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CURRENT STATE OF THE STUDY OF MICROBIAL LIPASES

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This review gives a comparative analysis of information accumulated over the past 15 years on the isolation, purification, properties, and use of lipases of microbial origin.

The choice of lipases (E.C. 3.1.1.3 triacylglycerol acylhydrolases) as objects of study is due to the following considerations. In the first place, they are of interest from the point of view of general enzymology – as enzymes acting at an interphase surface. In the second place, interest in them is due to numerous practical problems and, above all, the employment of lipases as special "tools" for studying the composition of membranes and their use for concrete biotechnological processes connected with the treatment of lipid-containing raw material [1].

The natural substrates for lipases are triacylglycerols; however, other insoluble or partially soluble esters of glycerol and some esters of other alcohols are also capable of being hydrolyzed by this class of enzymes. Soluble esters are hydrolyzed by lipases extremely slowly since they do not accumulate at a phase separation surface [2].

Lipolytic enzymes are widely distributed in Nature. Sources of lipases are animal and vegetable tissues and microorganisms. The last-mentioned group of producing agents is attracting the intense attention of scientists through their advantages over other sources [3].

The synthesis of many hydrolases, including lipases, by the producing microorganisms can be regulated and directed by the choice of appropriate conditions of cultivation and, in particular, the composition of the nutrient medium. Moreover, many microbial enzymes are formed in response to the action of an inductor added to the nutrient medium, the activity of the induced enzyme rising many times during the growth of the microorganism in response to the addition of a specific substrate, while in a medium without the appropriate inductor the enzyme is formed in minimal amounts [4, 5].

Microorganisms possess the capacity for synthesizing extracellular enzymes the activity of which many times exceeds the level of activity of intracellular enzymes; i.e., a capacity for performing "supersynthesis." All this determines in full measure the promising nature of the microbiological synthesis of lipases.

The progress achieved in the study of microbial lipases has made possible the solution of a whole series of practical problems [6, 7] dictated by the tendencies in the development of the oils and fats industry and the necessity of obtaining products with predetermined properties. A no less important role in the stimulation of investigations of microbial lipases has been played by the demands of the medical and pharmaceutical industry and of public health – indeed, the etiology, prophylaxis, and treatment of many diseases are connected with the functioning of lipolytic enzymes [8]. The possible spheres of employment of these enzymes expanded after it became known that, in addition to their hydrolytic capacity, lipases possess the capacity for catalyzing such reactions as the synthesis of glycerides, the acylation and alkylation of lipids, esterification and transesterification reactions, ammonolysis, oximolysis, thioacyl and thioalkyl exchange, etc. [9, 10]. Many of these reactions are possible only in nonaqueous media, where there are changes in the characteristics of the enzymes – specificity and optimum conditions of functioning, catalytic parameters, and demands on the chemical structure of the substrate [11-14].

Results obtained in the last 10-15 years show that microbial lipases are acquiring deserved employment in fine organic synthesis, petrochemistry, the manufacture of pharmaceuticals, and the production of basically new types of surface-active agents [15-17].

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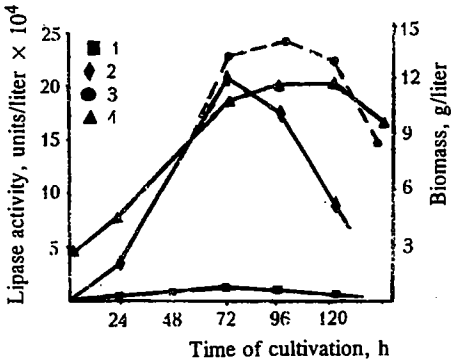

Fig. 1. Change in the level of lipase activity of the fungus *Mucor miehei* during its cultivation on the addition of various components to the medium. 1) Minimal medium with glucose (basal level); 2) medium with the addition of cottonseed oil (Ist threshold level); 3) medium with the addition of cottonseed oil and β-mercaptoethanol (IInd threshold level); 4) accumulation of biomass.

In addition to what has been said, the advances achieved in the development of effective methods for stabilizing and immobilizing microbial lipases have made realistic their use for the solution of concrete applied problems [17-19].

CLASSIFICATION AND SPECIFICITY OF LIPASES

According to the international nomenclature, lipases (E.C. 3.1.1.3 – triacylglycerol acylhydrolases) are hydrolases cleaving the ester bonds in a triglyceride molecule. In the classification of lipases the starting point is the fact that their substrates are triglycerides. On this basis, all lipases can be divided into three groups, depending on the positions and structures of the fatty acids (FAs) in the triglyceride molecule: 1) nonspecific lipases liberating any FAs from any position of a triglyceride (for example, lipases from *Candida rugosa* and *Oospora lactis*); 2) 1,3-specific lipases liberating FAs from position I or III of a triglyceride (lipases from *Rhizopus microsporus*, *Mucor miehei*, etc.); and 3) lipases liberating only a particular type of FA from any position of a triglyceride (for example, a lipase from *Geotrichum candidum* preferentially hydrolyzes *cis*-Δ9 unsaturated fatty acids). According to this classification, some lipases prove to be simultaneously in two groups (for example, the first and third) [20]. In order to resolve this contradiction, Jensen [21*] proposed an improved classification of lipolytic enzymes on the basis of their specificity. He distinguished: 1) substrate specificity (specificity to mono-, di-, or triglycerides (MGs, DGs, or TGs); 2) positional specificity (specificity for a definite position of a triglyceride); 3) fatty-acid specificity (for a definite type of fatty acid); 4) stereospecificity; and 5) a combination of 1) and 4). This classification, based on the specificity of lipases, is the most popular, the most frequently used, and most convenient; however, it does not take into account the fact that the substrates of lipases are not only glycerides but also other esters.

Some authors propose to classify lipases according to their specificity with respect to the chemical structure and positions of the alcohol moiety and the fatty acid residue of esters [22]. Thus, it is possible to distinguish lipases specific for an alcohol residue with respect to: 1) chain length; 2) structure (i.e., primary or secondary alcohol or polyol); 3) the position of the ester bond if the alcohol is a polyol; 4) branchings (if an aromatic substituent is present); and 5) stereochemistry (i.e., sn-1 or sn-3 position of a glycerol residue). And lipases specific for a particular carboxylic residue, according to: 1) chain length; 2) unsaturation (configuration of the double bonds); 3) branching (closeness to the carboxy group and nature of the branched groups); and 4) stereochemistry (i.e. S- or R-isomers).

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*[Sic], Ref. [21] does not refer to Jensen, who appears as the author of [22]. Other, similar, probable discrepancies in the numbering of the references have been detected and there are probably undetected ones as well – Translator.

TABLE 1. Purification of the Extracellular Lipases of the Fungus *Mucor miehei* UZLT-3

Stage of purification	Total amount of protein, mg	Lipase activity		Yield		Degree of purification
		specific, units/mg	total, units	protein	activity	
Filtrate of the culture liquid	5000	800	4000000	100	100	1
Precipitation with ammonium sulfate	1620	2050	3321000	32.4	83	2.5
DEAE-TSK 650M	325	9600	3120000	6.5	78	12
TSK-55 HW						
I (A)	120	16000	1920000	2.4	48	20
II (B)	76.6	12000	920000	1.5	23	15
G-100						
I (A)	35	48000	1680000	0.7	42	60
II (B)	30	24000	720000	0.6	18	30

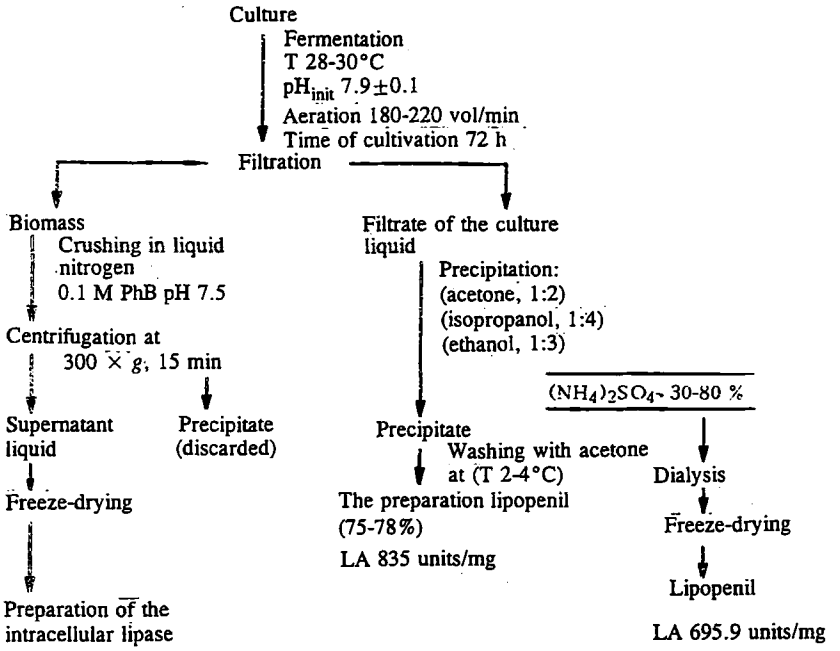

Fig. 2. Scheme for obtaining a complex preparation of microbial lipases.

Until now, the question of the interrelationship of the specificity and classification of lipases has remained open, and in the majority of cases a lipase is classified in accordance with the international nomenclature.

So far as concerns the specificity of lipases as such, this is a very important separate aspect. Of practical interest are not only the positional specificity but also the stereospecificity of the lipases, since the latter can be used to separate the optical isomers of a number of compounds [23, 24]. Stereospecificity in relation to derivatives of enantiomeric acylglycerols has been studied most carefully by Ransac et al. [25, 26].

It must be mentioned that the specificity of some microbial lipases can be regulated by changing the conditions of cultivation of the producing organisms. Below, we consider some features of cultivation exerting an appreciable influence on the productivity of the cultures and the characteristics of the enzyme synthesized.

CULTIVATION OF LIPASE-PRODUCING AGENTS

The cultivation of lipase-producing agents depends largely on factors of the medium (temperature of cultivation, composition of the nutrient medium, and, above all, the sources of nitrogen, carbon, and lipids, the concentration of inorganic

TABLE 2. Some Properties of Microbial Lipases

Producing agent	MM × 1000	pI	Amount of carbohydrates, %	A_{sp}, units/mg × 1000	Literature
Pseudomonas fluorescens	32	4.5	0	4.2	[85]
Ps.fluorescens 533-58	320		26		[86]
Asp. niger	25	4.6	10	24	[87]
Candida cylindracea	120	4.2	4.2	1.14	[88]
Geotrichum candidum	54	4.3	7.0	0.45	[89]
Humicola lanuginosa	27.5	–	0	1.5	[90]
Mucor javanicus		–	2.6	1.3	[90]
	21				
Rhizopus arrhizus					
	43	6.3	6.7	9.3	[91]
Rhizopus delemar					[93]
	41	4.2	–	4.0	
Penicillium roqueforti,	7.93	–	–	–	[94]
141	9.1				
	11.4				
Oospora lactis*	40.0	4.7	0	18	
	43	4.9	5.0	27	
	65	–	–	21	
Rhizopus microsporus*	28		12.1	30	
	39		8.5	21	
	43		15.8	45	
	63		5.2	15	
	69		10.1	27	Results from
Mucor michei*					
form A	43	4.9	–	48	the laboratory
form B	30	4.7	–	24	
Penicillium* sp.					of microorganic
form A	30	4.2	–	37	enzymes
form B	37	4.8	–	43	

*Results obtained by the authors.

salts, the availability of oxygen, etc.). The sources of carbon, nitrogen, and oxygen exert a particularly strong influence on the properties of the enzymes produced by the strains and on the ratio of the extra- and intracellular forms of the enzyme [27].

The synthesis of lipases is regulated by certain fatty acids, but it is impossible to choose the overall optimum concentrations of these acids in order to obtain different lipases [28, 29]. For example, during the fermentation of the lipase from *Streptococcus fecalis*, short-chain fatty acids exerted a stimulating effect, while long-chain unsaturated (oleic) fatty acids had no effect. A similar picture has been observed by other workers [30, 31].

The influence of polysaccharides on the elaboration of some lipases has been investigated in detail [32]. It was found that polysaccharide molecules act through some limited number of sites and not through electropolarity [33].

Inorganic ions such as Na^+, PO_4^{3-}, and Ca^{2+} are also basic for the growth of microorganisms [34, 35], while the action of iron ions on their growth has an ambiguous nature [36]. Experimental results on the influence of metal ions on the formation of lipases by microorganisms have hitherto been of debatable nature and the mechanism of their influence has not been discovered, although a number of hypotheses explaining this fact have been expressed.

We have investigated the influence of various classes of detergents and mercaptans on the secretion of lipases by microorganisms. It was established that some Tweens, Spans, and mercaptans cause a considerable increase in the secretion of lipases. This process is not due to an intensive accumulation of biomass or to the removal of the enzymes from the cell surface of the producing agents. In the strains of micromycetes that we studied, the synthesis of lipases is inducible and is subject to catabolite repression; nevertheless, when they were grown on minimal medium with glucose little synthesis of these enzymes (basal level) was observed (Fig. 1). In a medium with vegetable oils the secretion of a lipase is regulated by its level in the medium (first threshold level).

On the basis of the experimental results obtained, we have proposed the following hypothesis of the mechanism of the regulation of the synthesis and secretion of lipases in micromycetes. The genes responsible for the synthesis of lipases are probably included in one inducible operon (the lipase operon) with positive regulation. In the absence of an inductor (oil) only a background synthesis of enzymes ensuring their basal level in the medium takes place. On the appearance of an inductor (vegetable oil) an intensive synthesis and secretion of enzymes up to their first threshold level begins, leading to the saturation of the receptors of the cell surface, a signal from which showing a cessation of enzyme synthesis passes to the translation apparatus. Some detergents (Tweens, Spans) block these receptors nonspecifically (while mercaptans probably impair their capacity for binding the enzymes), the signal showing the cessation of synthesis is not formed, and synthesis with secretion continues up to the second threshold level.

Numerous investigations by other workers have confirmed the activation of the synthesis of lipases on the addition of vegetable oils to the medium for the growth of a number of producing fungi [37, 38]. However, other researchers [39] have reported a directly opposite effect.

It must be mentioned that the synthesis and secretion of lipases by microorganisms depend not only on the composition of the nutrient medium and the conditions of cultivation but also on features of the producing strain. Consequently, for each new strain that is a potential lipase producer it is necessary to carry out model investigations to select the composition of the nutrient medium and the conditions of cultivation.

PURIFICATION OF LIPASES

In the past few decades the demand for large amounts of microbial lipases has risen sharply. Their use in large-scale manufacture and in chemical and physical scientific investigations and for clinical purposes has stimulated a rapid growth of technologies for the high-efficiency extraction and purification of enzymes [40].

The final yield of an enzyme depends to a large degree on the choice of the actual source of the lipase and of the conditions for its growth.

Various methods are used for the purification of lipases, as also of other microbial enzymes: electrophoresis, electrofocusing, differential ultracentrifugation, zonal sedimentation, ultrafiltration, electrodecantation, chromatography, etc.

Intracellular lipases must first be isolated from the cells. Its own specific method of isolation is used for each microorganism [41].

We have developed a scheme for obtaining complex lipase preparations from many fungi (Fig. 2), including *Penicillium sp.* and *Mucor miehei*.

The purification procedure was a multistage process during which we used both traditional methods and the specific expedients required for obtaining purified preparations of just these enzymes (Table 1).

The first stage in the purification of the lipases from a filtrate of the culture liquid from the fungus *Mucor miehei* was performed by ion-exchange chromatography on DEAE-TSK 650 Toyopearl. Desorption of the proteins possessing lipase activity was achieved in a sodium chloride concentration gradient from 0 to 0.6 M in standard buffer. The active fractions were combined and were concentrated with the aid of polyethyleneglycol (PEG) having a molecular mass of 1500.

We then used gel filtration on TSK-HW-55 Toyopearl and Sephadex G-100. As a result, we obtained two forms of the lipases from *Mucor miehei*, differing in molecular mass and in a number of physicochemical properties.

The fungus *Penicillium sp.* also produces two forms of lipases. For their complete separation it is necessary to use repeated chromatography on various supports. The first stage in the purification of the lipase from the fungus *Penicillium sp.* was chromatography on Sephadex G-75, which permitted the separation of the high- from the low-molecular-mass proteins.

Ion-exchange chromatography was conducted on Q-Sepharose, which enabled us to eliminate a large amount of ballast proteins and to separate the two forms of lipases.

The following stage of purification was chromatography on DEAE-Sephadex A-50 (form A) and butyl-650 M (form B). Further purification of the lipases of form B was achieved by chromatography on hydroxylapatite.

The production of lipases from *Oospora lactis* and *Rhizopus microsporus* has been achieved by the large-scale growth of the fungi [42]. The extracellular lipase from *Oospora lactis* was purified 90-fold with an activity yield of 60%, and the periplasmic lipase 110-fold but with the low yield of 25%. To purify membrane-bound forms, the methods of biospecific chromatography were employed. Affinity chromatography, not previously used in the purification of microbial lipases, has proved to be an effective method enabling highly purified enzyme preparations to be obtained. The sorbent used for affinity chro-

TABLE 3. Primary Structures of Some Fungal Lipases

Rhizopus delemar	1	MVSFISISQGVCLLVSSMMLGSSAVPVS
Rhizomucor miehei	1	MVLKQRANYLGFLIVFFTAFLVEA VPIKRQ
Humicola lanuginosa	1	EVSQDLFNQFNLFAQYSAA
Penicillium camembertii	1	DVSTSELDQFEFWVQYAAA
Penicillium sp.	1	ATAATAAFPDLHRAAKLTSA
Rhizopus delemar	151	AYCRSVVPGNKWDCVQCQKWVPDGKIITTFT
Rhizomucor miehei	121	SYC RTVIPGATWDCI HCDA TEDLKIIKTW S
Humicola lanuginosa	20	AYCGKNNDAPAGTNITCTGNACPEVEKADATFLYSFED
Penicillium camembertii	20	SYYEADYTAQVGDKLSCSKGNCPEVEATGATVSYDFSD
Penicillium sp.		VYTG IGKAFDVT ITKRI Y
Rhizopus delemar	181	LLSDTNGYVLRSDKQKTIYLVFRGTNSF R
Rhizomucor miehei	151	LIYDTNAMVARGDSEKTIYIVFRGSSSI R
Humicola lanuginosa	58	SGVGDVTGFLALDNTNKLIVLSFRGSRSIEE
Penicillium camembertii	58	STITDTAGYIAVDHTNSAVVLAFRGSYSV R
Penicillium sp.	40	DLLTDTNGFVGYSTEKKTIAVIMKGAATA A
Rhizopus delemar	211	SAITDIVFNFSDYKPV KGAKVHAGFLSSYE
Rhizomucor miehei	181	NWIADLTFVPVSYPPV SGTKVHKGFLDSYG
Humicola lanuginosa	89	TGSGNLNFDLKEINDICSGCRGHDGFTSSWR
Penicillium camembertii	88	NWVADATFV HTNPGLCDGCLAELGFWSSWK
Penicillium sp.	64	AAAADIDIALITPEFP SDVKILAGVHRPWS
Rhizopus delemar	241	QVVNDYFPVVQEQLTAHPTYKVIVTGHSLG
Rhizomucor miehei	211	EVQNELVATVLDQFKQYPSYKVAVTGHSLG
Humicola lanuginosa	120	SVADTLRQLVEDAVREHPDYRVVFTGHSLG
Penicillium camembertii	118	LVRDDIIKELKEVVAQNPNYELVVVGHSLG
Penicillium sp.	97	AVHDTIITEVKALIAKYPDYTLEAVGHSLG
Rhizopus delemar	271	GAQALLAGMDLYQREPRLSPKNLSIFTVGG
Rhizomucor miehei	241	GATALLCALGLYQREEGLSSSNLFLYTQGQ
Humicola lanuginosa	150	GALATVADL RGNGY DIDVFSYGA
Penicillium camembertii	148	AAVATLAATDL RGKGY PSAKLYAYAS
Penicillium sp.	127	GALTSIAHVTLAQNFPRK SLVSNALNA
Rhizopus delemar	301	PRVGNPTFAAAVE STGIPFQRTVHKDIVP
Rhizomucor miehei	271	PRVGDPAFANVVV STGIPYRRTVNERDIVP
Humicola lanuginosa	173	PRVGNRAFAEFLTVQTGGTLYRITHTNDIVP
Penicillium camembertii	174	PRVGNAALAKYITAQ C NNFRFTHTNDPVP
Penicillium sp.	153	FPIGNQAWADFGT SQSGTFNRGNNVLDGVP
Rhizopus delemar	331	HVPPQSFGFLHPGVESWIKSGT SNVQICT
Rhizomucor miehei	301	HLPPAAFGFLHAGEEYWITDNS PETVQVCT
Humicola lanuginosa	204	KLPPREFGYSHSSPEYWILSGTLVPVTRN
Penicillium camembertii	203	KLPLLSMGYVHVSPEYWITSPNNATVS T
Penicillium sp.	184	NMYSTPLNFKHYGTEYY SSGT EASTVKC
Rhizopus delemar	361	SEI ET K DCSNSIV P FTSILDHLSYFDINEG
Rhizomucor miehei	331	SDL ET S DCSNSIV P FTSVLDHLSYFGINTG
Humicola lanuginosa	233	DIVKIEG ID ATGGNNQPNIPDIPAHLWYFGL IG
Penicillium camembertii	231	SDIKVIDGDVSFD GNTGTGLPLLTDFEAHIWYFVQ VD

matography was polyceramide, consisting of cephalin covalently bound with Kapron [polycaprolactam] granules, which has exhibited a high efficiency in the purification of phospholipases from various sources [43].

The fungus *Rhizopus microsporus* produces five forms of lipases, and schemes of purification have been developed for all of them, several schemes having been given for some of them, including methods of high-performance biospecific chromatography on polyceramide and also hydrophobic chromatography on a specially synthesized sorbent based on microcrystalline cellulose as support and *n*-butanol as ligand [44]. A preliminary stage in the purification of forms of the enzyme localized in vesicular structures was differential centrifugation in a Ficoll gradient, which enabled these vesicles to be concentrated.

As can be seen from the material presented, for purifying fungal lipases we have used the main direction by which the purification of the majority of microbial lipases proceeds – ion-exchange chromatography, gel filtration, chromatography on hydroxylapatites, and ion-exchange chromatography again. However, the use of hydrophobic and affinity chromatography as intermediate stages of the purification of lipases has an individual nature.

PROPERTIES OF LIPASES

Lipases purified to a homogeneous state (degree of purification checked with the aid of disk electrophoresis, electrophoresis in PAAG, ultracentrifugation, and the investigation of N- and C-terminal amino acid residues) are used for scientific and practical purposes.

Microbial lipases are acidic proteins with molecular masses of from 20 to 60 kDa. The specific activities of the pure proteins range from 500 to 10,000 units/mg of protein. The majority of microbial lipases have acidic or neutral isoelectric points.

Some lipases are glycoproteins, i.e., they contain a carbohydrate residue.

The properties of a number of microbial lipases are presented in Table 2.

The action of microbial lipases is regulated by numerous external factors, and therefore urgent attention is being devoted to the study of the mechanisms of their influence.

Since the activation of lipases by certain compounds, including Ca^{2+} and bile acids, has been noted by many authors, it would be desirable to dwell on this question. Ca^{+} ions exert a great influence on a whole series of microbial lipases. Various hypotheses explaining the activation of pancreatic lipase by Ca^{2+} ions have been put forward: 1) they stabilize the lipase structure [45]; 2) they bind FAs, forming the corresponding salts and thereby intensify hydrolysis proper in accordance with the law of mass action [46]; and 3) they enhance the adsorption of the lipase on the surface of the substrate [47]. Disputes over this question have not yet died down. It is most likely, in fact, that Ca^{2+} ions play an ambiguous role in the catalytic process, affecting both the catalytic center of the enzyme and its conformation and also the physical state of the substrate.

However, many ions inhibit the activity of lipases. For example, the hydrolysis of olive oil by an *Aspergillus niger* lipase is inhibited by iron ions many times more strongly than by other ions. The addition of even small amounts of iron ions to the medium appreciably inhibited lipase activity. Depending on the time of action of iron ions on the lipase, two types of inhibition were observed: 1) reversible inhibition taking place on brief contact with low concentrations of the ions; and 2) irreversible inhibition, taking place on prolonged keeping of the medium with high concentrations of iron. In the case of reversible inhibition, activity was rapidly restored on the addition of EDTA or sodium citrate. The effects of Fe^{2+} ions on the activities of five fungal lipases have been compared. It was found that a lipase from *Aspergillus niger* was rapidly inhibited on contact with even low concentrations of Fe^{2+}, regardless of the time of the action. The other lipases were inhibited to a smaller degree or were not inhibited at all by iron ions [45].

Apparently, the pronounced inhibition of some lipases by high concentrations of iron ions and their decomposition by proteases produced by the same microorganisms are the two main factors in the instability of lipases, and this must be taken into account in their commercial production.

We have established that the lipase from *Mucor miehei* is not a metal-dependent enzyme. The decrease in the catalytic activity of lipases in the presence of some salts ($ZnCl_2$, $CuCl_2$, $CdCl_2$) is possibly connected with a screening of the charges and a change in electrostatic interactions in the enzyme molecules, causing a disturbance of the catalytically active conformation.

It has been shown experimentally that the time of addition of an inhibitor or activator to the reaction medium is of definite importance. For example, the activating effect of $CaCl_2$ and $MgCl_2$ is enhanced when they are added to the reaction medium after a definite time from the beginning of the reaction, while the inhibiting capacity of $CuCl_2$ and $CoCl_2$ is suppressed under identical conditions. Apparently, the fatty acids produced by the time of addition prevent the inhibiting action of Cu^{2+} and Co^{2+} by forming the corresponding fatty acid salts [48].

Neither form of the lipase from the fungus *Penicillium sp.* requires the presence of bivalent metals in order to exhibit activity, while such cations as Zn^{2+}, Cu^{2+}, and Hg^{2+} are powerful inhibitors.

The addition of Co^{2+} and Ni^{2+} ions (10^{-2} M) to the reaction mixture for hydrolysis catalyzed by a *Rhizopus microsporus* lipase after a definite time from the beginning of incubation showed an inhibition of lipase activity proportional to the time for which the metal ions had been present in the medium. The observed inhibition obviously took place at the stage of the formation of an enzyme-substrate complex.

It is known that the addition of salts of bile acids accelerates hydrolysis. According to Borgstrom's hypothesis [49], bile acid salts form mixed micelles with the products of lipolysis, shifting the equilibrium in the direction of the formation of the products.

The influence of these compounds on the activity of the lipases from *Penicillium sp.* has been investigated. In the presence of sodium taurocholate at a concentration of 0.01-0.1%, an increase in the level of activity of form A by 15-20% and

of form B by 25-30% was observed, while in the case of a lipase from the fungus *Mucor miehei* medicinal bile and sodium cholate and dihydrocholate in the concentrations given above had no appreciable influence on activity, and sodium deoxycholate led to an inhibition of the enzyme [48].

The protective property of taurocholate and other bile acid salts is apparently connected with their lipophilic nature. It is just thanks to these properties that they are readily adsorbed at a phase separation surface and prevent the appearance of hydrophobic interactions between enzyme and substrate. At the same time, these compounds are fairly hydrophilic and therefore cannot themselves enter into a bond with the enzyme. However, on analyzing the functions of bile acid salts during lipolysis it can be stated that bile acid salts are not obligatory in the enzymatic hydrolysis of triglycerides either *in vitro* or *in vivo*.

It is known that the physicochemical and catalytic properties of any enzymes are determined by their structural features and it is, therefore, necessary to make a deep study and comparative analysis of the molecular organization of these enzymes and to elucidate the interrelationship of the structure and functioning of lipases under various conditions.

STRUCTURE AND FUNCTION OF LIPASES

The study of the structure of any protein begins with the elucidation of its primary structure, since this determines its spatial organization and, consequently, its properties. This also applies to microbial lipases [50]. Some scientists consider that lipases contain an exceptionally high proportion of hydrophobic amino acids and that it is precisely this property that is the reason for their preferential interaction with hydrophobic substrates. However, a careful study of the primary structures of lipases has shown that they are no more hydrophobic than any other enzymes [51]. Therefore, the true reason for interaction with a hydrophobic substrate is most probably hydrophobic "protrusions" on the surface of the lipase. These "protrusions" may also be the reason for the specific behavior of lipases in an aqueous medium. The primary structures of some microbial lipases are given in Table 3.

Many questions of the mechanism of catalysis and the molecular nature of activation have remained unanswered until now and will so remain until the tertiary structures of the lipases have been determined. The first attempts to obtain crystalline forms of lipases were made by Japanese workers as early as the 1970s, but the crystals that they obtained were not stable, apparently because of an inhomogeneity of the enzyme preparations, and they could not be used for determining structures [52].

Now, many lipases have been crystallized in the amounts necessary for performing x-ray structural analysis [53-55]. In spite of the fact that the majority of microbial lipases are glycosylated, scientists have obtained stable crystals of high quality giving diffraction.

The largest number of results on the determination of the tertiary structures of lipases have appeared only in the last 4-5 years. These results relate to a lipase from *Rhizomucor miehei* [56], human pancreatic lipase [57], a lipase from *Geotrichum candidum* [58], and some bacterial lipases [59]. Recently the structure of cutinase has been determined at the molecular level [60].

It has been shown that the lipases the tertiary structures of which have been determined have dissimilar amino acid sequences and form three families of proteins, while cutinase forms yet another family. But, in spite of the differences in their amino acid sequences, the tertiary structures of different lipases have much in common.

Crystals of a lipase from *Geotrichum candidum* have been investigated as a model characterizing lipase structure [61]. The molecule has an ellipsoidal form with dimensions of 50 × 50 × 70 Å. Adjacent to the active center there is a large "cleft." It is obvious that it is just within this "cleft" that the active site of the lipase is located, since it is large enough to accommodate a molecule of a large triglyceride (for example, triolein). The x-ray structural analysis of a lipase crystal at a resolution of 2.8 Å revealed the presence of nine α-strands and four β-sheets [62].

The chemical modification of the lipase has also revealed an interrelationship between the structure and function of the enzyme. Such investigations, performed with the lipase from *Geotrichum candidum*, permitted the conclusion that on the interaction of this enzyme with a substrate there is no involvement of an active serine residue, as in the case of pancreatic lipase and a number of others. Moreover, in contrast to lipases from *Rhizopus delemar* and *Humicolor lanuginosa*, the tryptophan residue in the molecule of the *G. candidum* lipase does not play a major role in catalysis, this function being borne by histidine and tyrosine residues.

In 1993, Swenson et al. [63] crystallized a proenzyme and a mature form of a lipase from *Rhizopus delemar*. They obtained crystals of different sizes, but for the x-ray structural investigations it was possible to use only one monoclinic crystal which, as further investigations showed, contained two molecules of the enzyme arranged asymmetrically with respect to one

TABLE 4. Characteristics of the N- and C-Terminal Amino Acid Sequences and the Active Centers of Some Lipases

Producing agents	M.m.	Number of amino acids	Amino acid sequence		Active center
			N-terminal	C-terminal	
Rhizopus delemar	44.0	398	MVSFISI	LESGEN	GHSLG
Rhizomucor miehei	32.0	269	MVLKQR	TCLGTN	GHSLG
Humicola lanuginosa	37.0	284		VRKFPL	GHSLG
Penicillium camambertii	27.5	272	DVSTSEL	LCTGI	GHSLG
Penicillium sp.	29.0	276	ATAATAAF	TGCGA	GHSLG

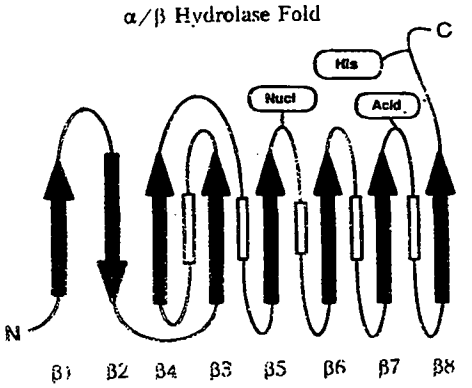

Fig. 3. Topological diagram demonstrating the mutual positions of the β-sheets in the α/β-proteins [61].

another and linked by a noncrystalline bond. The crystals of the proenzyme were orthorhombic and likewise consisted of two molecules in an asymmetric arrangement. To determine the correct orientation and position of the protein molecules in sections of the crystal the authors made use of known facts on the crystal structure of a lipase from *Rhizomucor miehei*.

The crystals from the latter fungus were used not only to elucidate the three-dimensional structure of the enzyme but also to establish the roles of its individual elements (in particular, the arginine residues) in catalysis [64]. It was found that Arg 87, localized in the "lid" covering the active site of the lipase, interacts with the "cleft" (polar cavity). The other polar arginine residues obviously also take part in the stabilization of the "open-lid" conformation of the enzyme. There are three arginine residues – Arg 30, Arg 80, and Arg 86 – in this stabilized complex, but Arg 30 and Arg 80 are located in strands round the polar cavity.

The observed molecular dynamics of the catalytic act, beginning with the "closed-lid" lipase conformation, shows that Arg 86 in the lid is the most important for stabilizing the "open-lid" conformation. Arg 86 is modified to a considerably larger degree than Arg 30 and Arg 80. Modification of the arginine residues by various agents (cyclohexane-1,2-diol, phenylglyoxal, guanidine) has permitted an understanding of the participation of the various arginines in the act of catalysis. In fact the enzyme is stabilized in the "open-lid" conformation by the hydrophobic interaction of the substrate itself with the hydrophobic side of the "lid," and Arg 86 participates only in maintaining the "lid" in the optimum position for catalysis. When Arg 86 is inhibited by guanidine it cannot form hydrogen bonds with the active site, and the "lid" does not assume its optimum position, as a result of which the activity of the enzyme falls.

Elucidation of the amino acid sequences of the "lid" region of the *Rhizopus delemar* lipase, human pancreatic lipase, and lipases from *Humicolor lanuginosa* and *Candida rugosa* showed the presence of arginine only in those lipases that were inhibited by guanidine. The impossibility of the inhibition of some lipases by guanidine indicates only that this inhibition is specific and is neither an artefact nor a common property of lipases.

An investigation of a lipase from *Humicolor lanuginosa* [65] showed that Trp 89, localized in the "lid," plays an important role in the manifestation of lipase activity. This fact has been confirmed by means of site-directed mutagenesis of this amino acid residue.

A similar investigation of a lipase from *Aeromonas sp.* led to the conclusion that the replacement of Try 230 by Phe causes the same loss of activity of the enzyme as its treatment with tetranitromethane. Tyr 230 is obviously necessary for the correct positioning of a phospholipid substrate in relation to the active site of the lipase. Some amino acid residues round the

active center exert a different action in the catalytic process. Thus, when Ser 18 was replaced by Val no protein at all was secreted, while when Phe 13 was replaced by Ser secretion was retarded, although the mutant protein remained active [66].

In spite of those mentioned above and a number of other differences in the tertiary structures of microbial lipases, they are all α/β-proteins (Fig. 3) [61]. The central part of the β-sheet consists of parallel chains round a serine active center and is maintained by a bond between the β_5 and β_8 chains.

In all these proteins, the serine active center is inserted into a supersecondary element that is similar in all of them: a β-strand-turn-α-helix. This common structural element has been detected not only in lipases but also in other hydrolytic enzymes cleaving ester bonds [67]. It is likely that all enzymes of the given class contain serine included in a similar supersecondary structure (Table 4).

The serine is present between a chain and an axis, assuming a conformation with helical twisting at an angle of 60-110°. Ollis [68] has suggested that such a conformation of a serine (or any other nucleophilic) group stores a large amount of energy in a small section remote from the nucleophile itself, which makes the triad Ser-His-Asp accessible for the substrate.

All lipases are characterized by the presence of a G-X-S-X-G sequence, in which the two glycine residues, located between a β-chain and an α-helix, play an important role in the existence of a compact structure.

During lipolysis, the conformation of the enzyme changes in a definite way. It has been established that the direct splitting of the substrate takes place on a surface covering the active center of the enzyme.

In the normal state, the active center of the lipase is inaccessible for a voluminous substrate. Substrates of the size of a triglyceride cannot enter the "cleft" to reach the active center. However, it is important to note that, for each of the enzymes, access to the active center is restricted differently. The size of the protecting part increases correspondingly with an increase in the size of the protein. Crystallographic investigations have revealed conformational changes permitting passage of the lipase from a state "closed" for the substrate to one "open" for it. In the "open" form the lipase increases its hydrophobic surface, which, in its turn, is involved in an interaction with the equivalent surface of the substrate.

Here it is appropriate to bring in results of investigations by Canadian [61] and American scientists who have made a detailed study of the binding of the substrate with the enzyme in the "open" conformation for the exemplary case of a *Candida rugosa* lipase [69]. The structure of this lipase was determined at a resolution of 2.06 Å in a state accessible for solvents. As compared with the crystal structure of a homologous lipase from *Geotrichum candidum*, in which the active site is covered by the surface of loops and is, thus, inaccessible for solvents, the structure of the *C. rugosa* lipase close to the active site has considerable differences. The three loops in this region have different conformations, and the interphase activity of this lipase is connected just with the conformational reorientation of these loops.

Scientists have proposed two statistical models of the activation of the lipase which explains the necessity of the presence of an interphase surface for stabilizing the "open" conformation of the lipase [70]. In the case of lipases from *C. rugosa, H. lanugosina,* and *Rh. delemar*, the lid is present in the "closed" state even in the presence of the substrate. However, the small amounts of a secondary alcohol used in the crystallization of the lipase from *C. rugosa* and the small amounts of detergent used in the crystallization of the lipases from *H. lanuginosa* and *Rh. delemar* may displace the "lid" from its "closed" position. That is, crystal packing factors may change the conformation of the "lid" in a definite manner. The authors consider that intermolecular contacts are the reason for the stabilization of the "open" form of the *C. rugosa* lipase. In the case of structural changes to the lipases of *H. lanuginosa* and *Rh. delemar*, the opening of the "lid" and access to the surface of hydrophobic interaction with a lipid is accompanied by a change in the spatial positions of amino acid residues. Conversely, in the *C. rugosa* lipase such a change in the positions of amino acid residues has an insignificant effect on the movement of the "lid." Gly 124 and Ala 210, present in its surface do not change their positions in either the "open" or the "closed" state or when various inhibitors act on the lipase.

Two theories explaining the activation of a lipase at an interphase surface exist: "substrate" [71] and "enzyme" [72] theories. According to the first theory, the state of the lipid–water interphase surface, the increased concentration gradient at the lipid–protein interphase surface, and the packing of the lipid molecules cause a rise in the activity of the enzyme. According to the "enzyme theory," the critical activation factor consists of conformational changes in the enzyme molecule. On the basis of these theories and later results on the structure of lipases, scientists have proposed a unified model of lipase action [69].

In the proposed model, the main point is the fact that the free energies of the "open" and "closed" forms of lipases are practically the same, and in an aqueous medium a fairly stable equilibrium of these two forms is preserved. The time during which the lipase is present in the open conformation varies in different lipases as a function of the size of the "lid," the nature of the amino acid residues, and other external factors. At the moment when the lipid concerned comes into contact with a lipase

in the "open" form, there is a close interaction of the substrate particle with the catalytic apparatus. This presupposes that hydrophobic residues present in depressions penetrate the lipid substrate and that the integrity of the protein structure is maintained by ion-pair interactions between polar groups present at the interphase surface. Thus, both lipase conformations and the nature of the interphase surface are important elements of the activation of a lipase at an interphase surface.

The determination of the three-dimensional structures of various lipases has confirmed that they belong to the serine hydrolases. Their active site consists of three residues: a serine residue linked by hydrogen bonds with a histidine residue and with a carboxylic residue, which, in its turn, may be represented by either aspartic or glutamic acid. The architectonics of the lipase catalytic triad greatly resembles that in serine proteases [73].

In the course of the reaction a tetrahydrate intermediate is formed which subsequently breaks down into an acylenzyme complex. The catalytic act proper takes place by the following scheme: the first nucleophilic attack of oxygen from the serine side on a carbon atom from the carbonyl-ester bond leads to the formation of a tetrahydrate intermediate. The histidine leads to an increase in the nucleophilicity of the serine hydroxy group. The histidine imidazole ring adds a proton and acquires a positive charge. The latter is compensated by the negative charge of the acid residue. The tetrahydrate complex is stabilized by two hydrogen bonds. This is followed by a nucleophilic attack of a hydroxyl ion, liberating the FA and regenerating the enzyme.

The study of the three-dimensional structures of lipases is the basis for a systematic investigation of the similarities and differences in related enzymes. On the other hand, the general similarity of the three-dimensional structures, in spite of the limited extent of the homologous sequences of the lipases, raises the question of their evolutionary links. The homologous amino acid sequence among lipases of various families is limited to the series G-X-S-X-G, which, in all known cases, includes the catalytic serine and, in all lipase structures, is present in β-folds surrounded by α-helices (i.e., in a supersecondary structure) [74]. Other authors [75], analyzing the stereochemistry of the supersecondary structure, have come to the conclusion that in all the lipases that have been studied the regions round the catalytic serines are identical.

LIPASES IN BIOTECHNOLOGY

Earlier, the main field of employment of lipases was the food industry, where they were used as aromatizers, emulsifiers, and so on [76]. The reason for such little interest in these enzymes was their high cost with a relatively limited possibility of production, particularly for such large-scale processes as the manufacture of detergents. But, thanks to a sharp jump in the development of technologies for obtaining lipases (including the cloning and expressing recombinant DNAs, with the aid of which it is possible to construct microbial strains producing various types of lipases), these enzymes have come into ever wider use in various fields of industry, and this applies in greater measure just to microbial lipases.

As we have shown, enzyme preparations of microbial lipases (lipolaktin, lipomikrosporin, lipomikhin, and lipopenil) and their immobilized forms, which differ in the specificity of their action, their heat and pH stability, and other properties, can be used in processes involving the hydrolysis of vegetable oils and wastes from them (soapstocks and hydrogenated fats) with the aim of obtaining fatty acids and glycerol; in the transesterification of vegetable oils and animal fats in order to create new fatty bases in the production of dietetic types of margarine and mayonnaises; in the clarification and stabilization of fruit juices and other biological liquids as components of enzyme compositions; in the defatting of wastes from the production of reeled silk, hides, and fur pelts; in the creation of new types of detergents, creams, shampoos, and toothpastes; as additives improving the food value and digestibility of fodders; in veterinary and medical practice for creating digestible drugs, for producing unsaturated and polyunsaturated fatty acids (prostaglandin precursors) in the processing of marine products; and for creating highly effective detergents for everyday chemistry – the purification of sewage, the cleaning of tankers, domestic pipes, etc.

It must be mentioned that in the food industry it is preferred to use just fungal lipases because of their low toxicity in comparison with bacterial lipases, which does not lessen the practical value of the latter in other fields.

For industrial purposes, use is made of microbial-lipase-catalyzed hydrolysis, esterification, and transesterification reactions of fats and oils.

The chemical splitting of fats is usually performed under severe conditions (240-260°C, pressure 60 bar) and has a number of undesirable effects – for example, the discoloration of the product and the degradation of some fatty acids. Today there are already examples of the competitiveness of microbial lipases in this field of application. Thus, Japanese scientists and the Miyoshi Oil and Fat Co. use lipases for the multitonnage production of soap [77].

Since the lipase is, however, a fairly expensive catalyst, its use in industrial processes is favorable only when the final yield of product is very high.

For a long time the chemical glycerolysis of oils and fats was used to obtain monoacylglycerols, which are emulsifying agents. Today it is already possible to speak of the replacement of this method by enzymatic glycerolysis using lipases. Thus, the lipase from *Pseudomonas* gives a mixture of mono- and diglycerides when a definite temperature below the critical level is maintained [78]; besides this, the final yield depends on the type of oil or fat used.

Since the main contribution to the cost of the lipase-catalyzed splitting of fats is borne by the price of the enzyme itself, attempts are being made to stabilize the enzyme in some way, to prolong its action, etc. The use of an immobilized lipase may permit the easy recovery of the enzyme after the process, which will reduce expenditure.

Although natural mixtures of triglycerides can be used directly for various purposes, it is frequently necessary to change their individual properties, especially their melting characteristics. In view of this, the oils and fats industry is developing chemical methods of hydrogenation (saturating double bonds) and a transesterification procedure. Sodium-catalyzed transesterification leads to a random distribution of fatty acid residues among the acylglycerol molecules [79]. Performing transesterification with specific lipases will enable lipids with predetermined properties to be obtained. A lipase is used particularly frequently in a transesterification reaction for obtaining triglycerides similar to cocoa butter. An immobilized lipase must be used for the commercial exploitation of the transesterification reaction. Thus, Bell and Patterson [80] have used a lipase bound with micelles for transesterification. But majority of workers use for transesterification a lipase included within some matrix or adsorbed on a definite support [81-84].

Lipases are attracting attention not only as catalysts of definite processes but also as potential functional agents in various mixtures. For example, as important additives to detergents, where they are effective in eliminating fatty spots, particularly at low temperatures. It is just in the creation of detergents that the future potential large-tonnage use of lipases appears to reside.

At the present time we may speak with confidence of broad prospects for the use of microbial lipases in medium- and large-scale industries, which will give a whole series of advantages over the use of physicochemical and chemical processes.

Numerous investigations of microbial lipases are opening up broad prospects of their use for both basic scientific and practical purposes. But, on the other hand, the incompleteness of the solution of a number of questions is dictating the necessity for further approaches in this field.

In particular, we may mention the problem of the stability of the lipases, especially in relation to the temperature factor. This problem can be solved by finding sources of lipases with high thermal stability and by isolating lipases from thermophilic sources, and it is possible to use gene-engineering approaches – namely the creation of recombinant DNA. And, finally, there is a need for the further development and perfection of the traditional methods of stabilization: immobilization and chemical modification.

The question of obtaining highly active lipases with the required specificity has not disappeared. For this purpose a search is being made for new lipase-producing strains and for improvements in the selection and mutagenesis of microoganisms, induction, etc.

REFERENCES

1. P. F. Fox, J. Soc. Dairy Technol., **33**, 118 (1980).
2. H. Brockerhoff, Biochem. Biophys. Acta, **159**, 296 (1968).
3. A. M. Bezborodov, The Biotechnology of the Products of Microbial Synthesis [in Russian], Agropromizdat, Moscow (1991), p. 238.
4. A. M. Bezborodov, The Biochemical Principles of Microbial Synthesis [in Russian], Legkaya i Pishchevaya Prom-st', Moscow (1984), p. 304.
5. F. Priest, in: Extracellular Enzymes of Microorganisms, J. Chaloupka and V. Krumphanzi (eds.), Plenum, New York (1987).
6. M. Ya. Tabak, Influence of the Conditions of Cultivation on the Biosynthesis of an *Oospora lactis* Lipase [in Russian], Dissertation ... Candidate of Biological Sciences, Tashkent (1979). p. 156.
7. T. R. Zubenko, The Biosynthesis of a Lipase by the Fungus *Rhizopus microsporus* [in Russian], Dissertation ... Candidate of Biological Sciences, Tashkent (1980), p. 122.

8. R. G. Jensen, Lipids, **9**, No. 3, 149 (1974).
9. A. Zaks and A. M. Klibanov, Science, **224**, 1249 (1984).
10. F. Ergan and M. Trani, Biotechnol. Lett., **13**, No, 1, 151 (1988).
11. K. D. Davranov, G. I. Meerov, A. M. Bezborodov, et al., Prikl. Biokhim. Mikropbiol., **21**, No. 2, 199 (1985).
12. H. S. Bevinakaytii and A. A. Banerji, Biotechnol. Lett., **10**, No. 6, 397 (1988).
13. A. R. Macrae, G. Ratledge, P. Dawson, and J. Rattray, Biotechnology for the Oils and Fats Industry, Amerivan Oil Chemists' Society, Champaign, Vol. 3 (1985), p. 189.
14. Y. Tsujisaka, S. Okumura, and M. Iwai, Biochim. Biophys. Acta, **489**, 415 (1977).
15. R. Shuch and K. D. Mukherjee, J. Agric. Food Chem., **37**, 1005 (1987).
16. R. A. Windom, P. Dunnill, and M. D. Lilly, Enzyme Microb., Technol., **6**, 443 (1984).
17. J. M. Muderhwa, M. Pina, and J. Graille, Oléagineux, **10**, 385 (1988).
18. H. L. Goderis, G. Ampe, and M. P. Feyten, Biotechnol. Bioeng., **30**, 253 (1987).
19. K. D. Mukherjee and J. Kiewitt, J. Agric. Food Chem., **37**, 529 (1987).
20. K. Yokezeki, S. Yamanaka, and E. Takinami, Eur. J. Appl. Microbiol. Biotechnol., **14**, 1 (1992).
21. A. Kilara, Proc. Biochem., **20**, 35 (1985).
22. R. G. Jensen, Lipolytic Enzymes. Progr. Chem. Fats Other Lipids, **11**, 347 (1971).
23. E. Charton, Doctoral Degree Report, Paris-Grignon (1991), p. 9.
24. T. Tzumi and S. Aratani, J. Chem. Technol. Biotechnol., **57**, 33 (1993).
25. T. Tzumi and S. Murakami, J. Chem. Technol. Biotechnol., **60**, 23 (1994).
26. C. Ransac, E. Rogalska, Y. Gargouri, A. M. T. J. Deveer, F. Paltauf, G. H. De Haas, and R. Verger, J. Biol. Chem., **265**, No. 33, 20,263 (1990).
27. E. Rogalska, C. Ransac, and R. Verger, J. Biol. Chem., **265**, No. 33, 20271 (1990).
28. U. Chander, B. Ranganathan, and M. Singh, J. Food Sci., **44**, 1566 (1979).
29. I. M. Khan, C. W. Dill, and R. C. Chandan, Biochem. Biophys. Acta, **132**, 68 (1967).
30. M. Simonen and I. Palva, Microbiol. Rev., **57**, 109 (1993).
31. A. Mates and D. Sudakevits, J. Appl. Bacteriol., **36**, 219 (1973).
32. U. K. Winkler and M. Stuchmann, J. Bacteriol., **138**, 663 (1979).
33. K.-E. Jaeger and U. K. Winkler, J. Bacteriol., **139**, 1065 (1989).
34. I. Okhuro, T. Komatsuzaki, M. Kawashima, and S. Kuriyama, Med. Biol., **97**, 171 (1978).
35. R. C. McKellar, K. Shamsuzzama, C. San Jose, and H. Cholette, Arch. Microbiol., **147**, 225 (1987).
36. L. Fernandez, C. San Jose, H. Cholette, and R. C. McKellar, Arch. Microbiol., **150**, 523 (1988).
37. J. J. Goodman, Science, **112**, 176 (1950).
38. Y. Tsujisaka, M. Iwai, J. Fukumoto, and A. Okamoto, Agric. Biol. Chem., **37**, 837 (1973).
39. J. A. Alford and J. L. Smith, J. Am. Oil Chem. Soc., **42**, 1038 (1965).
40. A. Atkinson, Proc. Biochem., August 9 (1973).
41. A. Atkinson, Biochem. J., **127**, 63 (1972).
42. K. D. Davranov, Lipases of the Fungi *Oospora lactis* and *Rhizopus microsporus* [in Russian], Author's Abstract of Dissertation ... Doctor of Chemical Sciences [in Russian], Tashkent (1984), p. 7.
43. R. Akhmedzhanov, M. M. Rakhimov, and B. A. Tashmukhamedov, Uzb. Biol. Zh., No. 2, 3 (1979).
44. Zh. Kh. Dierov, K. D. Davranov, M. É. Zakirov, and A. A. B. Paulyukonis, USSR Inventor's Certificate 877,933 (19) [sic].
45. E. J. Gilbert, A. Cornish, and C. W. Jones, J. Gen. Microbiol., **137**, 2223 (1991).
46. K. A. Brune and E. Gotz, in: Microbial Degradation of Natural Products, G. Winkelmann (ed.), VCH, Weinheim (1992), p. 243.
47. K.-E. Jaeger, F. J. Adrian, H. E. Meyer, R. E. W. Hancock, and U. K. Winkler, Biochem. Biophys. Acta, **1120**, 315 (1992).
48. B. Kh. Rozmukhamedova, Lipases of the Fungi *Mucor miehei* and *Penicillium sp.*: Isolation, Purification, Characterization [in Russian], Dissertation ... Candidate of Biological Sciences, Tashkent (1995), p. 86.
49. B. Borgstrom and R. L. Ory, Biochem. Biophys. Acta, **212**, 521 (1970).
50. J. Caplan, Work Term III Report, NRC of Canada, Biotechnology Research Institute, Montreal (1994), p. 3.
51. M. Cygler, J. Schrag, et al., Protein Sci., **2**, 366 (1993).

52. M. Fukumoto, M. Iwai, and Y. Tsujisaka, J. Gen. Appl. Microbiol., **9**, 353 (1993).
53. S. M. Franken, H. J. Rozeboom, K. H. Kalk, and B. Dijkstra, EMBO J., **10**, 1297 (1991).
54. K. Isobe, T. Akiba, and S. Yamaguchi, Agric. Biol. Chem., **52**, 41 (1988).
55. K. Isobe, K. Nokihara, S. Yamaguchi, T. Mase, and R. D. Schmid, Eur. J. Biochem., **203**, 233 (1992).
56. L. Brady, A. M. Brzozowski, Z. Derewenda, E. Dodson, G. Dodson, S. Tolley, J. P. Turkenburg, L. Christiansen, and J. Huge, Nature (London), **343**, 767 (1990).
57. F. K. Winkler, A. D'Arcy, and W. Hunziker, Nature (London), **343**, 771 (1990).
58. J. D. Schrag, Y. Li, S. Wu, and M. Cygler, Nature (London), **351**, 761 (1991).
59. R. Sarma, J. Daubman, A. J. Poulose, J. Bieten, and S. Power, in: Lipases: Structure Mechanism and Genetic Engineering, GBF, Monographs, Germany, **16**, 71 (1990).
60. C. L. Soliday and P. E. Kolattukudy, Biochem. Biophys. Res. Commun., **114**, 1017 (1983).
61. M. Cygler, J. D. Schrag, and F. Ergan, Biotechnol. Genet. Rev. (1993).
62. H. C. Hedrich, F. Spener, U. Menge, H. Hecht, and R. D. Schmidt, Enzyme Microb. Technol., **13**, 840 (1991).
63. L. Swenson, R. Green, R. Joerger, M. Haas, K. Scott, Y. Wei, U. Derewenda, D. M. Lawson, and Z. S. Derewenda, Protein: Structure, Function, Genetics, **18**, 301 (1994).
64. M. Holmquist, M. Norin, and K. Hult, Lipids, **28**, No. 8, 721 (1993).
65. M. Holmquist, M. Martinelle, I. G. Clausen, S. Patkar, A. Svendsen, and K. Hult, Lipids, **29**, No. 9, 599 (1994).
66. D. L. Robertson, S. Hilton, K. R. Wong, A. Koephe, and J. T. Buckley, J. Biol. Chem., **269**, No. 3, 2146 (1994).
67. J. D. Schrag, S. Wu, Y. Li, and M. Cygler, J. Mol. Biol., **220**, 541 (1991).
68. D. L. Ollis, E. Cheah, M. Cygler, B. Dijkstra, F. Frolow, S. M. Franken, and M. Harel, Protein Eng., **5**, 67 (1992).
69. P. Grochulski, Y. Li, J. D. Schrag, F. Bouthillier, P. Smith, D. Harison, B. Rubin, and M. Cygler, J. Biol. Chem., **268**, No. 17, 12,843 (1993).
70. B. Rubin, Struct. Biol., **1**, No. 9, 568 (1994).
71. H. L. Brockman, J. H. Law, and F. J. Keady, J. Biol. Chem., **248**, 4965 (1973).
72. C. Chapus, M. Semeriva, C. Bovier-Lapierre, and P. Desnuelle, Biochemistry, **15**, 4980 (1976).
73. S. Jager, G. Demelither, and F. Gotz, FEMS Microbiol. Lett., **100**, 249 (1992).
74. M. Cygler, J. D. Schrag, J. L. Sussman, M. Harel, K. Silman, B. P. Doctor, and M. H. Genry (1994), in preparation.
75. Z. S. Derewenda and U. Derewenda, Biochem. Cell. Biol., **69**, 842 (1991).
76. D. J. Stead, Dairy Res., **53**, 481 (1986).
77. M. M. Hoq, H. Tagami, T. Yamane, and S. Shimizu, Agric. Biol. Chem., **49**, 335 (1985).
78. G. P. McNeill and T. Yamane, J. Am. Oil Chem. Soc., **68**, 6 (1991).
79. A. R. Macrae and R. C. Hammond, Biotechnol. Genet. Eng. Rev., **3**, No. 6, 193 (1985).
80. G. Bell, J. A. Blain, J. D. E. Paterson, C. E. L. Shaw, and R. Todd, FEMS Microbiol Lett., **3**, 223 (1978).
81. A. E. M. Jansen, A. V. Padt, H. M. Van Sonsbeek, and K. Van't Riet, Biotechnol. Bioeng., **41**, 95 (1993).
82. Y. Dudal and R. Lortie, Biotechnol. Bioeng., **45**, 129 (1995).
83. S.-W. Cho and J. S. Rhee, Biotechnol. Bioeng., **42**, 204 (1993).
84. B. H. Junker, M. Bhupathy, and B. S. Buchland, Biotechnol. Bioeng., **42**, 487 (1993).
85. M. Sugiura and T. Oikawa, Biochem. Biophys. Acta, **489**, 262 (1977); **488**, 353 (1977).
86. N. A. Bashkatova, Author's Abstract of Candidate's Dissertation [in Russian], Moscow (1980).
87. Microbial Enzymes and Biotechnology [in Russian], Moscow (1986).
88. N. Tomizuka, Y. Ota, and K. Yamada, Agric. Biol. Chem., **30**, 576 and 1090 (1966).
89. Y. Tsujisaka, M. Iwai, and Y. Tominaga, Agric. Biol. Chem., **37**, 1457 (1973).
90. W.-H. Lui, T. Beppu, and K. Arima, Agric. Biol. Chem., **37**, 157 and 1349 (1973).
91. H. Ishihara, H. Okuyama, H. Ikosawa, and S. Tejima, Biochem. Biophys. Acta, **388**, 413 (1975).
92. M. Semeriva, G. Benzonana, and P. Desnuelle, Biochem. Biophys. Acta, **388**, 413 (1975).
93. M. Iwai, Y. Tsujisaka, Y. Okamoto, and J. Fukumoto, Biochem. Biophys. Acta, **37**, 929 (1973).
94. A. I. Marchenkova, Author's Abstract of Candidate's Dissertation [in Russian], Moscow (1980).