0273-2289/96/6101-02—0173$09.00

Research Activities in the Field of Enzyme Engineering in the Framework of the Russian State Scientific Program "Novel Methods in Bioengineering"

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ABSTRACT

The article describes the research activities in the field of enzyme engineering in Russia. The discussion is focused on fundamental studies of biocatalytic processes that expand utilization of enzymes, biocatalytic synthesis of organic products from renewable raw materials, enzymes for hydrolysis of cellulose and lignocellulose materials, immobilized cells, new enzyme-based drugs, enzymes in fine organic synthesis, bioanalytic devices, biosensors, and biofuels.

Index Entries: Enzyme engineering; biotechnology; biocatalysis; enzymes; biosensors; biofuels.

INTRODUCTION

Progress in modern technology is hardly possible without effective biocatalysts, which are characterized by high efficacy and responsible for a high quality of the final products. It is thus not surprising that studies in the field of fundamental and applied biocatalysis, which are carried out in Russia and in the countries of the ex-Soviet Union, are very broad. Research in this area has been very intensive in the past 20 years. Thorough studies of the research group headed by I. V. Berezin (1923–1987) (*1*) carried out

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at the Moscow State University resulted in formulation of the general principles of enzyme stabilization. This concept significantly stimulated a conversion of enzyme engineering into an independent branch of science in the 1970s. In 1974, Berezin, a bright pioneer in the field, founded the Division for Chemical Enzymology at the Department of Chemistry of Moscow State University, which later became the leading organization in enzyme engineering in the framework of the state program "Novel Methods in Bioengineering." Since 1989, several research projects in the field of enzyme engineering have received state financial support. The funding is run by the Scientific Council "Enzyme Engineering." One of its tasks is the distribution of funds on a competitive basis. As a result, approx 200 research groups are funded all over the country. The main areas that are believed to be the most important in terms of the funding are the following:

Selected fundamental investigations in the field of biocatalysis, aimed at elucidation of the enzyme structure and mechanisms of catalysis relevant to enzyme functioning in nontraditional media.
Biocatalysis in the organic synthesis.
Enzymes and enzymatic processes in hydrolysis of polysaccharides, lignin- and cellulose-containing materials.
Immobilized cells.
Enzymes in medicine.
Enzymes in analysis (immune and bioluminescent analysis and biosensors).
Biocatalytic processes in production of fuels.

FUNDAMENTAL STUDIES OF BIOCATALYTIC PROCESSES THAT EXPAND UTILIZATION OF ENZYMES

Up to now, the basic fundamental physicochemical reasons responsible for the high efficacy and catalytic activity of enzymes were not well understood. The elucidation of reaction mechanisms involves both the traditional routines of the steady-state and nonsteady-state kinetics, and the new ones, such as electrochemical methods and pulse radiolysis. The mechanisms of action of the following enzymes are under study: NAD-dependent dehydrogenase (*2*), pyrophosphatase (*3*), luciferase (*4*), laccase (*5*), hydrogenase (*6*), prostaglandin H synthase (*7,8*), lignooxidase (*9*), glyceraldehyde dehydrogenase (*10*), and neurotoxic esterase (*11,12*). The kinetic information obtained is often compared with that for related mutant enzymes, including NAD-dependent formate dehydrogenase (*2*), luciferase (*4*), peroxidase (*13*), and pyrophosphatases (*14*).

NAD-dependent formate dehydrogenase was modified using the site-specific mutagenesis to afford the enzyme with altered specificity with respect to NAD^+. The enzyme that is insensitive to the mercury ions

has also been created (*2*). Several enzymes that use DNA or RNA as matrices have been investigated by Lavrik et al. (Institute of Bioorganic Chemistry, Siberian branch of RAS) (*15*). Mechanistic and regulatory studies of prostaglandin-H synthase resulted in principally new mechanisms of the activity regulation via the substrate-induced inactivation of the enzyme in the reaction course (*7*).

Receptor-enzyme systems with a protein receptor for the regulation of the adenylate cyclase activity were investigated by the example of the neuropeptide-modified receptors of the central nervous system (*16*). Similar studies involved adhesive thrombocyte receptors (*17,18*), visual receptors (*19*), and enzymes of olfactory epithelium (*20*). Super cooperativity in the thrombocite receptor-enzyme system was discovered and investigated in detail. The dependence of the reaction rate on the concentration of the substrate and several inhibitors showed the abnormally high cooperativity coefficient of 30–40 (*18*).

Of extreme importance is the enzyme functioning in nonaqueous solvents. A detailed study was devoted to the solvent effects on activity and stability of enzymes (*21,22*). Reverse micelles in water-immisible solvents proved to be tremendously convenient microreactors for enzymatic reactions (*23,24*). Remarkably, the highest enzymatic activity in reverse micelles is displayed by the enzymes on the geometric correspondence of the protein and micelle size (*25*). The ways to regulate the catalytic activity of α-chymotrypsin and its modified forms, alkaline phosphatase, alcohol dehydrogenase, and many other enzymes were elaborated. The principles of micellar enzymology allow enzymatic reactions to be performed with water-insoluble substrates in systems with a very low water content.

The key question of enzyme engineering concerns enzyme stability. Thorough studies of the kinetics of inactivation of enzymes were performed by Poltorak and Chukrai (*26,27*). As a result, a kinetic model for the inactivation of oligomeric enzymes was developed. An approach to the stabilization of enzyme layers at the solid–liquid interphase was also proposed (*28*). The behavior of enzymes in the Langmuir-Blodgett films was also investigated (*29*).

Much work was devoted to the regulation of the stability and catalytic activity of enzymes by variation of their microenvironment in solution. By the example of β-chymotrypsin, a correlation between the stability at elevated temperatures and the chaotropic properties of the medium was found (*30*). It was demonstrated that different substances and their mixtures affect the enzyme stability in an additive manner. The general "upper" limit for the α-chymotrypsin stabilization occurs owing to the change in the rate-limiting step. New homogeneous solutions of hydrophilic proteins in nonpolar organic solvents were obtained. Enzymes in such systems are catalytically active and possess high stability. They withstand a prolong heating at 100°C without noticeable inactivation (*30*).

BIOCATALYTIC SYNTHESES OF ORGANIC PRODUCTS FROM RENEWABLE RAW MATERIALS: ENZYMES FOR HYDROLYSIS OF CELLULOSE AND LIGNOCELLULOSE MATERIALS

An important trend in research is related to the development of processes utilizing renewable raw materials. There are but two fairly capacious sources of renewable raw materials: (1) biomass with cellulose, hemicellulose, starch, pectins, and lignin as the main components; and (2) carbon dioxide, the reduction of which can provide various products (*31*).

Fairly deep investigations of enzymes for hydrolysis of cellulose and lignocellulosic materials are under way (*32–35*). The bioconversion of CO_2 was also explored and effectuated at the laboratory level (*see* Immobilized Cells and Biofuels).

Evaluation and optimization of the conditions for cultivation of cellulase-producing organisms afforded a notable increase in production of the cellulase complex enzymes. The high-productivity strain *Trichoderma reesei* was created. It was used to develop and master the technology for industrial production of cellulases (*36*). The effect of the cellulase complex was studied kinetically to develop a mathematical model of the process (*36*).

Much research was performed on preprocessing of raw materials and on construction of efficient reactors for enzymatic hydrolysis. The most optimal process is the pretreatment by the "vapor explosion" (*37*). Two new hydrolysis tanks were designed: (1) a reactor with vibroagitation, and (2) a reactor with electromagnetic agitation (rotating magnetic fields and metallic ferromagnetics). In the latter reactor, the lightest productivity of insoluble cellulose was achieved (*38*). Bekker and Sinitsin have been examining the enzymes of ligninase type (*39*).

A principle advance step seems to be the high-temperature cellulose hydrolysis by thermophilic cellulases, which occurs at high rates, and the product is sterilized owing to low rates of cellulose (*40*).

Velikodvorskaya and Mosolova searched for thermophilic cellulases and transferred the thermophile genes into *Escherichia coli* (*40*).

Mosolova et al. also transferred the genes of endo- and exocellulase genera from *Clostridium thermocellum* (*41*). Van Syaoyui and Sinitsina studied cellulose hydrolyzates as substrates for microbiological production of amino acids (lysine and tryptophan) and ethanol as well for catalytic conversion of bioethanol into ethene (*42*).

IMMOBILIZED CELL

Complex biosynthetic reactions can be effected by use of immobilized cells incorporated in diverse natural or synthetic matrices. A traditional

procedure seems to be the entrapment of cells into various natural alginate agar-agar and gelatin gels, as well as in synthetic polymers, such as polyacrylamide gel (PAG). The latter procedure leads to the biocatalysts with "dead" cells usable for reactions involving one or a few enzymes. This approach was used to modify steriods (*43*).

The entrapment of the microbial vegetal and animal cells in the genes of synthetic polymers by cryoimmobilization was developed (*31,44,45*). The procedure is grounded on cooling the cell suspension in a polymeric solution down to 20 to −30°C to form the cryostructures stable up to 70–80°C. The biocomplexes obtained are advantageous since:

1. Cryoimmobilization results in high-porosity structures affording an efficient transfer of substrates, metabolites, and proteins; up to 10% of the total volume of cryogels can be occupied by immobilized cells;
2. The pellets are mechanically fairly strong, stable in a wide range of pH and concentrations of metal ions; and
3. Cryogels are stable up to 70–80°C and, therefore, suitable for immobilization of thermophilic microorganisms.

Using this technique, Rainina and Varfolomeyev obtained the catalysts for ethanol production by immobilized cells, for synthesis of lysine and riboflavin (*46,47*). The production of acetic acid by deep reduction of CO_2 by H_2 in the presence of immobilized cells of *Clostridium kivui* seems to be promising. The biocatalyst does not change its activity in a year (*31*). On the basis of cryoimmobilized cells, ecologically fair catalysts for degradation supertoxic of organophosphorous pesticide, such as Sarin and Soman, were created (*48,49*).

NEW ENZYME-BASED DRUGS

Immobilization of pancreatic inhibitor and some proteases on textile carriers was used to work out dressings for treatment of burns and purulent wounds. The Pharmacological Committee of Russia has issued permission for a clinical application of the preparations (*50,51*). The Bowman-Birk inhibitor from soybeans proved to be highly efficient in suppressing elastase and cathepsin G enzymes from leukocytes. It was chemically modified to enhance the tropism and to prolong the activity (*52,53*).

Proteinase inhibitors (soybean inhibitor, heparin, aprotinin, and its conjugate with dextran) were studied for suppressing the activity of the influenza viruses. (A and B types). An aerosol form of aprotinin was elaborated and clinically tested. The Russian State Center for expertise of drugs issued it for clinical applications (*54,55*). The inhibitor of α-glucosidases "Hypoglukin" was isolated from a microbial material and tested as a drug for diabetes (*56*).

Kost et al. studied the key enzyme regulating blood pressure, angiotensin-converting enzyme, to evaluate its inhibiting activity in burns of the eyeball. The damage was shown not to develop deeper and healed much faster on application of the inhibitors (*57*).

A bacterial transformation of γ-aminoglutamic acid into a basic component of cardiodrugs, "gamalon" (γ-aminomaleic acid) was performed (*50*). New dressings were elaborated on the basis of "collitin," the mixture of proteinases and nucleases of cattle pancreas immobilized on textile carriers (*50*). Clinical experiments showed an exceptional efficiency of these dressings in the therapy of contaminated wounds. The mechanism of interaction between the hydrophobic ribonuclease, as a potential anticarcinogenic drug, and cell was studied. The procedures for the enzyme entrapment in the cellular matrix are being searched for (*58*).

A new thrombolytic, viz. urokinase immobilized on thrombin, was elaborated at the Institute of Experimental Cardiology. The efficient preparation showed a high tropism to thrombus, a prolonged activity, and a notable decrease in by-effects. Maximenko optimized the isolation and purification of the initial components (*59*). Other thrombolytic preparation, viz. a complex of acylated plasminogen with streptokinase, showed a notable prolonged effect and the absence of by-effects. The synthesis of the plasminogen-acylating agents was optimized. The stability of protein preparations and their action, separate and as a mixture, were explored (*60*).

Komov developed the technology to isolate superoxide dismutase from medicinal herbs. The enzymatic preparations obtained were pharmacologically tested to show the lack of allergic aftereffects (*61*).

Studies of newly synthesized physiologically active low-mol wt thrombogenesis inhibitors, substituted 3-pyridilysoxasolines, are in progress. Vrzheshch designed a kinetic model describing the action of preparations in terms of the theory of signal transfer in the chain of secondary mediators (*62*). The recombinant protein-C was isolated, fostering the decrease in thrombogenesis resulting from stresses. A new concept was advanced and proved to obviate thrombogenesis by use of peptide inhibitors of thrombin (*63*).

New results were obtained concerning a possible synthesis of ecdysterone in cell cultures of plants for its subsequent modification to obtain a cardiotropic prolonged drug (*64*).

ENZYMES IN FINE ORGANIC SYNTHESIS

There has been a successful advance in designing the coupled biocatalytic reactors to obtain the labeled (α-, β-, and γ-phosphorus-32 and 33) nucleoside triphosphates. In 1994, the enzymes were coimmobilized to synthesize γ-phosphorus-33 labeled ATP on a nitrocellulose membrane. Kozlov tested a sample with the immobilized enzymes under the

conditions of industrial radioactivity (*65*). The first industrial syntheses in the suspension reactor proved that the interrelated system of elementary bioreactors based on immobilized enzymes is promising.

The first successful attempt to synthesize stereoisomers of fluorine-substituted β-amino acids was reported. Svedas synthesized new aminophosphonic and aminophosphonous acids, as well as aspartic acid analogs (*66,67*). Biocatalytic technologies for enzymatic synthesis of ampicillin and cephalexin are under way (*68–71*).

The enzymatic synthesis of peptides is in progress. New chromophore substrates of proteinases were developed for medical assays and enzyme production. Stepanov was successfully developing the method to apply enzymes for introduction and removal of protecting groups in peptide synthesis (*72*).

BIOANALYTICAL DEVICES

In the framework of the Russian National Program on Enzyme Engineering, the research is performed to improve the enzyme immunoassay and to develop analytical procedures based on bioluminescence (*73,74*).

The work was continued on elaboration of test systems to define the S-triazine herbicides (simazine at razine and 2,4-dichloro-phenoxyacetic acid) (*75,76*). The procedures to monitor the quality of antibodies were elaborated (*77*). Studies of interactions between antibodies and fluorescent labeled antigen offered the procedures for assaying herbicides. This was performed by the method of polarization fluoroimmunoassay and membrane chemiluminescent assay. The detection limit was 0.3–1 μg/L, and the assay time was 2–20 min. The herbicides could be detected in surface and subsoil waters (*78*).

The membrane immunoassay test with visual two-color semiquantitative detection of myoglobin was developed. Hallows were developed to differentiate the norm-pathology states during acute myocardial infarction (*79*). Based on immobilized firefly luciferase, the method was developed for specific express assay of intestinal bacteria in milk products. The industrial trials were performed on ice cream and smears taken from the industrial equipment of a diary factory (*80*). Bacterial luciferase and luminescent bacteria were used for environmental monitoring (natural and tap waters). The bioluminescent biotests were shown to be the most informative and to correlate well with other methods of ecological monitoring.

BIOSENSORS

Biosensors attract a fairly large amount of attention of researchers in various institutions in Russia. The research is directed at:

1. The theory of biosensors;
2. The development of analytical methods; and
3. The design of biosensor models with amperometric, potentiometric, optical, and gravimetric detection.

As a biologically sensitive component, antibodies, tissue sections, enzymes, DNA, and cells were used.

An appreciable role in development of this domain is played by the phenomenon of bioelectrocatalysis (*81,82*). In his reviews Varfolomeyev showed that the active sites of redox enzymes adsorbed on electron conductors and semiconductors were capable of direct electron exchange with the electrode. The direct electron fransfer becomes the basis for some reagentless amperometric and potentiometric biosensors (*83*). These works underlie wide research on this subject (*84,85*).

Kurganov developed a theory of functioning of mono- and two-enzyme amperometric sensors affected by various parameters (substrate concentrations, reaction product, inhibitors, membrane thickness, and so on) (*86*).

The methods were developed to obtain the ordered monolayers of biomolecules (enzymes and antibodies) by the Langmuir-Blodgett method. The film technology in use yielded the reproducible layers of biologically sensitive material, which increased the sensitivity and reproducibility of assays using biosensors. This method is especially important for designing "direct" biosensors based on recording the interactions of detectable ligand with receptor (*87*). Varfolomeyev and Bachurin published the first successful attempts on entrapment of enzymes into organic semiconductors and organic systems in the late 1970s (*88,89*).

The research is being performed on designing the amperometric and potentiometric biosensors based on enzymes entrapped in polymer semiconductor matrices. A procedure was developed to obtain electrodes covered with the polyanyline films toned with the Berlin blue dye. The modified electrode affords both potentiometric and amperometric measurements (*90*).

Yaropolov et al. elaborated a flow-injection method to quantify phenol compounds based on coimmobilized laccase and tyrosinase, which affords quantification of phenols up to $10^{-8}M$ (*91*). Ignatov et al. optimized the conditions for cultivation of bacterial cells used to detect phenol. Thus, a biocatalyst with a high and stable activity in phenoloxidation was created. The linear range of detectable phenol concentrations was 0.1–1 mg/mL; the biosensor response time was not more than 1 min, and the assay time was 5 min (*92,93*).

To develop biosensoric assay of thermophilic microorganisms, Reshetilov adapted the immunosensor on the basis of the field-effect transistor. The procedure provided an efficient sensor signal, about 5–10 mV/s. The assay is promising for express estimation of the content of anaerobic microorganisms (*94*).

Yevdokimov et al. explored the liquid-crystalline dispersions formed from DNA complexes with three porphyrins of different structure. Liquid-crystalline DNA dispersions were formed that had three anomalous bands in the CD spectrum. This fact opens a principal possibility for constructing a biosensor containing the stained compounds, which can differently respond to the presence of various biologically active species in a test sample (*95,96*).

Among the biosensor models that were created are an analyzer of glucose in dissolved blood based on immobilized glucosidase for express assay in the range of glucose concentrations 2–20 mmol/L (the instrument has the technological documentation; tentative medical trials have been performed); a multichannel gravimetric sensor for "direct" assay of various antigens up to 10^{-8}g/cm^2 (*97*); "Multiferm," a new modification of flow-injection analyzer for assaying various compounds by use of cryoimmobilized cells and enzymes (*98*); "Optical immunosensor," based on monolayers of antibodies labeled with fluorescent compounds (*99*); an optical biosensor on liquid-crystalline DNA complexes (*100*); an electrochemical biosensor for nervous toxins involving "direct" electron transfer (*101*); and an optical fiber biosensor with oxygen detection from fluorescence change (*102*).

A series of publications from the Ryabov group is also closely related to the modeling of biosensors (*103–107*). They are based on a combination of enzymes with organometallic compounds (*108,109*).

BIOFUELS

Biofuels are interesting because of a progressing depletion of mineral fuels and a growing contamination of the environment by organic wastes and carbon dioxide.

Gogotov et al. studied hydrogenases from various sources and found the strains showing a high hydrogenase activity (*110*). Some hydrogenases were isolated and thoroughly characterized, such as *Thiocapsa roseopersicina* (*111*) and *Alcaligenes eutrophus* (*112*). A homogeneous hydrogenase preparation was obtained from an extremely thermophilic sulfur-reducing bacterium. The preparation consists of two subunits. The enzyme activity increased threefold on increasing the temperature from 85–100°C. Hydrogen-producing activity increased 50-fold on temperature elevation from 50–100°C.

Varfolomeyev and Karyakin studied hydrogenases that split hydrogen heterolytically (*113,114*).

Photoprocesses occurring in semiconductors (CdS and TiO_2) were compled with hydrogenases (*115*). Photogenerated cadmium metal was involved in the electron transfer from the inorganic semiconductor to

hydrogenases (*116*). The mechanism of photoformation of hydrogen and photoeeduction of nicotinamide coenzymes using the inorganic semiconductor CdS and enzymes (*T. roseopersicina* hydrogenase or NAD-dependent hydrogenase *A. eutrophus*) was investigated (*117*).

The photocatalytic system was obtained for the NAD photoreduction in the course of mediatorless coupling of the inorganic semiconductor CdS and *A. eutrophus* hydrogenase (*118*).

Kalyuzhnyi and Nojevnikova conducted a systematic study of the mechanism of methane generation by the methane-producing cultures (*119*). They examined a wide range of organic substrates, such as aliphatic alcohols and acids, amino acids, carbohydrates, and proteins. The kinetics of the process was investigated in detail. Varfolomeyev and Kaluzhny put forward a generalized mathematical model of the system (*120*). The systems were designed to purify organic sewage in the presence of methane-producing bacteria (*121*).

A global contamination of the environment by carbon dioxide and carbon monoxide encourages the development of biocatalytic systems for CO and CO_2 conversion into useful products. The Russian National Program on Enzyme Engineering involved three projects:

1. Elaboration of the systems for CO and CO_2 conversion to obtain molecular hydrogen;
2. Biocatalytic reduction of CO_2 and CO into acetic acid; and
3. Production of carbohydrates.

The strains of anaerobic thermophilic bacteria were isolated and have shown that CO is the only source of carbon and energy. The cells of the strain Z-2906 were found to contain highly active enzymes of lithotrophic metabolism, including formate dehydrogenase, hydrogenase, and CO-dehydrogenase. The CO-dehydrogenase strain 2906, possessing high activity and stability, was characterized; the enzyme retained the activity up to 135°C, the optimum was at 109°C. The cell suspensions were capable of active evolution of H_2 70°C according to the equation $CO + H_2O = CO_2 + H_2$ (*122*).

A semicontinuous process was designed to obtain acetic acid via a deep reduction of CO_2 by molecular hydogen (*31*) in the presense of immobilized thermophilic acetogenic bacteria *Acetogenium kivui*. The catalyst is active within a year without loss in catalytic activity at 65–70°C. The possibility of CO_2 reduction in the biocatalytic mode was analyzed. Bioelectrosynthesis was considered an alternative to photosynthesis (*123*).

Interesting pioneer works were performed on reduction of CO by hydrogen into carbohydrates, alcohols, and organic acids. The production of carbohydrates $C_8 - C_{24}$, methanol, ethanol, and acetic acid by the cell-free extracts from *Desulfovibiro* was shown to be feasible (*124,125*). The authors studied the influence of various physicochemical parameters of the reaction (temperature, pressure, CO concentration, pH, reaction time, enzyme-substrate ratio) on the catalytic activity of homogeneous

biocatalysts in the synthesis of organic compounds from CO and H_2. To obtain a stable biocatalyst and to incrase the yield of reaction products, the cell extracts were immobilized on a carbon fiber and amberlite. Active preparations were obtained, and their basic properties studied, such as capacity, specific activity, and thermostability. Immobilized biocatalysts were tested in CO transformation. The overall yield of organic compounds was 10-fold compared to noimmobilized extracts, whereas the productivity of the biocatalyst (0.18–0.2 g product/g catalyst/h) was comparable with the industrial catalysts (*126*). The research on potentials of this process by use of immobilized cells is under way.

The analysis of the studies performed and the results obtained in the field of enzyme enzymology showed that the most promising areas are

1. Organic and microbial synthesis based on immobilized enzymes and cells and using renewable raw materials;
2. The elaboration of novel bioanalytic methods and devices for medicine, agriculture, and the monitoring of technological processes and environment; and
3. The creation of new physiologically active compounds and drugs.

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