sources (plants, animals, bacteria, yeasts and fungi), pH optima (acid, neutral and alkaline), specificity (stereospecificity, positional specificity, substrate specificity) and so on. Enzymes commercially available now in Japan and enzymes being extensively studied at academic institutions in Japan are introduced. Some useful biochemical reactions catalyzed by these enzymes are reviewed: hydrolysis of fats and oils by lipases, transesterifications (acidolysis, alcoholysis and interesterification) of fats and oils by lipases, hydrolysis of lecithins by phospholipase A_2, transphosphatidylations of phospholipids by phospholipase D and preparation of prostaglandins by prostaglandin synthetase are discussed. Applications of these biochemical reactions to food industries and to medical fields are mentioned. Activities of research and development in this field in Japanese industries are introduced. With reference to their applications, states with which enzymes and microorganisms are used in microaqueous solvent system (i.e., in low water-activity media or in nearly anhydrous solvent) are described. Some configurations of microaqueous bioreactors are shown, and the importance of optimal moisture content control is emphasized. Finally, problems involved in the enzyme technology for lipid industry (operational stability, cost, etc.) and future prospects of enzymes for lipids industry are mentioned.

Session I: Biotechnological Modification of Oil Plants: Fat Metabolism and Genetic Implications

1.1 Cellular Compartmentation for Seed Oil Storage

Roland Theimer, Gerhard Wanner and **Rolf Eggersmann**
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Variable amounts of triacylglycerols are stored in seeds (cotyledons, endosperm), fleshy fruits (pericarp) or even

tissue cultures of certain plant species. In electron micrographs of such cells, the triacylglycerols appear to be in distinct spherical granules called lipid bodies (spherosomes, oil bodies, oleosomes). They exhibit an amorphous matrix lacking any interior structures but stain with osmium tetroxide in some tissues. In cross sections, they appear surrounded by an osmiophilic single layer that evidently consists of phospholipids and hence is interpreted to be a "half-unit-membrane." Analyses of purified lipid body fractions show that they are comprised of up to 94% triacylglycerols, a few electrophoretically separable protein fractions and up to 11% phospholipids. During maturation of oil-storing seeds, massive production of triacylglycerols from fatty acids occurs in the endoplasmic reticulum (ER). Fine structural examination of maturing seeds from numerous oil plant species revealed lipid accumulation within the bilayered unit membranes of the ER. It was therefore proposed that lipid bodies develop between the lipophilic phospholipid moieties in the ER membranes, pushing them apart and eventually budding off, still being surrounded by the phospholipid layer. It obviously prevents the individual lipid bodies from coalescing even when densely packed as in the mature oilseed tissues. During germination, the oil stores are hydrolyzed with the help of the enzyme lipase whose activity is low in resting or imbibed seeds of most plant species but shows a dramatic increase thereafter. Incubation of a purified lipid body fraction under appropriate conditions leads to autolysis, i.e., liberation of fatty acids from the fat contents. Most (if not all) of the lipase activity is recovered from lipid body coats obtained after ether extraction of the fats from purified lipid bodies. *In vivo*, the "empty" lipid body coats may be observed in electron micrographs of cells whose storage fat has been exhausted. Such structures that still show lipase activity have been isolated, e.g., from the cotyledons of rape seedlings.

1.2 Biosynthesis of Triglycerides in Plant Storage Tissue

Allan Keith Stobart
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Sten Styme
Plant Physiology, Swedish University of Agricultural Science, Uppsala, Sweden

The lecture will deal with our present knowledge regarding the synthesis of fatty acids in relation to triacylglycerol (vegetable oil) production in the developing seeds of oleaceous crop plants. It is now generally accepted that the fatty-acid synthase system is located, solely, in the plastid-organelles present in the cells of the maturating seed-cotyledons. Palmitate (C16:0), stearate (C18:0) and oleate (C18:1) that are produced in the plastid, are transported to the membranes of the endoplasmic reticulum (E.R.) in which reside all the enzymes necessary for the formation of C18-polyunsaturated fatty acids, triacylglycerol assembly and oil deposition. In recent years, it has been established that the substrates for linoleate (C18:2) and linolenate (C18:3) synthesis are the oleate and linoleate residues, respectively, in microsomal phosphatidylcholine. The mechanisms whereby the oleate moieties enter phosphatidylcholine for desaturated and the polyunsaturated fatty acid products are made available for triacylglycerol formation will be discussed. Where appropriate, emphasis will be given to those reactions which regulate the fatty acid quality of the final oil in order to underpin future ventures in acyl-manipulation through programs of plant breeding and genetic engineering.

1.3 Fatty Acid Synthesis in Plant Cells

J. Brian Mudd
The Plant Cell Research Institute, 6560 Trinity Court, Dublin, CA 94568, USA

Fatty acid synthesis (*de novo*) in higher plants is confined to the plastid. The enzymic steps of the synthesis are catalyzed by proteins that are soluble and separable. The system of fatty acid synthesis in higher plants is therefore more like that in bacteria than that in animals where fatty acid synthesis is catalyzed by multifunctional proteins. Control of fatty acid synthesis can be placed in two categories: chain elongation and desaturation. The most common chain lengths are C16 and C18 fatty acids which are preponderant in membranes. In seed oils, there is considerably more variation, particularly well exemplified in the genus *Cuphea* where different species accumulate C8, C10, C12 or C14 fatty acids in the seed oil. It is important from the view of biotechnology to understand the factors that control chain length. The control of desaturation is also an important commercial consideration. In the leaves of higher plants, there is a diurnal variation in fatty acid unsaturation. This is probably dependent on the effects of photosynthetic activity on desaturation reactions. Some herbicides have a profound effect on desaturation. In the fats and oils industry, control of fatty acid desaturation in seed oils is a much more important consideration. There have been notable successes in changing contents of unsaturated fatty acids in seed oils, particularly rape and sunflower. There has been considerable effort put into changing the proportions of unsaturated fatty acids in soybean. Success in these modifications has so far come from selection and breeding methods using either intact seeds or tissue culture methods. Potential for the application of genetic engineering techniques to the modifications of fatty acid chain length and unsaturation will be discussed.

1.4 Recent Advances in Oil Crops Breeding

Paul Knowles
Professor Emeritus of Agronomy, University of California, Davis, California, USA

Plant breeding began more than 10,000 years ago when primitive societies began the process of crop domestication. Though the process was slow, it yielded most of our crop plants in essentially their modern form. One of the objectives of modern plant breeding is, or should be, to continue the process of crop domestication with particular reference to species producing oils of use to the fats and oils industry. Plant breeding continues to provide cultivars that are: better adapted to different environments, thus higher in yield; higher in oil content; changed in oil quality through changes in fatty acid composition; and more resistant to diseases, insects and other pests. Plant breeding procedures and technology that will facilitate achieving those objectives are: germplasm collection, screening and preservation programs; development of hybrid cultivars for all oil crops; manipulation of the environment to shorten the life cycle of plants, thus providing more generations per year; use of mutagens to change genes, particularly those affecting adaptation, oil quality, and resistance to pests, pesticides and herbicides; and genetic engineering, also to change genes, and to facilitate the transfer of genes to oil crops from other species, other genera, and other families. In addition, genetic engineering should facilitate interspecific hybridization, and generation of doubled haploids through microspore and anther culture, thus abbreviating the duration of breeding programs. To be most effective in providing benefits to the fats and oils industry, plant breeding and modern biotechnology must be closely merged. Increasingly, plant breeding, augmented by genetic engineering, is being done in the private sector, leading to increased financial investment and with it, more rapid and abundant cultivar development, but increased protection of the investment through restricted germplasm exchanges and patents of plant-breeding products and processes.

1.5 Fatty Acid Biosynthesis: Sites of End Product Regulation and Potential for Genetic Engineering

Daniel J. Guerra
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The synthesis of fatty acids in plants is under developmental and tissue specific control. The molecular genetics of plant lipid metabolism are essentially uncharacterized. However, the biochemical framework has been adequately

described. In photosynthetic tissues, fatty acid synthesis (FAS) *de novo* is localized in the chloroplast. The central cofactor/cosubstrate for C_8 to C_{18} fatty acid synthesis is acyl carrier protein (ACP). Evidence from photosynthetic tissue suggests that acyl chain length and *in situ* distribution may be regulated by ACP isoform structure. In developing oilseed, the plastid is most probably the site of ACP mediated FAS. To potentially engineer triacylglycerol synthesis, we must ascertain the relationships between ACP mediated *de novo* FAS and the Kennedy pathway. Several research laboratories are involved with aspects of this complex developmental system. ACP gene manipulation in oilseed crops has become a major focus. A general overview and some specifics of research target agendas will be described. Discussion topics include the following: (a) fatty acid synthesis *de novo*: enzymology and points of regulation; (b) specific desaturation/elongation reactions; (c) branch point and end point metabolism; (d) recombinant acyl carrier protein—expression in E. coli, characterization *in vitro* and potential as a molecular probe; and (e) oilseed modification via genetic engineering.

1.6 Genetic Transformation for Oil Crop Improvement

R.B. Horsch, J.E. Fry, A. Barnason, S. Metz, S.G. Rogers and R.T. Fraley
Monsanto Company, 700 Chesterfield Village Parkway, St. Louis, MO 63198, USA

We have produced transgenic *Brassica napus* cv Westar plants with a vector system that harnesses the natural gene transferability of *Agrobacterium tumefaciens*, the causative agent of crown gall disease. The disease is the result of the transfer and expression of phytohormone biosynthesis genes from the tumor-inducing (Ti) plasmid of *A. tumefaciens* into the chromosomes of infected plant cells. The transferred DNA segment (T-DNA) is defined by a specific sequence of 25 bases present as a direct repeat just outside both ends of the T-DNA. Since none of the tumor-causing genes found in the T-DNA is involved in the DNA transfer process, those genes can be replaced by a benign vector that does not cause tumors or otherwise interfere with plant morphogenesis, growth or reproduction. The genes that control the DNA transfer process are located in an adjacent region of the Ti plasmid, but are not transferred to the plant cell. Thus the DNA becomes a permanent part of the plant genome, losing all capacity to move again. We have deleted the phytohormone biosynthetic genes that cause crown gall disease from the Ti plasmid and substituted a vector that contains a selectable marker, neomycin phosphotransferase (NPT II) that confers resistance to kanamycin, and a scoreable marker, nopaline synthase (NOS) that produces a unique and easily scoreable metabolite, nopaline. The vector system also permits addition of other genes for co-transfer into transformed plant cells. The vector plasmid is engineered in *E. coli* cells and then mated into *A. tumefaciens* where it is then capable of being transferred into infected plant cells. Transformed *Brassica napus* plants have been produced by means of a simple stem segment transformation and regeneration method that couples the gene transfer mechanism of *A. tumefaciens with kanamycin selection and de novo* shoot regeneration. Surface-sterilized stem segments were inoculated with *A. tumefaciens* strain containing our vector and cultured for two days. The explants were then transferred to regeneration medium containing kanamycin. Shoot regeneration occurred within 3-6 weeks and transformants were confirmed by a leaf callus assay on medium containing kanamycin. Progeny of the transgenic plants inherited the new genes in the expected Mendelian patterns of 3:1 or 15:1. This method should be adaptable to many species that are within the host range of *A. tumefaciens* and where regeneration of plants from culture is possible. The ability to alter genes and then reintroduce them into plants opens the door to unprecedented structure-function analysis in genetics, biochemistry and developmental biology. The first generation studies have examined gene structure and expression and are quickly being followed by analysis of altered expression of proteins. The next generation of experiments will likely examine the effects of altered proteins on biochemical pathways, and will lead to insights into physiological systems. We can now monitor expression of genes at different times and places during plant development and will soon be able to change the time and place of expression of such genes by changing promoters. Ultimately, directed changes in genes will lead to a better understanding of how molecules give rise to the growth and developments of plants. This understanding and technical capability will revolutionize how we can alter/improve plants for human benefit. One of the first quality traits that may be possible to change will be oil quality. Recent breakthroughs in the biochemistry and genetics of oil production coupled with rapidly advancing molecular genetics of the genes involved are major topics of this meeting.

1.7 Commercial Development of Oil Palm Clones

L.H. Jones
Unilever Research, Colworth/Welwyn Laboratory, Colworth House, Sharnbrook, Bedford, England

The laboratory techniques for clonal propagation of oil palms by tissue culture have been available for the past 10 years. Production of commercially useful oil palm clones which can be propagated economically requires integrated programs of factory development and of palm breeding and selection. The laboratory techniques require optimization and scale-up to enable cost effective production of uniform good quality plants. Successful clones depend on selection of the best genetic material from current breeding programs. Since genetic and environmentally induced variation both contribute to individual palm performance, each clone must be field tested over several years in different environments before final clone selections are made and they enter commercial production. A number of clones in current commercial trials show promising yield increases compared with seedling palms. Several clones subjected to large-scale production are producing abnormal flowers resulting in bunch failure. Current research is concentrated on discovering the cause of the abnormality and providing quality control and cultural methods to avoid its induction and to guarantee production of normal palms.

1.8 Biotechnology for Coconut Improvement

Yukio Sugimura, Kazuya Otsuji, Shinta Ueda and Kikuhiko Okamoto
Kao Corporation, Tochigi Research Laboratories, 2606 Akabane, Ichikaimachi, Haga, Tochigi 321-34, Japan
Myrna J. Salvana
QC/R&D Department, Philipinas Kao Incorporated, Cagayan de Oro City, Misamis Oriental, Philippines

Coconut palm is at present propagated exclusively from seeds. Since this palm is generally cross-pollinated and heterozygous, the resulting genetical variation between seedlings is a serious problem. In order to provide clone materials from proven, high-yielding, disease-resistant coconut, the development of rapid means of clonal propagation is of prime importance. Tissue culture techniques would be useful to solve this problem. The study of embryo culture was initiated for the aim of propagation of "Makapuno" coconut which do not germinate under natural conditions. Plantlets could be regenerated by culturing isolated embryos *in vitro*. Conditions for culture in medium and subsequent transplanting to soil were investigated by several research groups. This success may enable us not only to propagate "Makapuno" seedlings, but also to exchange and preserve genetic resources in the state of embryo itself. Callus induction was observed from various tissues such as embryo, stem, leaf, root, endosperm and inflorescence. However, differentiation from these calluses is not yet achieved consistently. Using excised embryo, two types of callus were found during culture for around three months. While nodular callus may be initiated from meristematic zone in plumule and radicle, strand-like callus may take place from procambial parts. For these responses, it was required to incorporate 100 ppm 2,4-D into modified Y-3 medium in the presence of activated charcoal. When finely sliced tissue from rachilla was used as explant, small nodular masses appeared in about one month after initial culture in the medium containing 20-30 ppm, 2,4-D and continued to grow during sub)culturing. For high frequency of callus induction, it is essential to select particular size of rachilla and to include activated charcoal for minimizing tissue browning. The solidified agent "Gelrite", instead of "Bacto-agar", provided better growth of callus. During further sub-culture in low concentrations of 2,4-D, green shoot-like structure was developed from well grown callus under fluorescent lamps.

1.9 Genetic Modification of Polyenoic Fatty Acid Composition in Flax (Linseed)

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Linoleic acid and linolenic acid are the only two polyenoic fatty acids present in significant concentrations in the major economic seed oils. Linoleic acid (C18:2), the principal component of polyunsaturated oils, is an essential component of the human diet. Linolenic acid (C18:3), although also essential in human nutrition, is an undesirable component in edible oils due to flavor reversion problems associated with its oxidation instability. Plant breeders successfully have modified the proportions of these two fatty acids in a number of oilseed crops in order to improve their quality as edible oils. These changes have been achieved through genetic modification of the activities of the desaturase enzymes that sequentially convert oleic acid (C18:1) to linoleic and linolenic acids. The conversion of linseed oil from an industrial oil into an edible oil is a striking example of such genetic modification. Following EMS treatment of seeds of the high-linolenic (45-50%) linseed cultivar Glenelg, two mutants, M1589 and M1722, having reduced levels of linolenic acid (28-30%) were isolated. By recombining the M1589 and M1722 mutations into a single genotype, linolenic acid content was further reduced to around 1%. This reduction was associated with an equivalent increase in the highly desirable linoleic acid to 62%. Proportions of other fatty acids remain unaltered. The mutations thus appear to block the linoleic acid desaturation step of fatty acid biosynthesis. The reduction in linolenic acid content has greatly improved the oxidative stability of the oil, making it suitable for edible uses. An additional 6% increase in linoleic acid content has been achieved by backcrossing the mutations into other genetic backgrounds.

1.10 Biotechnology for Soybean Improvement

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Nearly a quarter of the world supply of edible fats and oils is from soybeans. Though not as valuable as oil, soybean protein and carbohydrate co-products are also of significant economic importance in the oilseed industry. Despite their obvious market value, soybeans suffer a number of constraints. Some of these problems might be eliminated by application of conventional or non-conventional genetic approaches, and result in soybeans tailored for specific end uses. Topics to be discussed from this perspective during the presentation include (a) strategies directed toward elimination of undesirable tastes, odors and antinutritional factors; (b) manipulation of the oil composition to modify stability and physical properties; (c) engineering of enzymes and other proteins to improve production efficiency or the quality of soy products; and (d) extractability and bioavailability of seed carbohydrates to mono-gastric animals.

1.11 Biotechnology for *Brassica* and *Helianthus* Improvement

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During the past 15 years, *Brassica* and *Helianthus* have enjoyed substantial growth, both in total production and in new markets. In agronomic terms, this market expansion translates to improved total yield, environmental fitness and modified oil and meal components. Market opportunities continue to exist for new genotypes improved in these three areas. Of particular interest for *Brassica* are agronomic traits such as even seed maturity, shattering resistance and effective hybridization. Resistance to drought, disease and cold temperatures, and stability of oil profiles under variable growing conditions are important to both *Brassica* and *Helianthus*. For both oilseeds, intensive interest exists for fatty acid profiles tailored to meet specific food and industrial market needs. Cell culture, plant regeneration and genetic transformation techniques are highly developed for *Brassica* species; they are less so for *Helianthus*. New phenotypes created by a few discreet mutations, especially a decrease in enzyme activity, are readily obtainable through mutagenesis and somaclonal variation regimes. Because these mutations occur randomly, effective selection or large)scale screens greatly enhance the chance of success. While such schemes are straightforward for sunflower, the amphidiploid nature of *B. napus* and *B. juncea* makes rapeseed modification more complex. For the introduction of traits requiring new or enhanced enzyme activity, genetic transformation is the best approach. However, detailed molecular knowledge of key enzymes, structural genes and regulatory elements required for transformation, is lacking for most useful phenotypic changes. The acquisition of this knowledge presents a major challenge to biochemists, geneticists and plant breeders.

Session II: Biotechnology: New and Unique Oil Sources and Byproducts

2.1 Genetic Diversity of Lipids in Plant Germplasm

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Lipid chemists have recognized the existence of over 500 different fatty acids and other lipid structures, most of them in the plant kingdom. This number appears to be the ultimate in natural chemical diversity, but when we consider that only about 10,000 of the approximately 250,000 species of plants have been studied in any depth, we realize that this number of different compounds is probably only the tip of the proverbial iceberg. In general, we have little idea why one plant will produce long-chain fatty acids and another, in the same environment, will biosynthesize fatty acids of shorter chain length

than the usual C18 acids. In fact, the role that fatty acids and other lipid structures play in the life cycle of plants is, in most part, only beginning to be understood. We consistently find these compounds to be markers that point to a particular genus, family or order. For example, the presence of cyanolipids seems to be limited exclusively to the family Sapindaceae and the delta-5 C20 and C22 monoenoic fatty acids are found only in the genus *Limnanthes*. In this paper, I will point out the diversity of lipid structures and suggest their use as chemotaxonomic markers.

2.2 Development of New Industrial Oil Crops

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Unusual fatty acids such as those present in the seed oils of rapeseed, coconut or palm kernel, tung nut or castor bean have been utilized as raw materials since the early days of the chemical industry. These oils form a stable and valuable, although still relatively small, fraction of the world oil market. Changes in agricultural production, diversification of industrial applications and the unreliable supply of mineral oils as petrochemical raw materials prompted a search for new potential oilseed crops possessing sufficient agronomic performance for an economical production of seed oils with such unusual fatty acids. The first large screening program for new industry plant oils was started in 1959 by the U.S. Department of Agriculture. But it took until the 1970s before interest increased to intensify agronomic investigations in the production of the respective plant species. Jojoba even created a speculative boom when its wax was shown to be a useful substitute for whale sperm oil. Substitutes also were desired for the former supply of long-chain fatty acids after rapeseed had been bred worldwide to zero-erucic content for nutritional uses. Lauric acid always has been in chronic shortage on world markets. Crop development involves several steps: (a) collection and (b) evaluation of germplasm, (c) chemical and utilization studies, (d) agronomic evaluation, (e) breeding work, (f) production and processing scale-up, and (g) commercialization. Costs and time requirements depend on the level of domestication of the species in question, on its ecological adaptation and its seed yield potential. In each case, there is a minimum requirement for quite a number of traits and if only one character of these is of unsatisfactory performance, the highest value of others can not compensate for such deficiency. This multi-step improvement of plant production by breeding as well as

by agronomic means will be exemplified referring to recent work with *Cuphea* (to produce medium-chain triglycerides), *Calendula* (trienoic F.A. with conjugated double bonds), *Lesquerella* (hydroxy f.a.), *Crambe* (long-chain F.A.), and other imaginable new industrial crops.

2.3 Implications of Modern Biotechnology to Plant Lipids

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In contrast to the relatively few fatty acids found in plant membrane, fatty acids of plant storage oils exhibit a rich diversity. Such diversity is the basis for several industrial uses of plant oils and suggests that plants will tolerate genetic manipulation of their storage triacylglycerols. Oilseed varieties with altered fatty acid compositions have been obtained with conventional breeding techniques and even greater modification may be obtained in the future using molecular genetic techniques. However, application of these techniques is limited by the lack of information concerning the biochemistry of fatty acid synthesis (FAS). It appears that most seed specific unusual fatty acids are first synthesized by the plastid and then modified. Little is known about regulation of the subsequent modification steps and the specific targeting of unusual fatty acids to triacylglycerols. We have been studying the organization and regulation of oilseed fatty acid synthesis using acyl carrier protein (ACP) as a representative of the fatty acid synthetase. Plants contain at least two forms of ACP which are expressed differently in seeds and leaves. In developing soybean seeds, both the ACP protein levels and the amount of messenger RNA for ACP increase during the period of rapid fatty acid synthesis, suggesting that oil production is controlled at least in part by turning on FAS genes. We have constructed a synthetic ACP gene which is being used to examine the structure, regulation and function of ACP at both protein and nucleic acid levels. The recent isolation of native ACP genes in other labs provides methods for seed and organelle specific targeting of foreign proteins in oilseeds and may allow modification of seed fatty acid metabolism.

2.4 Genetic Control of Fatty Acid Biosynthesis in Yeasts and Other Lower Fungi

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In an attempt to investigate basic principles underlying the molecular architecture and functioning of fatty acid synthetase (FAS) multienzyme complexes, the FAS genes from various selected organisms currently are studied in our laboratory. In this paper, we report the complete nucleotide sequence of the multifunctional FAS1 and FAS2 genes from *Saccharomyces cerevisiae*, of the FAS1 gene from *Yarrowia lipolytica* and the FAS2 gene from *Penicillium patulum*. In all three organisms, the FAS multienzyme complex is an $\alpha 6\ \beta 6$ hexamer of the penta-functional subunit β and of the trifunctional subunit α. Subunits α and β are encoded by FAS2 and FAS1, respectively. The obtained nucleotide sequences were used to deduce the coding region, amino acid sequence and size of the corresponding gene products together with other protein chemical parameters. All FAS genes studied exhibited a remarkably high degree of homology (60-80%) at the level of both DNA and protein structure. The order of catalytic domains in FAS1 is acetyl transferase (N-terminal), enoyl reductase, dehydratase and malonyl/palmityl transferase. In FAS2, the peripheral (cysteine) and central (pantetheine) SH groups were localized according to their known amino acid context, while the β-ketoacyl reductase domain was localized in the N-terminal part of the gene by deleting mapping. The *Penicillium* GAS2 gene contains two small (45 bp) introns while the yeast genes were intron-free. From the presence of several non-homologous deletions and insertions in the fungal FAS genes as well as from their size in comparison to that of the corresponding vertebrate gene, it is concluded that the DNA content of FAS1 and FAS2 in yeast may be considerably higher than is absolutely required for the catalytic process. By in vitro mutagenesis, it was intended to discriminate between functionally essential and non-essential parts of genes.

2.5 Technical and Economic Aspects and Feasibility of Single Cell Oil Production Using Yeast Technology

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There has been intermittent interest in the production of lipids by microorganisms for at least the past 50 years. Many authors have shown that the three principal groups of microorganisms—yeasts, bacteria and fungi—can all be induced to accumulate substantial quantities of intracellular lipid by nutrient manipulation. Of these, yeasts produce lipids more like conventional edible oils from plant sources than the other two groups. Yeasts were probably the first microorganisms used by man to produce food products over a period of the last several thousand years. The technology for growing and harvesting these organisms on a large scale is simple and well established and they are aesthetically acceptable to consumers as food materials. As with many biotechnology processes, production of the microorganisms is only part of the story. Extraction of the lipid from the cell by an efficient yet economic process is crucial to the overall process economics, but little published information has appeared on this important area. One solution to this problem developed by a New Zealand group is bead milling dried yeast cells in hexane which appears to

satisfy these criteria. Production of one ton of lipid requires about five tons of carbon substrate (sucrose, glucose or similar) and this cost is the most important economic element in the process. Availability of a cheap carbon source will make production of a wider range of edible oil substitutes possible than with higher-priced carbon sources which restrict the possibilities to higher-priced specialty lipids such a cocoa butter substitutes. At the present time, although it is technically feasible to produce substitutes for the cheaper vegetable oils by yeast fermentation, it is not economic to do so unless a very cheap carbon source is available.

2.6 Production of γ-Linolenic Acid by Fungi

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A very efficient microbiological method has been developed for the production of a lipid rich in its content of *gamma*-linolenic acid and for the preparation of *gamma*-linolenic acid therefrom. Strains of *Mortierella* fungi in a liquid culture medium produce a fungal body containing a large amount of lipid rich in its content of *gamma*-linolenic acid. The growth of the fungal mass of these strains was very rapid even in a conventionally high concentration of the carbon source of 200 g/liter or more to give a yield of the fungal mass of 80 to 156 g/liter on a dry basis. The fungal mass contained 37% to 58% by weight of the lipid, corresponding to a yield of lipid of 30 to 83 g/liter. The content of *gamma*-linolenic acid in the fatty acids of thus obtained lipid was as high as 4% to 11% by weight. The extraction method of lipids from the fungal mass and the isolation of *gamma*-linolenic acid from extracted lipids investigated, as well as the industrial production process that has been developed in Japan based on these studies, will be discussed.

2.7 Microalgae as a Source of EPA-Containing Oils

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Recent epidemiological and clinical investigations have implicated eicosapentaenoic acid (EPA), a polyunsaturated fatty acid in certain fish oils, in reduced incidence of coronary heart disease and cancer. It is unclear at this point, however, whether the EPA in fish oil is synthesized directly by the fish, or accumulated through the food chain from that produced by many species of marine phytoplankton (microalgae). Our recent work has focussed on screening, selecting and improving the EPA yields of several oil-producing microalgal species as a prelude to determining if this source of EPA-containing oil may be of economic importance to the food or pharmaceutical industries. Our results from growing certain strains under a variety of culturing conditions have demonstrated the plasticity of lipid and EPA content of these microalgae. For example, a 10 C decrease in the culture temperature can double the EPA in some cases. Strain selected organisms in culture are now producing EPA to levels of one quarter of their total fatty acid complement. These organisms grow quickly (doubling time of 6 hr) and are amenable to controlled, fermentation-like culture by using newly developed photobioreactor technologies. As strain selection strategies continue to improve the oil content of these organisms while retaining their high EPA levels, this biotechnologically based source of EPA-containing oil is becoming more attractive as an alternative to fish oil for commercial use in the food industry.

Session III: Biotechnological Modification of Fats, Oils, Fatty Acids and Glycerol

3.1 Immobilized Lipases in Organic Solvents

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Lipases from various sources are able to catalyze hydrolysis, synthesis and interesterification of different classes of esters. To apply lipases to ester synthesis or ester exchange reaction, it is essential to carry out the reaction in an organic solvent to avoid hydrolytic reaction. Immobilization on or in suitable supporting materials renders the enzymes to resist to the denaturation to be caused by organic solvent. We have developed novel methods to immobilize biocatalysts including lipases by entrapment in synthetic polymer gels prepared from photo-crosslinkable resin prepolymers and urethane prepolymers. These methods are very useful for the bioconversions of lipophilic compounds, such as substrates for lipases, because of easy choice of hydrophilicity-hydrophobicity balance of gels, which affects seriously the diffusion of lipophilic compounds inside gels. Interesterification of triglyceride (olive oil) with stearic acid was mediated successfully in water-saturated *n*-hexane by *Rhizopus delemar* lipase entrapped with a hydrophobic photo-crosslinkable resin prepolymer after adsorbtion on Celite. The entrapped enzyme preparation was found to be more stable than the Celite-adsorbed enzyme. Esterification of terpenoids could be catalyzed by various kinds of lipases. A primary alcohol, citronellol, served as a good substrate for lipases, although stereoselective reaction was not observed. Secondary alcohols, such as menthol and borneol, were stereoselectively esterified by entrapped lipase from *Candida cylindracea* in water-saturated isooctane or cyclohexane. Long-chain fatty acids were good substrates for esterification but not for stereoselective reaction. Medium-chain fatty acids, especially 5)phenylvaleric acid, were found to be satisfactory acyl donors from both points of view. Effects of water content in the gels and kinds of organic solvents on the esterification activity are also discussed, together with the effect of chemical modification of the enzyme on the activities of diverse reactions.

3.2 Enzymatic Fat Splitting

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A brief review is given of the more recent research and technical literature on enzymatic fat splitting. It was found that the rate of enzymatic hydrolysis is directly proportional to the logarithm of reaction time and also to the logarithm of enzyme concentration. The lipase from *C. rugosa* is commercially available and appears to be more suitable for fat splitting. Different fats or oils will hydrolyze at different rates, probably due to differences in chemical structure. Temperature has little influence on reaction rate. Addition of electrolytes, albumin or other additive either has no effect or adversely affects hydrolysis. A novel assay method based upon the above logarithmic relationship is described. The most favorable aspects of the enzymatic approach are low energy input, high quality and relatively low capital cost. Its practicality has been demonstrated by the existence of industrial batch processes as well as continuous processes described in the patent literature.

3.3 Ester Synthesis with Immobilized Lipase

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The use of immobilized lipases for the preparation of a variety of esters will be reviewed. Fatty acid ester products can be waxes, emulsifiers and flavors. Biochemical synthesis offers a number of advantages compared to chemical catalyzed processes, such as selectivity, gentle reaction conditions leading to pure products, and no use of toxic catalysts. Examples of thermostable, microbial lipases useful for the synthesis of primary and secondary alcohol esters will be given. It will be illustrated that lipases may accept a number of acid and alcohol components apart from their natural fatty acid/glyceride substrates. Lipase stereoselectivity may be used in the preparation of optically pure substances. The benefit of immobilization in terms of enzyme activity, stability and handling will be demonstrated. Lipase development also has included process technology such as a simple and efficient vacuum system for making wax esters. Furthermore, the use of substrate derivatization or solvents does not exclude the use of lipases in ester synthesis reactions.

3.4 Enzymatic Conversion of Diglycerides to Triglycerides in Palm Olein

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The purpose of this study is to improve the purification yield of crude palm oil (CPO) and the quality of palm oil (PO). For instance, crude PO includes rather high concentration of diglycerides (ca. 8%) and fatty acids (ca. 5%). These fatty acids are removed during purification, resulting in low recovery of oil. On the other hand, diglycerides (DG) remain in palm oil even after purification. These remaining DG are known to be one of the causes for instability in PO when heated and for the delay of crystallization when used as a component of margarine. To resolve these problems, enzymatic conversion of DG and FA to triglyceride (TG) in crude PO has been studied. As the substrate, crude palm olein was used instead of crude palm oil. Enzymes were immobilized into celites. Lipase P (originated from *Pseudomonas fluorescence*) was selected because it shows some activity even at less than 100 ppm of water in oil. The reaction seemed to follow this equation: $DG + FA = TG + H_2O$. Turning point of hydrolysis and synthesis as for the water concentration in crude palm olein was determined to be around 100 to 150 ppm. By keeping water level at less than 100 ppm, TG was increased from 87% to 95% within 48 hours. The factors to promote reaction speed were investigated. Those are the addition of various fatty acids, the adsorption ratio of the enzyme to celites, the use of highly purified enzyme, and so on. Finally, the activating factor of the enzyme at such ultra micro aqua level as less than 100 ppm of water was found out. These results made possible a much shorter conversion time.

3.5 Omega-Hydroxylations

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Hydroxycarboxylic acids constitute a chemically very interesting class of compounds since they carry two different functional groups within the same molecule. Thus, hydroxy carboxylic acids can serve as precursors for polymers or cyclic lactones which are used in fragrance and antibiotics. Moreover, hydroxy acids are constituents of some biological surfactants. Although it is feasible to synthesize hydroxy fatty acids by chemical means, the procedures involved are complicated and extensive purification is needed. On the other hand, hydroxy fatty acids occur widespread in vegetable oils. However, omega hydroxy fatty acids are rather exceptional. It is known that alkane-degrading microorganisms can hydroxylate alkanes or fatty acids to the respective alcohol or hydroxy fatty acid. However, although several patents have been filed for microbial hydroxy acid formation, no such products are sold to a large extent. The reason might be that fermentation yields are too poor to sustain a rentable process. For the selection of strains suitable for omega-hydroxy fatty acid production, a detailed knowledge of the biochemical reaction involved in fatty acid metabolism is required. Besides omega-hydroxylation, OH groups might also be introduced at other places in the molecule. Thus, beta-hydroxy fatty acids are a common byproduct of fermentations leading to dioic- or hydroxy acids. This can be explained by the biochemical reactions involved. For hydroxy-acid production, alkane-degrading microorganisms as yeasts of the Candida-type or Corynebacteria are more suited. In order to prevent further degradation of the product formed, these microorganisms are blocked in the alkane/fatty acid metabolism. A block within the beta-oxydation system might result in the formation of 3-OH acids. The terminal hydroxylation is accomplished

by monoxygenase systems which most commonly contain a cytochrome P450 components. The further oxidation of the alcohol formed in this reaction proceeds through dehydrogenases of different specificity. These reactions are usually much faster than the hydroxylation and have to be slowed down to obtain hydroxy-acid formation. Alternatively, enhanced levels of the monoxygenase system must be obtained.

3.6 Production of Dicarboxylic Acids by Fermentation

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It has been well known that the production of long-chain dicarboxylic acid (DCA) by chemical synthesis or by chemical oxidation is not easy and that most of the DCAs produced by these methods are of relatively short chain length. On the other hand, there have been many research papers which reported that the DCAs could be produced from n-alkanes by microorganisms. Also, a number of chemical companies have claimed inventions in the field of fermentative production of DCAs. However, there has not been a commercial process for producing DCAs by fermentation until recently. We have developed the fermentation process for producing long-chain DCAs from n-alkanes using *Candida tropicalis*. The improvement of the microorganism by mutation and the optimization of fermentation conditions have resulted in 140g/l of DCA accumulated. This fermentation process can produce DCAs from various kinds of raw material, e.g., n-alkanes, n-alkenes fatty acids, and fats and oils. Brassylic acid (DCA-13) is now being commercially produced in the amount of 150 tons a year only by this fermentation process.

3.7 Enzymic Modification at the Mid-chain of Fatty Acids

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Natural fatty acids commonly show a range of mid-chain modifications including mono- to polyunsaturated, hydroxyl substituents and methyl branches. Enzymes responsible for these modifications and others involved in degradation are found in most enzyme classes, with oxidoreductases predominating. Unsaturated fatty acids are the most common substrates giving hydroxy-, epoxy- and hydroperoxy- products after oxygenase attack. Further enzyme reactions can lead to keto- and dihydroxy- fatty acids and lipoxygenase can oxidize arachidonic acid to a prostaglandin precursor. Unsaturated fatty acids also can be reduced by enzymes found in rumen microorganisms. A mixed bacterial culture can fully reduce alpha-linoleic acid to stearic acid by a series of reactions mimicking the hardening process currently used by the oils and fats industry. Most individual chemically catalyzed mid)chain modification processes are efficient and controllable but their enzymic counterparts display regiospecificity and, perhaps more significantly, stereospecificity which could be the key to novel products. Some chemical processes such as ozonolysis have no identified enzymic parallel although there are alternative biotechnological routes to the same products. Consideration of the differences between chemical catalysis and possible biocatalytic routes highlights the need to solve the problem of reductant supply which is common to most oxidoreductases, or to find alternative routes. Progress is being made in using whole cells for these types of biotransformations where the sequential and spatial requirements for complex reaction sequences and recycling of cofactors have not been disrupted by isolating the enzymes. Derivatization or immobilization of cofactors such as NAD+ shows promise for the repeated use of isolated redox enzymes although further development is needed. A limited range of mid-chain products may be made using enzymes which do not require cofactors. Potential processes using whole cells or isolated enzymes will need to be operated intensively with essentially non-aqueous lipid feedstreams if they are to compete with chemical catalysis.

3.8 Competition in Lipase-Catalyzed Reactions: Application to a Simple Test for Lipase Specificity

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Abstract not available at press time

3.9 Engineering of Lipases and Proteases for Improved Transesterification

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Serine hydrolases can catalyze the addition of nucleophiles to ester bonds. We have identified a region in the enzyme subtilisin which is associated with nucleophile specificity and shown that amino acid substitutions in this region can dramatically alter the transesterification properties of the enzyme. This led us to investigate the effects of similar changes in a lipase. Although this enzyme is not homologous to subtilisin, it is a serine hydrolase and is mechanistically similar to subtilisin. We found that changes made to an analogous region of the lipase resulted in alterations in transesterification properties similar to those observed for subtilisin. These types of changes in the properties of lipases could have direct applications in the area of fat and oil chemistry.

Session IV: Biotechnology and the Preparation of Unique Fats, Fatty Acids and Biosurfactants

4.1 Unusual Fats and Fatty Acids: Occurrence and Function

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Whereas as little as seven types of fatty acids account for more than 90% of the fatty acid-derived lipids occurring in nature, an astonishing variety of unusual fats and fatty acids have been reported from many genera of animals, plants and microorganisms. In fact, more than 1,000 different fatty acid structures easily were compiled from publications concerning chemotaxonomical markers. In most cases, the biological significance of such unusual fats and fatty acids is unknown. In a few cases, however, their biological role has become clearer. It is inferred that from a more systematic investigation into the relationship between their structure and biological function, industrial applications of such compounds and their derivatives might be derived.

4.2 Production of Arachidonic Acid and Eicosapentaenoic Acid by Microorganisms

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Arachidonic acid (eicosa-5,8,11,14-tetraenoic acid) and eicosapentaenoic acid (eicosa-5,8,11,14,17-pentaenoic acid, EPA) are fatty acids that are important as precursors of prostaglandins and show themselves several unique physiological activities. Several protozoal and fish oil products are available as the sources for these fatty acids. From an economical point of view, however, these sources are not so suitable for large-scale preparation. We recently have found that fungal mycelia are rich sources of these fatty acids, which would make preparation of arachidonic acid and EPA far simpler. A soil isolate, *Mortierella elongata 1S-4*, was found to show high productivity of arachidonic acid through our screening for wide variety of microorganisms. The production of arachidonic acid reached 3.6 g/l (80 mg/g dry cells) when the fungus was grown in a medium containing 2% glucose and 0.5% yeast extract as main carbon and nitrogen sources. The fungus produced EPA together with arachidonic acid when grown at low temperatures. Therefore, we assayed the productivity of EPA in various fungal strains under the growth conditions of low temperatures and found the *M. alpina* grows well at low temperatures (5-24 C) and accumulates more than 0.5 g/l of EPA (26.6 mg/g dry cells). These fatty acids were found to accumulate especially in polar lipids in mycerial membrane. These fatty acids were isolated as either oil forms or highly pure fatty acid forms from the mycelia in high yields through a simple extraction procedure. High productivity of the fatty acid shows practical significance of these novel fatty acid producers.

4.3 Biotransformation of Oleic Acid to Ricinoleic Acid

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Various microorganisms were tested for their ability to transform oleic acid to ricinoleic acid. Organisms were grown in the medium containing 6.0% glycerol, 1.0% $(NH_4)_2HPO_4$, 0.2% K_2HPO_4, 0.65% yeast extract, 0.1% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.008% $MnSO_4 \cdot H_2O$, 0.0008% $ZnSO_4$, 0.01% nicotinic acid and 0.5% oleic acid. The pH of the medium was adjusted to 7.2 for bacteria and to 5.5 for yeasts and fungi. After incubation at 30 C for 72-96 hours, the reaction products were extracted with ethyl acetate under acidic conditions. Ricinoleic acid formed routinely was analyzed by thin layer chromatography and reverse phase high performance liquid chromatography, and identified also by gas chromatography-mass spectrometry. We selected three fungal strains, four yeast strains and six bacterial strains as producers of ricinoleic acid. A strain of soil bacterium, BMDI20, which was incapable of utilizing oleic acid as a sole carbon source, formed the highest amount of ricinoleic acid from oleic acid in the culture fluid. When the cells of BMDI20 were grown in the medium containing 1.5% polypetone, 0.2% KH_2PO_4, 0.2% K_2HPO_4, 0.5% NaCl, 0.01% $MgSO_4 \cdot 7H_2O$, 0.01% yeast extract, 0.01% meat extract, 0.1% glucose and 0.5% oleic acid at 30 C with shaking, ricinoleic acid was produced most abundantly. Approximately 3.6 mg/ml of ricinoleic acid was produced when oleic acid was added to the fermentation broth at the stationary phase. Accumulation of ricinoleic acid began about 48 hours after addition of oleic acid. Resting cells grown on the medium containing oleic acid catalyzed conversion of oleic acid to ricinoleic acid. The immobilized cells also probably are applicable to the transformation of oleic acid.

4.4 Temperature Effects in the Biosynthesis of Unique Fats and Fatty Acids

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Temperature, directly or indirectly, is one determinant in altering the biosynthesis and distribution of fats and fatty acids. Both in nature and in the laboratory, a decrease in temperature may cause liquids to solidify and an increase in temperature may cause solids to liquefy. These gel/liquid crystalline transformations are an integral part of the phase changes by which naturally occurring lipids may respond to an environmental stress such as temperature. In many instances, these phase modifications may be detrimental to the routine performance of life functions and perhaps even to life itself. Living systems have devised response networks to retain suitable membranous liquidity or phase relationships and function within their tissues by homeoviscous or homeophasic adaptions. One of these biochemical mechanisms in times of thermal stress is an adjustment of lipid unsaturation: low temperature survival favoring an increase in lipid unsaturation, and high temperature survival favoring a decrease in lipid unsaturation. Lipid changes in response to temperature alteration transcend effects on the degree of unsaturation. They may include, for example, changes in chain lengths of fatty acids, levels of fatty acid branching and cyclization, and the distribution and relative proportions of members of the glycolipid and phospholipid families. At least in some

cases, there still remains the question of whether effects of temperature on lipid unsaturation and other structural responses are direct on genetic or biochemical expression, or whether the temperature effect is indirect, with the direct mediator being oxygen availability or cell growth rate, for example. These reactions to synthesize modified structures and proportions of fats and fatty acids often occur in microorganisms, plants and animals in a universal response to thermal stress. While this affords a survival advantage to living systems, in many instances, such as in production of polyunsaturated fatty acids, unsaturated cooking oils and unsaturated wax esters, there is the promise of commercial relevance.

4.5 Lipids of Acinetobacter

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The alkane-utilizing microorganisms, *Acinetobacter* species HO1-N, synthesize a number of unique lipids when grown at the expense of long-chain alkanes, fatty alcohols, fatty acids and symmetrical long-chain diaklyl ethers. The metabolism of n-alkanes and fatty alcohols yields wax esters as primary end products. The characterization of peroxypalmitate in lipid extracts of hexadecane-grown cells plus an enzyme which converts peroxypalmitate to palmitaldehyde have led to the possible existence of a new pathway in alkane dissimilation by *Acinetobacter*. Alkane-inducible fatty alcohol dehydrogenase is absent in *Acinetobacter*, whereas a NADP-dependent fatty aldehyde dehydrogenase and a fatty acyl-CoA reductase are induced by growth on n-alkane, fatty alcohol and fatty aldehyde. Isotope dilution experiments support fatty aldehyde as a central, branch-point intermediate, with fatty alcohol a metabolic end product rather than a metabolic intermediate. A double mutant of *Acinetobacter* converts fatty acid to short-chain alkane and decanedioic acid. These products result from an internal carbon-carbon scission of the endoperoxide, 10-hydroperoxy-*trans*-hexadec-8-enoic acid. Further, *trans*-hexadec-9-enoic acid and *trans*-hexadec-9-enoic accumulate in the culture broth. Extension of these studies to the metabolism of long-chain, symmetrical alkoxyalkanes (C_{14}-C_{20}) demonstrated the oxidation of a homologous series of diaklyl ethers to alkoxyacetic acids and dibasic acids. The reaction mechanism involves an internal carbon-carbon scission 2 carbons removed from the ether oxygen. The lkoxyacetic acids accumulate in the culture broth as non-metabolizable end products. Similar bioconversions result from the oxidation of phenoxy-substituted fatty acids to dibasic acids and phenoxy-substituted short-chain acids. A general mechanism will be presented for the synthesis of unique lipids in *Acinetobacter* resulting from the metabolism of alkanes and fatty acids.

4.6 Strategies for Biosurfactant Production

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The growth of microorganisms on hydrocarbons or other lipophilic substrates often is associated with the synthesis of biosurfactants. These metabolites are mostly involved in the mechanism for the initial interaction of the lipophilic substrates with the microbial cell. Biosurfactants are amphiphilic compounds. The hydrophilic part in the molecule can be a carbohydrate, a cyclic peptide or an amino acid. The hydrophobic part contains long-chain fatty acid, hydroxy-fatty acid or α-alkyl-β-hydroxy-fatty acid. In many cases, the synthesis of these hydrophilic and hydrophobic parts of a biosurfactant derives directly from the primary metabolism. This is the reason that there are some common rules concerning the biosynthesis and the regulation of these metabolites. For the microbial preparation of biosurfactant hydrocarbons, vegetable oils, glycerol or carbohydrates can serve as carbon substrates. Dependent on the microorganisms used, the biosynthesis can be cell-growth associated, enhanced by growth-limiting conditions or by the addition of a precursor. Further, the production of biosurfactants is influenced by different egulation mechanisms such as induction or catabolite repression. Another aspect, dealing with the physical properties of biosurfactants, is the alteration of the product composition from a microorganism by variation of the carbon substrate or the culture conditions. These results pointed out the possibilities to change the structure of a certain biosurfactant for tailoring the surfactant properties.

4.7 Biosurfactants as Food Additives

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This paper gives a general review of the use of biosurfactants in food featuring recent work on transphosphatidylation of soy lecithin. A biosurfactant is defined as an abundant natural substance that lowers interfacial tension and stabilizes emulsions. The structure and properties of proteins and lecithins conforming to this criterion are reviewed in relation to their emulsifying abilities. Milk proteins, which despite their relatively moderate surface activities are referred to as components of a refined emulsion system, are reviewed in relation to their emulsifying abilities. Milk proteins, which despite their relatively moderate surface activities are referred to as components of a refined emulsion system, are similarly discussed. Many attempts at chemical modification of both proteins and lecithins, which cause a marked change in surface activities, are introduced. With respect to lecithins, their naturally thorough emulsifying power, production, market, structure, composition, analysis (including a new and improved method), processing and modification are overviewed. In addition, a new biosurfactant, transphosphatidylated lecithin, in which most of the phosphatidylcholine and phosphatidylethanolamine has been converted to phosphatidylglycerol by phospholipase D is introduced. The properties and screening of the enzymes, the conditions of transphosphatidylation in an aqueous system or an inorganic solvent system, and the emulsifying properties of the transphosphatidylated products are detailed. Although the safety of adding chemically modified products to food is questionable, transphosphatidylation,

which is an enzymatic modification, may be acceptable. A possible future application of this reaction is also described.

4.8 Biosurfactants for Petroleum Recovery

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Chemically synthesized surfactants are currently being evaluated by the oil industry for enhanced oil recovery applications. The primary purpose of a surfactant flood is to lower the interfacial tension between the injection fluid and the oil in order to displace crude oil that cannot be mobilized by water alone. The petroleum sulfonates have been the favored group of surfactants for this application due to their ability to lower interfacial tension against crude oil from 30 to less than 10^{-3} mNm^{-1}. These compounds, however, show optimal activity over a narrow range of temperatures and salinities and can therefore rapidly lose their effectiveness as the surfactant "slug" moves through a reservoir. A wide variety of surfactants is produced by microorganisms. Many of these compounds have been identified and characterized but only recently have quantitative assessments of biosurfactant performance for enhanced oil recovery been described. Certain biosurfactants have been reported to display some very promising properties for oil recovery. Crude bacterial culture broths can reduce interfacial tension against oil to 10^{-2} mNm^{-1} and in the presence of al alcohol cosurfactant, interfacial tensions as low as 10^{-5} mNm^{1} have been measured. The injection of a biosurfactant into a water flooded laboratory sandpack recovered up to 87% of the residual oil. Biosurfactants are effective at low concentration and can be made from cheap sources of sugar. In the future, they may find applications in many other industries in addition to the oil industry.

4.9 Biosurfactants in Cosmetic Applications

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As the first step for the development of biosurfactants, we focused on glycolipid surfactants, the development of which has been delayed due to some limitation in raw materials and difficulties in production technology in the field of industrial synthetic surfactants. We started our research by finding a variety of oligo-glycolipid biosurfactant producing microorganisms from nature. We first collected soil samples, separated hydrocarbon-utilizing microorganisms and then screened oligo-glycolipid-producing microorganisms. As a result, we obtained 23 oligo-glycolipid-producing strains. One of these strains, a yeast, *Torulopsis bombicola* KSM-36, produced 10g or more of glycolipids per liter. As a result of an analytical study on the structures of these glycolipids, it has been found that they are a series of derivatives containing, as their backbones, sophorolipid (SL) structures in each of which ω- or ω-1-hydrooxyfatty acid has been ω-glucosylated with a sophorose group obtained by bonding two moles of glucose together. We succeeded in determining production conditions under which sophorolipid can be produced at a stable rate of about 100g to 150g per liter of a combined carbon source of palm oil and glucose. We prepared alkyl-SL esters through esterification between a group of long-chain fatty alcohols and SL. On the other hand, being attracted by the hydroxyl group of sophorose group of SL, we prepared P-SL by subjecting a propylene oxide and SL to addition polymerization. And it has been clear that Oleyl-SL and P-SL, the latter being an addition-polymerized product of one mole of SL and about 12 moles of propylene oxide, have specific compatibility to the skin. They have found commercial utility as skin moisturizers.

Session V: Biotechnology: Engineering and Scale-up in Fat and Fatty Acid Biotransformations

5.1 Bioreactors for Hydrolysis of Fats and Oils

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Lipases that can be mass produced have been developed through studies on microbial lipase that have been conducted since the 1960s. Especially, the highly active lipase from *Candida cylindracea* has been widely used in studies for the commercialization of the hydrolysis of oils and fats. The hydrolytic reaction has been investigated mainly using the two-phase reaction system. Various bioreactors including stirred tank, packed bed and membrane systems have been progressively developed. Especially, the membrane-type bioreactor equipped with many disperser-membranes has been evaluated as a highly efficient and compact reactor. For high-melting fats, the solid phase static process has been evaluated.

5.2 Biosensors for Lipids

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The determination of lipids for the precise control of industrial processes is very important. Quantitative determination of lipids was conventionally carried out colorimetrically. However, complicated pretreatments were required before the measurement. Therefore, the development of rapid and simple determination of lipids is required. In this study, novel biosensors for lipids such as neutral lipids, phosphatidylcholine, cholesterol

and free fatty acids, are examined. These sensors are based on electrochemical methods using enzyme membranes. Neutral lipid was determined by lipoprotein lipase-immobilized membrane and pH electrode. The measurement was based on sensing pH change caused by hydrolysis of neutral lipids. Phosphatidylcholine was determined amperometrically, measuring oxygen consumption by sequential enzyme reaction (phospholipase D and choline oxidase). Cholesterol and free fatty acid also were determined amperometrically by using enzyme-immobilized membrane and oxygen electrode. These methods require no special pretreatment of the sample. Furthermore, measurements were completed within 10 minutes. These sensors appeare to be promising and attractive methods for the routine measurement of lipids.

5.3 Modifications of Fats and Oils in Membrane Bioreactors

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Membrane bioreactors offer promising possibilities for the efficient integration of bioconversion and separation processes. The possibility to perform biocatalytic conversions between two immiscible phases which are separated by a membrane makes this technology of special interest to the oil and fat industry where the processing of emulsion is common. Examples are the hydrolysis of oils and the esterification of fatty acids and glycerol using lipases. For the esterification of fatty acids, a controlled water activity in the enzyme's vicinity is important. Using membrane bioreactors, the water activity can be roughly controlled. There are several approaches for operating membrane bioreactors: (a) An emulsion of a lipase solution with fatty acid is pumped through the fibers of a hydrophilic membrane reactor. Glycerol is pumped through the shell side of the reactor; (b) Lipase dissolved in a glycerol phase is pumped through a plate membrane unit equipped with a hydrophobic membrane. Fatty acid is passed through the unit at the opposite side of the membrane; (c) Lipase, either physically or covalently immobilized onto a hydrophilic membrane, catalyzes the esterification of fatty acid dissolved in organic solvent. The oil/organic solvent phase and glycerol phase are separated by the membrane. Some research data with respect to the esterification of fatty acids with glycerol via these approaches will be presented and advantages and/or disadvantages discussed. Clearly, optimization of a membrane bioreactor (network) in terms of its biocatalytic and economic performance requires extensive research. A simplified general cost-price calculation procedure will be presented. Such a procedure can be used to identify factors that greatly influence the economics and is helpful in setting research targets.

5.4 Mass Transfer in Bioreactors

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The term bioreactors is used to describe fermentation vessels and enzyme reactors. While fermentation processes could eventually impact fats and oils technology, much of the current research interests in this area have centered around enzymatic systems. Consequently, this presentation will focus on the mass transfer occurring in enzymatically catalyzed reactions. Mass transfer or, more precisely, mass transfer limitations occur at interfaces. In reaction systems containing immiscible liquids, there will be mass transfer limitations at the liquid-liquid interfaces. If an enzyme is immobilized on a solid support, there will be a liquid-solid interface through which mass transfer must occur. If the solid support is smooth, then mass transfer occurs by convective flow. If the support is a porous solid or membrane, then, depending on pore size, internal diffusion would control mass transfer. The effect of mass transfer limitations is to alter the observed or overall reaction rate. This rate is what is measured during a particular experiment with a given set of experimental conditions. It is the superposition of mass transfer effects on intrinsic kinetics. Changing an experimental parameter such as temperature changes the observed rate by affecting both intrinsic and mass transfer rates. However, changing such parameters as stirring rates, vessel size or enzyme support pore size will not affect the intrinsic kinetic rates but could affect the mass transfer rates and consequently change the observed rate. The variability of the mass transfer rates complicates the scale-up of reaction systems. To properly size a batch, continuous stirred tank or packed bed reactor from laboratory bench-scale data, the intrinsic kinetic rates must be determined and the mass transfer rates must be determined and the mass transfer rates must be cast in dimensionless form. In certain systems, it may be possible to use mass transfer limitations as an advantage. In a sequential reaction where the desired product is an intermediate, mass transfer constraints can act to selectively increase production of the intermediate over the final product. In mixtures of competing substrates, a potential advantage of pore diffusion is that by selecting the proper enzyme support pore size, a particular substrate can be preferentially selected for reaction.

5.5 Continuous Use of Lipases in Fat Hydrolysis

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In comparison to other enzymatically catalyzed reactions, especially in the pharmaceutical field, the product-added value from the fat-splitting process is low. On the other hand, fatty acids have a remarkable market volume. Thus, continuous processing with reuse of the enzyme seems to be the method of choice. In order to achieve high space-time yields, carrier fixation of a lipase may be unfavorable due to mass transfer limitations, especially in the case of a two-phase reaction system. Therefore, the aim of this investigation was to develop a method for the continuous use of lipases without carrier fixation. Since the enzyme is enriched ("immobilized") at

the phase boundary (fat/water) where the reaction takes place, a micro-emulsion is desirable for the reaction, and a phase separation is needed for product isolation and the recovery of the enzyme at the boundary layer. This was accomplished by the use of two continuously operating centrifuges. The conditions were selected so that about 90% of the pure aqueous phase containing glycerol (first stage) and about 90% of the pure fat phase containing fatty acids (second stage) were separated. By this series of two incomplete separations, it was possible to recycle about 90% of the enzyme with phase boundary layer. There is a small loss of enzyme in the aqueous phase. This can be minimized by ultrafiltration and recycling the concentrate. This procedure allows a kinetically and thermodynamically desirable countercurrent flow of the fat phase and the aqueous phase. The feasibility of such a process was demonstrated with a continuous soybean hydrolysis in two stirred tank reactors (3 l) and two centrifuges. Although technically feasible, the approach is not yet economical.

5.6 Economic Aspects of Lipid Biotechnology

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The production of various lipids by microbial fermentation is now a technically feasible alternative to the continued dependence upon plant-derived oils and fats. Despite the particular attraction of this option to countries which face heavy import costs for lipids, and recent developments in large-scale fermentation technology, commercial exploitation has been slow. Economic barriers exist for all but the specialty lipids which command higher values. This paper examines the raw material costs and the various scale-related plant and processing costs for the batch production of microbial lipids. The sensitivity of production costs to the use of alternative carbohydrate sources, improvements in key process parameters and the utilization of fed)batch or continuous processing routes are considered to identify where developments in technology are required. Comparison of these costs with the current market values of lipids illustrates possible process opportunities.

5.7 New Process for Purifying Soybean Oil by Membrane Separation and Economical Evaluation of the Process

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A feasibility study has been conducted on introducing membrane separation technology into the refining process for edible oils. In general, vegetable oils for edible use, such as soybean oil or rapeseed oil, are purified and produced by chemical treatment with alkali agents. The conventional chemical process has disadvantages of considerable loss of oil and highly contaminated effluents; instead physical refining process has been desired. Membrane separation can provide an answer to this need. Ultrafiltration makes it possible to manufacture purified oil by the following process: degum crude miscella by ultrafiltration, obtain degummed oil by removing hexane from the permeated miscella by distillation, carry out bleaching and deodorization of the degummed oil. The solvent-resistant polyimide ultrafiltration membrane, which is available from Nitto Electric Industrial Co. Ltd., designated as NTU-4200, is effective for processing soybean miscella at 40-50 C, and the molecular weight cut-off value 20,000 of the polyimide membrane shows good rejection of gum materials. As a result, the complete process for physical refining of soybean oil has proven to be practicable. Based on economical estimation with a bench-scale test plant, the physical refining of soybean oil by use of membrane separation is clearly profitable when compared to the conventional chemical refining. This may have significance to the oil-processing industry.

5.8 Commercializing Biotechnology

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Commercializing biotechnologically adapted products in the fats and oils industry requires careful and early attention to a range of production technology, product specification and marketing feasibility issues. Our experience indicates that many companies fail to adequately assess all facets of their new development plans on a timely basis. Resources committed to such evaluation are frequently minuscule relative to the costs of the development program and risks inherent in the marketplace. Evaluation is often initiated far too late in the development process to prevent substantial waste of resources in pursuing uneconomical business opportunities or inappropriate technological avenues. New competitive factors threaten to change the established industry patterns and may result in structural changes in the industry. New opportunity areas will be defined. The relative roles of patents and trade secrets may be altered. Marketing issues will become more important. Long-term commitment to technological strategies will become more important, even critical. Inter-industry boundaries will be blurred, especially between fats and oils and seed. Higher product development risks will be required by biotech companies, fats and oils companies and fats and oils users, with highly exclusionary technology being the stake. The ante will go up. Those who wait and see may not survive. Premiums will accrue to market control and integrated downstream marketing savvy. There will be critical marketing success factors. Errors will be extremely costly, not only in economic terms but in customer confidence and supplier image. Some key questions are: Can established players adapt? Can small players survive? Will the lawyers get the whole pie? Is market research still valid or even possible? Can customers know what they need? Who will be left in 1999? Can we learn from analogs?

Session VI: Regulatory Aspects of Biotechnology in the Fats and Oils Industry

6.1 Analytical Tools for Monitoring Biotechnology Alterations of Fats and Oils

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Recent advances in chromatographic technology and detector instrumentation have greatly improved our ability to separate, identify and quantify low levels of structurally similar molecules that may arise by intended or incidental alterations through new biotechnologies. For example, the development of rugged wall-coated open tubular (WCOT) capillary columns in conjunction with multi-dimensional gas chromatography provides the capability to easily separate molecules with only subtle structural differences. Technological advances in pumping systems and in the production of uniform, small diameter column packing materials have made high performance liquid chromatographs (HPLC) common analytical instruments for both the research laboratory and the quality control laboratory. The development of chromatographic instrumentation which uses supercritical fluids as eluants i.e., carbon dioxide in liquid state, has provided the chromatographer with another tool to separate and quantify molecular species in complex matrices. Advances in detector technology, e.g., uv-visible photodiode array, FTIR, MS and their respective interfaces to chromatographic systems, have dramatically increased the information available to the analyst relative to peak purity and molecular structure. These new analytic tools will be discussed in terms of their impact on biotechnology as it relates to specific components of fats and oils.

6.2 Toxicological Evaluation of Biotechnology Products: A Regulatory Viewpoint

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For drugs produced by biotechnological techniques, it is now understood that toxicity testing by routine protocols may be inadequate, unpredictive or even impossible to perform. Judgment of safe use of such products may depend, to a larger degree than for chemically synthesized compounds, on the availability of other data, e.g., obtained from clinical studies. On the other hand, quality, safety and efficacy will have to be established as for any other drug before registration. In the European Community, Council Directive 87/22/EEC has been enacted on July 1, 1987. To put this directive into action, two Notes for Guidance on quality aspects (monoclonal antibodies of murine origin; products derived by recombinant DNA technology) have been finalized, whereas the Note for Guidance on Pre-Clinical Biological Safety Testing still awaits such finalization (Draft III/407/87-EN; revision 3-April 1987). In this Note for Guidance, proposals for testing requirements are outlined for the following product groups: (a) hormones and cytokines and other regulatory factors; (b) blood products; (c) monoclonal antibodies; and (d) vaccines. It is understood that quality (including problematic areas of identity, purity, determination of contaminants possibly encountered from the novel processes used in their manufacture and from the complex structural and biological characteristics of the products) be assessed, namely not by unsuitable toxicological techniques but by analytical tests. Depending on e.g., existing/non-existing pharmacological activity, the status of the patient and the disease to be treated, physiological or large non-physiological doses expected to be used, foreseeable single or repeated administration of the product, the extent to which the biological effects of the substances are characterized, a flexible, case-by-case approach for safety testing will have to be sought. Major weight will often lie on (general) pharmacodynamic studies. No battery of safety tests can be prescribed. The usefulness of performing various test combinations, including toxicity and pharmacokinetic studies, has to take into account the development of immunological incompatibility of the product with the animal species used.

6.3 Toxicological Evaluation of Biotechnology Food Products

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Biotechnology enables preparation of a diverse range of products, which may be end products for the user or processing agents. Toxicological evaluation requires greater attention to some potentially harmful properties, but does not differ in essentials from standard considerations and test requirements. Products of biotechnological processing may be considered in terms of (a) examination for potential hazards for the products'user, (b) protection of the environment from contamination by organisms derived by genetic engineering, and (c) public emotional responses to biotechnological products and processes. This presentation is mainly concerned with the first issue. Examination for potential hazards requires consideration of the product-forming microorganisms, whether or not the product is the recipient of genes derived from other organisms. If gene transfer has been made, detailed information is required on the identity of the gene, identity and properties of the source and recipient organisms, and transfer of the expression vector into the host. The examination of the fermentor product will be considered in detail, particularly with reference to the detection of any unknown toxins and potential mutagens. First considerations are the stability of the organisms used, and the identity and purity of the gene product. Subsequent examination for mutagenic activity depends upon compatibility with *in vitro* assay. These include bacterial mutation, primary rat heptocytes (toxicity, DNA repair, inhibition of protein synthesis) and Chinese hamster V79 cells (toxicity and cytogenetics).

Parallel tests may be made of digests of food products. If the test product is not compatible with *in vitro* assays, *in vivo* tests for micronucleus formation, metaphase chromosome analysis and DNA repair are available as substitutes. Apart from this screen for mutagenic activity, additional tests follow standard procedures for safe andling and feeding tests, pharmacokinetics and teratology, all supported by full pathology.

6.4 General Regulatory Aspects of Biotechnology: Europe

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Biotechnology is any technique that uses living organisms to make or to modify products, to improve plants or animals, or to develop microorganisms for specific uses. Like any other technology with implications for the health of the public and the integrity of the environment, it requires some form of regulatory control. The major areas of application of biotechnology, including genetic engineering, relate to novel foods, industrial enzymes, certain pharmaceuticals, some animal feeds and new processes. Within Europe, the Commission of the European Economic Communities is making efforts to develop a harmonized approach, acceptable to all member states, for the regulation of biotechnological processes and products. Certain countries, such as the United Kingdom and Germany, already have set up some national machinery to deal with the problems. For example, the United Kingdom has published guidelines on the procedures for approval of novel foods. In a similar manner, the Federal Republic of Germany has developed guidelines for the approval of single-cell proteins in animal feeds. Many national efforts are directed toward the creation of control systems for products of genetic engineering, particularly in the pharmaceutical areas. These controls also are concerned with the conditions under which research, experimental trial and the environmental releases of products or organisms resulting from recombinant DNA technology may be carried out. The need for greater uniformity in the regulatory approach to the control of biotechnology will be discussed.

6.5 The United States "Coordinated Framework" for the Regulation of Biotechnology Research and Products

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On June 26, 1986, the final part of the U.S. government's "Coordinated Framework for the Regulation of Biotechnology" appeared in the *Federal Register* (FR 51, No. 123, pp 23302-23393). The policy guidelines are based on generally accepted scientific principles and, therefore, provide a rational basis for regulation. At the time of publication, we felt that we had finally achieved the appropriate regulatory balance necessary to deal with a new and rapidly developing industry and hoped that the principles established in this policy will be suitable as a model for other countries. The U.S. government's "Coordinated Framework" is a broad and complex policy that explains the application of existing statutes to the regulation of biotechnology and outlines our approach to interagency coordination, which is so vital in this field. The first part of the two-part policy appeared in the Nov. 14, 1985, *Federal Register*, (FR 50, No. 220, pp 47174-47195), and announced the establishment of the Biotechnology Science Coordinating Committee (BSCC). The committee, which consists of seven senior government officials, operates as a scientific coordinating committee among the regulatory agencies as established in its charter and reinforced by a Memorandum of Understanding signed by each of the members. The final part of the policy, which appeared on June 26, 1986, consisted of six elements: the preamble and statements of policy from the Food and Drug Administration, the Environmental Protection Agency, the U.S. Department of Agriculture, the Occupational Safety and Health Administration and the National Institutes of Health. Also published at the same time were proposed U.S. Department of Agriculture rules governing the "Introduction of Organisms and Products Produced through Genetic Engineering which are Plant Pests" and an "Advanced Notice of Proposed Guidelines for Biotechnology Research," also from USDA. While each of the agency statements stands alone, the *Federal Register* notices show how the separate agencies' policies form a coordinated regulatory framework.

6.6 Policy in Japan for Securing Safety in the Application of Recombinant DNA Technology

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R-DNA technology was published for innovative technology as improving organisms over the species wall. It was pointed out that organisms produced by r-DNA technology have potential risks for environment and health soon after r-DNA technology was published. So, governments began setting guidelines for experimental work to secure the safety of r-DNA technology. The Japanese government also made guidelines, in 1979, estimating potential risk based on knowledge available at that time. Now that r-DNA technology has made rapid progress and has grown to be used in industrial processes, the Japanese Ministry of International Trade and Industries (MITI) proclaimed "Guidelines for Industrial Application of Recombinant DNA Technology" in June 1986. The guidelines provide that the organizer of a working organization can request the Minister of International Trade and Industry to authorize the industrial plan if it conforms with the present guidelines. The MITI already has authorized 68 plans through June of 1987. Application technology of r-DNA organisms in open areas, such as purification of wastewater, is developing now. We are researching to improve prediction,

evaluation and monitoring of the outcome of applications of r-DNA organisms in these situations.

6.7 Toxicology of Biotechnology Products: Dietary Fat in Relation to Cancer and Other Chronic Diseases

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Consumption of high-fat diets has been associated with increased risk from a number of chronic diseases, including cancer and cardiovascular disease. The high caloric density of high-fat diets is also conducive to obesity. With respect to cancer, there is little evidence that dietary fats contain carcinogens, but animals fed high-fat diets after exposure to carcinogens develop tumors of the mammary gland, colon, pancreas and skin more readily than those fed low-fat diets. Polyunsaturated vegetable oils promote tumorigenesis more effectively in animals than saturated fats or polyunsaturated fish oils, apparently because n-6 polyunsaturated fatty acids are required for tumor promotion. In epidemiological data, however, mortality from cancer at sites such as the mammary gland, colon and prostate shows the strongest positive correlations with total fat and/or unsaturated fat and little or no correlation with dietary polyunsaturated fatty acids. This may be because most national diets supply adequate amounts of n-6 fatty acids. The positive correlation with total fat may be related to a separate, non-specific requirement for a high level of dietary fat observed in experiments on promotion of tumorigenesis in animals. The mechanisms by which dietary fat promote tumorigenesis are not known. Dietary n-6 fatty acids may alter the composition and properties of cellular membranes and this, in turn, can affect intercellular communications and modify physiological responses to antigens, hormones or other stimuli. The effects of n-6 fatty acids may be mediated by eicosanoids, which include prostaglandins, thromboxanes and leukotrienes. The non-specific promotion of tumorigenesis by high levels of dietary fat may be mediated by an increase in calorie intake or by altering metabolism in other ways that encourage cellular proliferation.

6.8 Dilemma of Patenting for Oilseed Breeders and Biotechnologists

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In most countries, the rapid biotechnological advances in crop plants, and oil crops in particular, has outpaced the present policy and legal framework designed to handle intellectual property rights. The present legal interpretation of what biotechnology innovations are patentable is still evolving and varies widely from country to country, with the U.S. and Japan having the most liberal interpretations. One of the major problem areas for crop plants is at the interphase between patent legislation and plant breeders' rights (PBR), the latter being the proprietary right to a crop variety as legislated under the International Union for the Protection of New Varieties of Plants (UPOV). Some have advocated replacing PBR with patent law. The vast majority of plant breeders believe that the patenting of sexually propagated plant varieties is neither appropriate nor practical. However, some system is required to provide proprietary protection for engineered genes inserted into crop plants. Similarly, biotechnology must recognize the value and rights associated with protected varieties which are the vehicle for the commercialization of the patented gene. Fortunately, the legal and moral dilemmas presented by biotechnology manipulation of plants have and are continuing to bring officials into direct contact with interested scientific circles, a process long overdue. These and other current plant gene patenting problems will be discussed and possible solutions presented.

Abstracts for Poster Presentations

P-1 Microbial Transesterification of Sugar Corynomycolates

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During growth on glucose, fructose, mannose, sucrose or maltose, *Athrobacter spec.* and the bacterium *M9b* synthesize the corynomycolic acid esters of these carbohydrates. The yield was 100-300 mg/g biomass (dry weight). Experiments with resting cells in a buffer without N-source showed that the microorganisms transferred the lipid moiety from the original sugar to another polyhydroxy compound. Labeling studies proved that the alpha-branched-beta-hydroxy fatty acid of the new glycolipids came from the exogenous carbon source. This transesterification, involving the C-6-position of sugars, only succeeded with mono-, di- and trisaccharides where the adjacent hydroxy group of the C-4 atom was in equatorial conformation. With additional free C-6-hydroxy groups, highly esterified molecules could be isolated from the cells. Structural carbohydrates such as cellobiose also were esterified with fatty acids by an insoluble esterase. In general, the yield of new glycolipids by resting cells was not higher than growing cells. Therefore, the influence of sugar corynomycolates added at the beginning of resting cell experiments was investigated. Monoesters of disaccharides reduced the interfacial tension of water against n-hexadecane to 1 mN/m at a CMC of 20 mg/l.

P-2 Production of Eicosapentaenoic Acid and Arachidonic Acid by the Alga *Porphyridium cruentum*

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The red microalga *Porphyridium cruentum* contains two rare fatty acids, eicosapentaenoic acid and arachidonic acid. Eicosapentaenoic acid was shown to be effective in reducing cholesterol levels in blood. Fish oils, the current source for eicosapentaenoic acid, contain cholesterol and have several fatty acids of similar structure that make purification more expensive. The fatty acid composition of *Porphyridium* is highly dependent on environmental conditions. *Porphyridium* strains were cultivated at 25 C and high light intensities for eicosapentaenoic acid; or 30 C, low light intensities and nitrogen starvation for arachidonic acid production. Eicosapentaenoic acid was concentrated in the glycolipids while arachidonic acid was mainly found in neutral and phospholipids. The respective glycerolipids were fractionated from each treatment and transmethylated. The obtained fatty acid methyl esters were treated with urea and yielded ca. 75% pure eicosapentaenoic acid or arachidonic acid.

P-3 Enzymatic Esterification of Ferulic Acid with Sterols

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Reactions were performed by mixing the lipase obtained from microorganisms, feruric acid, and sterols (cholesterol, beta-sitosterol, stigmasterol) in a buffer solution or in cyclohexane at 40 C. Reaction products were separated by silica gel column chromatography. IR and 13C-NMR spectra of the products confirmed that steryl-ferurates were formed by the enzyme reactions. HPLC analyses of the reaction products showed that steryl-ferurate esters were produced more readily in cyclohexane than in the buffer solution. It also was observed that the lipase from *Candida* was the most active of the lipases examined.

P-4 Bio-interesterification Process for the Preparation of *trans*-free PUFA, Vanaspati and Other Valuable Products

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Enzymatic processes for industrial use have many advantages over the conventional chemical techniques. Several patents have been taken by Macrae and others for ester-ester interchange reactions. In the present study, attempts have been made to conduct ester-ester interchange reactions using a non-specific catalyst *Candida scylindracea* with mowrah oil, sal (*Shorea robusta*), soybean oil, mowrah plus rice bran oil, or rice bran plus palm oil plus sal plus soybean oil. The products after recovery were subjected to TLC and GLC analysis. The reaction periods were varied from 24 to 93 hours. Slip point and dilatation studies were conducted in addition to chemical analysis of products. Results indicated substantial differences in product characteristics. Various other combinations of oils were subjected to treatment. Applications of the method will be discussed.

P-5 Biochemical Characterization of a Genetic Trait for Low Palmitic Acid Content in Soybean

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Recurrent mass and within half-sib selection methods have been used to develop soybean germplasm (N79-2077) exhibiting low palmitic acid content. The palmitic acid content of N79-2077 (47.8 mmol/kg dry weight) was ca. 2.3-fold lower than typical soybean cultivars. Studies of glycerolipid composition and metabolism have shown that this trait was expressed only in triacylglycerol; normal levels of palmitic acid were found in phospholipid and diacylglycerol. Saturation kinetics with acetate revealed that the apparent Km for synthesis and incorporation of saturated fatty acid into glycerolipid was 3.7-fold greater for triacylglycerol than phospholipid. Triacylglycerol molecular species composition and the activity of purified diacylglycerol acyltransferase with various substrates were determined in N79-2077 and the commercial cultivar "Dare." These studies have demonstrated technological advances toward development of soybeans with less than 5% (w/w) total saturated fatty acid content.

P-6 Substrate Specificity of Diacylglycerol Acyltransferase Purified from Soybean

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Soybean germplasm has been developed with genetic traits for high oleic, low linoleic, low linolenic acid concentration (N78-2245); and low oleic, high linoleic, low linolenic acid concentration (PI 123440). These germplasm exhibited significant difference in triacylglycerol molecular species composition. Biochemical evidence has shown genetic mutation of oleoyl-phosphatidylcholine desaturase activity in N78-2245, and linoleoyl-phosphatidylcholine desaturase activity in PI 123440. Hence specific diacylglycerol molecular species were derived from phospholipid metabolism. The inherent capacity of diacylglycerol acyltransferase to accept certain substrates was investigated. Kinetics of diacylglycerol acyltransferase purified from imbibed seed of N78-2245 and PI 123440 were determined with all possible combinations of seven molecular species of sn-1,2 diacylglycerol and four species of acyl-CoA. Results indicated genotypic differences in substrate utilization. It was concluded that the substrate specificity of diacylglycerol acyltransferase from these germplasm influenced triacylglycerol molecular species composition of the seed.

P-7 Oleic Acid Conversion to 10-Hydroxystearic Acid by *Norcardia* Species

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Five species of *Norcardia* catalyzed the formation of 10-hydroxystearic acid from oleic acid. Complete conversion was observed in 4 hr., and the yield of product was 40% to 53% based on GC analysis of the reaction products with methyl palmitate as an internal standard. 10-Kerostearic acid was a secondary product that appeared to be formed from oxidation of the primary product. The ratio of 10-hydroxystearic to 10-ketostearic acids varied among species. Oleic acid was apparently hydrated to form the product as evidenced by the formation of 9-deuterio-10-hydroxystearic acid when deuterated water was present. The enzyme system was specific for oleic acid; elaidic, erucic and *cis*-8-octadecenoic acids were not converted to hydroxy acids. Triolein was partially hydrolyzed by lipase. The released oleic acid was converted to hydroxystearic acid. Very little, if any, linoleic acid was transformed to hydroxy octadecenoic acid.

P-8 Molecular Biological Studies of Plant Lipoxygenases

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Preliminary studies indicated that tobacco DNA contains sequences homologous to soybean lipoxygenase (LOX) cDNA. Therefore, a tobacco genomic DNA library cloned in the lambda phage Charon 32 was screened with the soybean cDNA, coding for LOX. One Charon 32 clone was found to contain sequences very homologous to pLX10 and therefore was most likely a LOX gene. Most of this putative gene has been sequenced. In addition to its homology to soybean LOX cDNA, this gene contained the same apparent intron/exon splicing sites as the corresponding soybean genomic DNA, indicating a high degree of conservation of the soybean and tobacco LOX genes. To elucidate the physiological roles of LOX, the full length of cDNA of the LOX gene from soybean (pLX10) in the "sense" and "antisense" orientation was cloned into a vector suitable for the transformation of plants (pKYLX7). LOX RNA levels, proteins, and enzymatic activity will be evaluated with these constructs. Those plants with altered LOX activity will be investigated for changes in volatile lipid peroxidation product formation by different tissue organs.

P-9 Genetics of High Oleic Sunflower

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The segregation of heterozygotes (F_1) sunflower plants for high and low oleic acid content in the seed was studied at three different temperatures. The results show three different segregation patterns, 9:7, 27:37 and 3:1, which suggests that at least three genes are involved in oleic acid inheritance. A new analytical method was developed that could be used instead of the half seed method to identify germinating seeds from a pool of different phenotypes. This method was used to select seeds with special characters by analyzing part of one cotyledon.

P-10 Catalysis by a Lipase-Bearing *Rhizopus arrhizus* Mycelium in Fluorinated and Halogeno-Fluorinated Hydrocarbons

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A lipase bearing dead mycelium of *Rhizopus arrhizus* was found to be very active on fluorinated and halogeno-fluorinated hydrocarbons. Ester linkage synthesis and hydrolysis (included polyols), acidolysis and alcoholysis of glycerides, interesterification of fats, amide and thioester linkage synthesis were demonstrated in both batch and continuous conditions. This technology has several advantages over classical organic reaction media including: total non-inflammability, total non-explosivity, and very low toxicity. Under these conditions, the lipase exhibited high activity and good aging properties. A continuous fixed-bed reactor was run continuously for two months with tricholorotrifluoroethane as medium for tallow and sunflowerseed oil interesterification. The loss of lipase activity was only about 15% per month.

P-11 *Candida cylindracae* Lipase Catalyzed Interesterification of Butterfat and Butterfat Solid Fraction/Rapeseed Oil Mixtures

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Butterfat and butterfat solid fraction S_{24}/rapeseed oil mixtures were subjected to *Candida cylindracae* lipase catalyzed interesterification reactions. The lipase was immobilized by adsorption on Celite and the reactions were carried out in a medium of low water content to produce fats with low content of lipolysis products. To follow the nonspecific lipase catalyzed interesterification reaction, a method was developed to quantitatively determine triacylglycerols separated on capillary columns by acyl carbon number and level of saturation. To overcome the problems of irreversible adsorption, polymerization and degradation, silylation was achieved during on-column injection. Columns of low and medium polarity were studied. The effect of silylation on the degradation of the higher triacylglycerols was corrected by empirical correction factors. Coefficients of variation were only fair. However, the change in the triacylglycerol composition induced by nonspecific lipase catalyzed interesterification was so pronounced that analysis on capillary columns was a useful method.

P-12 Interesterification of Lipids Using an Immobilized *sn*-1,3 Specific Triacylglycerol Lipase

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The performance of an immobilized lipase (Lipozyme) from *Mucor miehei*, that specifically hydrolyzes the acyl moieties from the *sn*-1,3 positions of triacylglycerols, has been investigated in several interesterification reactions in the presence of hexane at 45 C. Triacylglycerol from ucuhuba (*Virola surinamensis*) that contained 90% 12:0 and 14:0 was used for interesterification with methyl esters of 13:0, 15:0, 17:0, 19:0 and 20:0; unesterified 17:0; trioleoylglycerol (sunflowerseed oil and corn oil); octadecyl alcohol and glycerol. Exchange (transfer) of acyl moieties occurred exclusively at the *sn*-1,3 positions of the triacylglycerols and the rates of interesterification with various substances were of the order: long chain alcohol $\gg$ fatty acid > triacylglycerol > methyl ester $\gg$ glycerol. Rate of interesterification of methyl esters decreased with increasing chain length. Melting behavior of the triacylglycerol formed by interesterification suggested potential uses in various food and dietetic products. In addition, these studies showed the wide utility of immobilized lipases for the production of a large variety of lipids, such as monoacylglycerols, diacylglycerols and wax esters.

P-13 Novel Methods for Studying Lipase-Catalyzed Interesterification of Lipids Using Radiochemical Techniques

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Reactions involving lipase-catalyzed interesterification of lipids have been monitored and assayed by radiochemical methods. Medium chain (C_{12} plus C_{14}) triacylglycerols were reacted in the presence of an immobilized lipase from *Mucor miehei* and hexane at 45 C with methyl [1-^{14}C]-oleate, [1-^{14}C]oleic acid, [carboxyl-^{14}C]trioleoylglycerol, [1-^{14}C]octadecenyl alcohol, and [U-^{14}C]glycerol. The rate of incorporation of radioactivity in acyl moieties of triacylglycerols (from methyl oleate, oleic acid, and trioleoylglycerol), alkyl moieties of wax esters (from octadecenyl alcohol), and into glycerol backbone of monoacylglycerols and diacylglycerols (from glycerol) was determined.

P-14 Kinetic Studies on Interesterification of Oils and Fats Using Dried Cells of Fungus

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Kinetic studies on enzymatic interesterification of oils and fats, using acetone dried cells of *Rhizopus chinensis* as the lipase catalyst, have been investigated in a batch operation. Several model systems based upon the ratio of triacylglycerol/fatty acid and water content were proposed to describe the reaction mechanism under the various conditions. A reaction between olive oil and stearic acid methyl ester was used to determine parameters in each model system. Experimental data was compared to values predicted with the model. The accuracy of the models was verified with data obtained from reactions with stearic and palmitic acid methyl esters where the ratio of triacylglycerol/fatty acid and water content were varied. Based on the first order kinetics of the triacylglycerol enzyme complex, the predictive value of the models was greatly useful under a wide range of reaction conditions. These data suggested triacylglycerol was formed during interesterification of oil and fats.

P-15 Oleic Acid Ester Synthesis with Enzymes

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Oleic ester synthesis was studied with lipase from *Mucor miehei* in a batch reactor where the lipase was immobilized on a macroporous anion exchange resin. The lipase was specific toward the sn-1,3 ester bonds of triacylglycerol and catalyzed esterification and interesterification reactions. The activity was 23 BIU/g. The degree of conversion was determined by measurement of acid values. The influence of time and pressure on the yield of oleic acid esters with short chain fatty alcohols was studied with equimolar solutions of fatty acid and alcohol. Sufficient yields were obtained with alcohols having more than three carbon atoms. The enzyme activity was achieved with primary alcohols and decreased with iso)alcohols. The lowest enzyme activity was achieved with secondary alcohols. Vacuum affected reaction rates and equilibrium due to removal of coproduced water from the high boiling substrates. Addition of n-hexane had no influence on the reaction yields.

P-16 Enzymatic Acidolysis Reaction of Some Fats

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The present work has investigated the modification of composition and properties of some fats by incorporating a desired fatty acid in appropriate amount and selectively in the triglyceride molecules by acidolysis with the aid of *Mucor miehei* lipase. Sal (*Shorea robusta*) fat and mango (*Mangifera indica*) kernel fat which are highly deficient in

palmitic acid content have been enriched in their palmitic content by acidolysis reaction with palmitic acid in presence of *Mucor miehei* lipase. Slip melting point and SFI have been significantly changed by this process. Fats from mowrah (*Madhuca latifolia*) and palm (*Elaeis quineensis*) have been enriched with stearic acid in the presence of *Mucor miehei* lipase. As a result of increase stearic acid content, the slip melting point and SFI properties of the two modified fats became different. The enzymatic acidolysis reaction involving the use of *Mucor miehei* lipase showed considerable promise in altering fats for making value-added confectionery and shortening products. Reaction parameters, chemical composition and properties of the acidolysed products and the original fats will be presented.

P-17 Palm Oil Splitting by an Immobilized Lipase

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Palm oil is becoming an increasingly important source of oleochemicals. Conventional splitting of palm oil utilizes physical and chemical methods that often are energy intensive. Modern enzymic biotechnology would greatly reduce the cost of this operation. In our laboratory, an enzymic method has been devised to split RBD palm oil, palm olein and palm stearin completely within a few hours at room temperature. A yeast (*Candida rugosa*) lipase was successfully immobilized onto acid-treated Florisil by an adsorption mechanism. The properties and performance of the immobilized lipase in batch experiments and column reactors could be regenerated and reused several times without significant loss of performance. The acid-treated Florisil also could be recovered and reused. The significance of this method in relation to the splitting of oils and fats is discussed.

P-18 CALO (Low-Calorie) Fat Development

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The announcement that the U.S. Food and Drug Administration will review olestra which will replace 35% to 70% of triglycerides and whose basic patent will expire in a year brings into focus the subject of Calo fats. Eighty existing technologies and several products, including one from tapioca and one from potato starch, already are marketed. Reach Associates estimates that 16% of the edible fats and oils industry in two decades will be of the Calo and Cal variety. A scenario of various technologies and marketing aspects for various products will be given, as well as the effect of recent patents such as U.S. 4,626,441. Biotechnology and special properties required will be stressed in the panorama including their impact on the supermarkets, prepared foods, fast foods and restaurants.

P-19 Microbial Production of Different Sophorose Lipids and Their Biochemical Conversion

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Under nitrogen-limiting conditions in a complex nutrient media, the yeast *Torulopsis bombicola* overproduces biosurfactants. Using combinations of glucose with either soybean oil, oleic or stearic acid methyl ester or oleic acid up to 70 g/L, pure glycolipids were excreted into the medium. The structural elucidation of these products revealed different sophorose lipids of nonionic and anionic character. Depending on the carbon source, a saturated or unsaturated hydroxy fatty acid was glycosylated to the sophorose. In some compounds, the acid was free; in others, linkage of the acid at the C-4-position of the sophorose led to lactonic forms. Most of the sophorose lipids carried one or two acetate groups. Two approaches were used to produce lactonic lipid without acetate groups: (a) deacylation without cleavage of the lactonic ring by use of acetyl esterase and the lactonic diactoxy sophorose lipid, and (b) acidic desacetoxy glycolipid products were used for re-lactonization by chemical and biochemical methods. Esterases of *Tornlopsis bombicola* and *Mucor miehei* were tested in detail.

P-20 Surface Active Properties and pH-Sensitive Conversion of Molecular Aggregation in Rhamnolipid Biosurfactant

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Studies were carried out to evaluate rhamnolipid B [2-O-(2-O-α-decenoyl-α-L-rhamnopyranosyl)-α-L -rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoic acid] and its precursor as a microbial biosurfactant. These compounds, produced by a hydrocarbon)assimilating bacterium *Pseudomonas aeruginosa* BOP 100, elicited large micelle formation, small critical micelle concentration, and reduced surface or interfacial tension comparable to synthetic surfactants. Moreover, highly efficient gross effects were obtained in dispersing, emulsifying, foaming and penetrating actions. It was found that altered pH of the medium caused reversible changes in the molecular aggregates of these membranous constituents, from micelle to vesicle.

P-21 Characteristics of Spiculisporic Acid as a Polycarboxylic Type Biosurfactant

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Spiculisporic acid, 4,5-dicarboxy-4-pentadecanolide, (S-acid) may be produced from glucose by *Penicillium spiculisporum* Lehman (No. 10-1) with a yield of 110 g/liter of the culture broth. This acid and the analogous compound, 3-hydroxy-1,3,4-tetradecane-tricarboxylic acid (O-acid) were converted to amphiphiles by neutralizing with inorganic or organic alkali, and by amidating with long chain alkylamines. Sodium salts of S-acid decreased surface tension ($gamma_{cmc}$=32 mN/m) and showed unique properties for emulsion polymerization for cosmetics. Alkylamine salts had greater ability to lower critical micelle concentration, greater gel)forming properties and higher dispersing actions for solid particles than sodium salts. Furthermore, the vesicle formation of octylamine salts and polyaklyl derivatives were observed by means of negative stain transmission electron microscopy, polarized and fluorescent microscopy and differential scanning calorimetry. It was concluded that spiculisporic acid had unique properties as a polycarboxylic surfactant.

P-22 Downstream Processing of Fatty Acid/Lipid Mixtures Using Membranes

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Fat hydrolysis and esterification are important processes for the food and agricultural industry. Technologies for using lipase enzyme reactions in membrane reactors have been developed for this purpose. A mixture of fatty acids and glycerides was produced from triacylglycerol hydrolyzed in a lipase hollow-fiber reactor. The rate of the hydrolysis reaction decreased with increasing fatty acid content. Therefore an on-line removal step was needed to keep the fatty acid content of the oil stream as low as possible. Hydrophobic microfiltration membranes used in series were tested and shown to achieve this need. An instable emulsion was formed by adding a solution of sodium hydroxide and sodium chloride to the oil. The emulsion droplets were forced through a hydrophilic microfiltration membrane, which was impermeable to the emulsion phase. The effect of adding alcohols to this system was investigated. These results and other methods will be discussed.

P-23 The Production of Fatty Acids by Lipase Immobilized in a Membrane Reactor

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Triacylglycerols may be converted to fatty acids and glycerol by lipase enzymes. For economic reasons, industrial application of this process requires the lipase to be immobilized in the reactor on a membrane. The membrane consists of hollow fibers made of cellulose. The enzyme is fixed on the inner side of these fibers through which the lipid phase is circulated. The water phase, outside the fibers, impregnates the fiber wall supplying the reaction water. The enzyme is maintained at the interface of the two phases for optimum reaction rates. The glycerol formed during reaction diffuses to the outside of the fibers and is carried in the aqueous phase, while fatty acids remain in the oil phase. Several batch and continuous experiments, which give information about the kinetics of the reaction, were performed. The influence of temperature, composition of the water phase and flow rates on the process were determined. Results with different enzymes of varying purity will be discussed.

P-24 Membrane Reactor for Immobilized Enzyme Esterification of Mono-, Di- and Triacylglycerol

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The food industry requires special acylglycerols of quality standards that are not always present in the original feedstock. Chemical processing of these acylglycerols involves a complex process often at both high temperatures and pressures. Enzymatic processing, using less extreme conditions, may offer great advantages. However, in simple stirred tank reactors, the high surface activity of the protein causes a very stable emulsion to be formed. An investigation was conducted on the enzymatic production of acylglycerols using an immobilized enzyme membrane reactor. In a model system, decanoic acid in organic solvent was circulated through the fibers, allowing the synthesis of acylglycerols at the membrane surface. The membrane prevented the emulsification of the acylglycerols/fatty acid and the glycerol/water phases. The esterification was dependent on the type of lipase used. Synthesis of specific products may be obtained by optimizing the process conditions.

P-25 Destruction of Foams and Recovery of Liquids in Bioreactors with the Aid of Centrifugal Forces

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The major purpose of bioreactors could be described as a mass transfer process that is based on microbiological reactions. Biological catalysts—enzymes—can play an important role in such processes. However, it is inherent to the process that a sufficient amount of oxygen be made available to ensure the expected growth of the microorganisms. This results in the formation of foam

that may obstruct further processing of the biomass. The degassing centrifuge as described in U.S. Patent No. 4,030,897 may be used to extract gasses, such as air, nitrogen and carbon dioxide from viscous liquids produced in bioreactors. A specially designed model of the centrifuge may be used to destruct the foam and to recover the liquid contained in the foam. Some applications of the degassing centrifuge will be presented.

P-26 Continuous Glycerolysis of Fat by Lipase with Membrane Bioreactor and its Scaleup

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Continuous glycerolysis of edible oil by *Pseudomonas fluorescens* lipase was performed in a microporous hydrophobic membrane bioreactor. Two kinds of flat membranes, polypropylene and poly-(tetrafluorethylene), were compared. Poly-(tetrafluorethylene) gave a somewhat higher conversion than polypropylene at all flow rates. Water concentrations higher than 4% (crude enzyme) and 3% (pure enzyme) were used. On-line continuous measurement of electric conductivity was used to monitor the moisture content of the microaqueous glycerol containing the enzyme. The microporous hydrophobic membrane bioreactor was scaled up with a 16-fold increase of units. Total area of the membrane ranged from 0.0726 m_2 to 1.16 m_2. Two operational modes, parallel and series oil flow, were compared. Results demonstrated no change in the relation between conversion rate and oil flow rate per sheet of the membrane. The product contained 5-25% monoacylglycerol, 30-50% diacylglycerol and 25-35% triacylglycerol. Half life of the enzyme during the continuous reaction was ca. 3 weeks at 40 C.

P-27 Fundamentals of Process Engineering for Application of Immobilized Lipases

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The development of an application for commercial usage of immobilized lipases requires systematic investigation of engineering and reaction kinetic boundary conditions. Important parameters include the reaction velocity, external and internal mass transfer limitations, backmixing of the fluid in the reactor, and pressure drop of the immobilized enzyme bed. Studies were performed in small fixed bed reactors. The pressure drop of the biocatalyst bed was determined as a function of the temperature of the fluid (olive oil), the flow rate, the height of the fixed bed, the average particle diameter of the biocatalyst, and the diameter of the reactor. Mathematic models were developed and used to scale-up immobilized enzyme systems. Enzyme kinetics for an interesterification reaction involving two intermediate steps were calculated and compared to experimental results. The overall effectiveness factor for a spherical porous biocatalyst was described by an axial dispersion model with boundary conditions for a closed system. Predicted conversions and experimental results will be discussed.

P-28 Large-Scale Separation of Unsaturated Fatty Acids and Their Esters by Centrifugal Partition Chromatography

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Highly purified unsaturated fatty acids and their esters have been separated by centrifugal partition chromatography in preparative scale from cereal, fish and microbial oils. This system, which was designed and constructed in our laboratories, consists of a flow-through type centrifuge equipped with liquid-liquid partition micro-cells, high pressure constant flow pump, UV monitor and fraction collector. The system is applicable for preparative or industrial separation through liquid-liquid partition chromatography (normal and/or reversed phase) using no solid support (resins) for the stationary phase. Fatty acids and their esters (linoleic, linolenic, arachidonic, icosapentaenoic and docosahexaenoic) were separated in hundred gram quantities from their crude materials with more than 98% purity. Feasibility studies of separation processed were performed using this technology and other conventional separation methods. Results will be discussed. Centrifugal partition chromatography also may be used as a bioreactor for the enzymatic esterification and hydrolysis of the fats and oils.