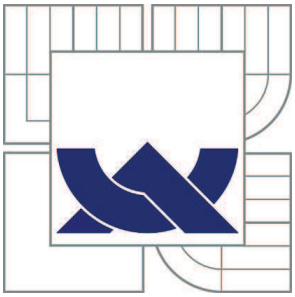

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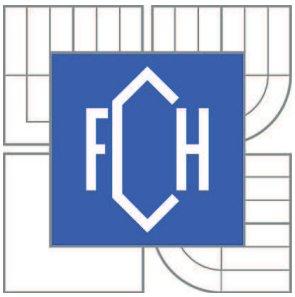

FAKULTA CHEMICKÁ
ÚSTAV CHEMIE POTRAVIN A BIOTECHNOLOGIÍ

FACULTY OF CHEMISTRY
INSTITUTE OF FOOD SCIENCE AND BIOTECHNOLOGY

BIOCATALYSTS BASED ON LIPASES, THEIR IMMOBILIZATION AND CHARACTERIZATION

BIOKATALYZÁTORY NA BÁZI LIPÁZ, JEJICH IMOBILIZACE A CHARAKTERIZACE

BAKALÁŘSKÁ PRÁCE
BACHELOR'S THESIS

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Název bakalářské práce:

Biokatalyzátory na bázi lipáz, jejich imobilizace a charakterizace

Zadání bakalářské práce:

V teoretické části bude zpracována literární rešerše problematiky imobilizovaných enzymů používaných pro biomedicínské a průmyslové aplikace. Pozornost bude věnována jak základním metodám imobilizace, tak typům heterogenních nosičů a dalším metodám vyvinutým v posledních letech. V experimentální části bude stanovena aktivita a charakterizace základních vlastností (pH optimum, teplotní optimum a tepelná stabilita komerční lipázy z Rhizopus arrhizus. Příprava nerozpustné formy komerční lipázy bude provedena imobilizací na polyetylentereftalát. U získaného nerozpustného enzymového preparátu bude stanovena relativní aktivita a v případě úspěšné imobilizace i ostatní charakteristiky.

Termín odevzdání bakalářské práce: 4.5.2012

Bakalářská práce se odevzdává ve třech exemplářích na sekretariát ústavu a v elektronické formě vedoucímu bakalářské práce. Toto zadání je přílohou bakalářské práce.

Anna Bančáková	Mgr. Soňa Hermanová, Ph.D.	doc. Ing. Jiřina Omelková, CSc.
Student(ka)	Vedoucí práce	Ředitel ústavu

V Brně, dne 31.1.2012

prof. Ing. Jaromír Havlica, DrSc.
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ABSTRACT

The thesis deals with the issue of immobilized enzymes. In the theoretical part, principal and novel methods of immobilization and types of carriers are reviewed together with practical applications of immobilized enzymes. Particularly, recent utilization of immobilized enzymes in a variety of industries such as food industry, medicine, chemical analysis, bioremediation, and biodiesel production is summarized. Different methodologies including adsorption, entrapment, covalent binding and cross-linking are discussed from the viewpoint of their dis/advantages resulting from the extent and the character of binding and the maintenance of enzyme activity. Among new carriers, the research work done on the utilization of graphene as a novel carrier for enzyme immobilization is particularly reported.

In the experimental part, immobilization of commercial preparation of lipase (*RA*) isolated from microscopic fungi *Rhizopus arrhizus* was performed by adsorption on polyethylene terephthalate as a carrier. The basic parameters as lipolytic activity, temperature optimum, pH optimum and thermal stability of both soluble as well as immobilized enzyme were determined spectrophotometrically using p-nitrophenyl laurate (pNPL) as a substrate.

Both the soluble and immobilized lipase showed maximum activity at pH 7.2 and a decrease in activity was observed above pH 8 or below pH 6.5. The dependence of activity on pH of reaction medium was more pronounced in the case of immobilized form of enzyme. The soluble and immobilized lipases exhibited maximum activity at a temperature of approximately 30 °C. A drop in activity values was observed when the temperature was increased up to 50 °C and above this temperature the stability of the soluble lipase sharply decreased. On the contrary, thermal stability of immobilized *RA* lipase was significantly improved. Immobilized form of enzyme possesses activity 3.7 % in comparison to the soluble form.

KEY WORDS

Lipase, immobilization, *Rhizopus arrhizus*

ABSTRAKT

Bakalárska práca sa zaoberá problematikou imobilizovaných enzýmov. V teoretickej časti sú zhrnuté najnovšie používané metódy imobilizácie, typy heterogénnych nosičov a taktiež praktické využitie imobilizovaných enzýmov v potravinárskom priemysle, medicíne, chemickej analýze, pri produkcii bionafty a bioremediácii. Uvedené sú tiež výhody a nevýhody jednotlivých metód imobilizácie (adsorpcia, zachytenie, kovalentné spojenie a zasieťovanie) plynúce z charakteru spojenia enzýmu a nosiča a výslednej aktivity imobilizovaného enzýmu. Z hľadiska nových typov heterogénnych nosičov práca konkrétne uvádza využitie grafénu, ktorý je pre svoje špecifické fyzikálno-chemické vlastnosti často používaným a skúmaným nosičom v posledných rokoch.

V experimentálnej časti bola prevedená imobilizácia komerčného preparátu lipázy *(RA)*, izolovanej z mikroskopickej plesne *Rhizopus arrhizus*, adsorpciou na polyetyléntereftalát, ktorý bol použitý ako nosič. U voľnej i imobilizovanej formy enzýmu boli stanovené základné parametre ako lipolytická aktivita, teplotné optimum, pH optimum a tepelná stabilita. Aktivita enzýmu bola meraná spektrofotometricky pri vlnovej dĺžke 420 nm. Ako substrát bol použitý roztok p-nitrofenyllaurátu.

U voľného aj imobilizovaného enzýmu bolo stanovené pH optimum 7,2, pokles aktivity bol zaznamenaný pri pH nad 8 a pod 6,5. Väčšia závislosť výslednej aktivity na pH prostredia bola dokázaná u imobilizovanej formy enzýmu. U voľného aj imobilizovaného enzýmu bola pozorovaná najvyššia aktivita pri teplote 30 °C. Pokles aktivity bol pozorovaný po zvýšení teploty nad 50 °C. Po dosiahnutí tejto teploty stabilita rozpustného enzýmu prudko klesala. Avšak tepelná stabilita skúmanej lipázy sa po jej imobilizácii výrazne zlepšila. Imobilizovaná forma enzýmu mala v porovnaní s voľnou formou aktivitu 3,7 %.

KĽÚČOVÉ SLOVÁ

Lipáza, imobilizácia, *Rhizopus arrhizus*

BANČÁKOVÁ, A. *Biocatalysts based on lipases, their immobilization and characterization.* Brno: Brno University of Technology, Faculty of chemistry, 2012. p. ***38*** Supervisor Mgr. Soňa Hermanová, Ph.D.

DECLARATION

I declare that the diploma thesis has been worked out by myself and that all the quotations from the used literary sources are accurate and complete. The content of the diploma thesis is the property of the Faculty of Chemistry of Brno University of Technology and all commercial uses are allowed only if approved by both the supervisor and the dean of the Faculty of Chemistry, BUT.

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Student's signature

ACKNOWLEDGEMENT

Hereby I would like to thank my supervisor Mgr. Soňa Hermanová, Ph.D. for helpful advice and comprehensive assistance in creating my bachelor work. I would also like to thank doc. Ing. Jiřina Omelková, CSc. for her patience and help with experimental part of my work.

CONTENT

1 INTRODUCTION

Enzymes are subject of intense research since they have been discovered. They are utilized extensively in many industries such as agro-chemistry, medicine, and pharmaceutics or food science as highly specific and highly enantioselective and regioselective catalysts.

Triacylglycerol ester hydrolases, lipases (EC 3.1.1.3) are considerably utilized biocatalysts in organic chemistry. They are produced by microorganisms, animals, plants and fungi, and play essential role in the digestion, transport and processing of lipids in most living organisms. At present about 4 000 lipases are known and about 200 of these are used commercially [1].

Lipases are used in food science as food additives, where they influence the taste and smell of food. They are included for example in production of cheese, where mixed culture containing lipolytic microorganisms is used. They are also used to create characteristic taste of chocolate. Formation of degradation products of fats which have properties of emulgators, results in their application in bread production (longer durability of bread) [2].

Other applications of lipolytic enzymes concern the separation of racemic mixtures in production of optically active compounds and the biodegradation of plastics as well. Lipases can be also used to degrade fats present in wastewater.

Among the applications of lipases their utilization as an ingredient in detergents is commercially the most important one. Lipase content in detergents is about 0.4 – 0.8 wt.% . The task of enzyme is to reduce the temperature of washing and to increase the washing effect [3].

Despite all the advantages of practical applications of enzymes, there are also some restrictions that should be mentioned. Enzymes are excellent reactions catalysts but without any improvement thus in pure state, they are not very suitable for use in reactors in industrial scale. Enzymes may be instable due to spontaneous oxidation, self-digestion, or denaturation and they work properly only on natural substrates and under physiological conditions. They are soluble in water and also in organic solvents and can be strongly inhibited by excess amount of substrate, product formed during enzyme-catalyzed reaction and however by certain by-products of the reaction.

The development of efficient and economical biotransformations often requires sophisticated technologies for the immobilization of biomolecules or cells. Immobilization of enzymes can enhance their thermal and operational stability, and reusability, which are important for industrial applications. Immobilized enzymes used in industry have to be also highly selective to reduce side reactions, able to meet safety regulations and last but not least innovative for recognition as intellectual property [4]. Today most of lipases, which are used in industry, are in their immobilized state. Due to all facts already mentioned, enzyme immobilization represents with no doubt a big challenge in modern biotechnology.

2 THEORETICAL PART

2.1 Rhizopus

Regarding to scientific classification the genus *Rhizopus* belongs to kingdom of fungi, division zygomycota, class mucorales, and family mucoracea. Genus *Rhizopus* is very widespread in nature. It causes spoilage of fruits and some other foodstuffs. *Rhizopus* is a cosmopolitan fungus determined in soil, old bread or house dust [5]. We can observe lipolytic activity on species *R. oryzea, R. niveus, R. delemar, R. japonicus, R. arrhizus, R. chinensis* [6] .

From the microscopical point of view their hyphes are broad with few or no septa. They have fibres more than 1 cm long. 2 or 3 long sporangiophores grow from hyphes or stolons. The sporangiophores are terminated with a dark, round sporangium that contains a columella and several oval, colorless or brown spores. At the point where the stolons and sporangiosphores meet, the brownish bodies called rhizoids are produced [5].

Temperature optimum growth of Rhizopus was reported to be about 30 °C. The pathogenic species grow well at 37 °C [5].

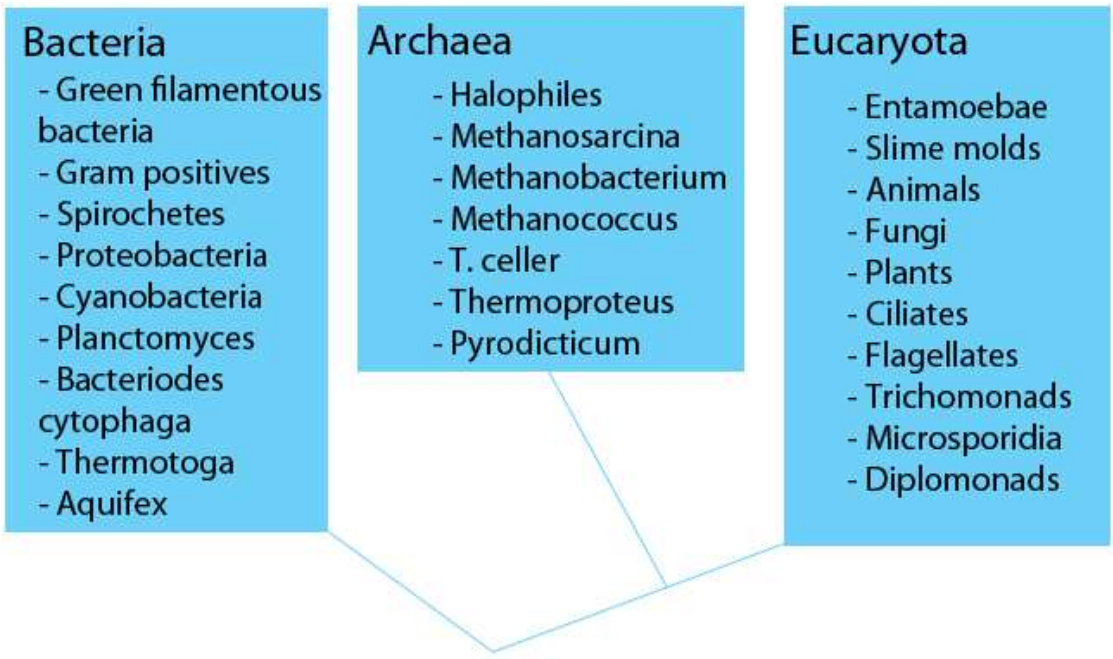


Figure 1 *Phylogenetic tree of life* [7]

2.1.1. Classification of microscopic fungi *Rhizopus arrhizus*

Rhizopus arrhizus is a phycomycetous fungus belonging to the order *Mucorales.* Some species cause soft-rot diseases of the fruits and their spores are common in the atmosphere and cause well known black bread mold [8]. It belongs to one of the most significant producers of commercial lipases. Most of microorganisms are able to produce it, but not all of them are suitable for commercial utilization. The main disadvantages are related to low production, difficult isolation from the culture medium, and so forth.

Colonies of *Rhizopus arrhizus* grow very fast, maturing within four days. Colonies are typically white or grey looking like cotton wool and they turn yellowish brown in time [5].

There is also notable effect of initial pH on enzyme production. In published studies, maximum activity was obtained at pH 5.5 ± 0.5 of the medium.

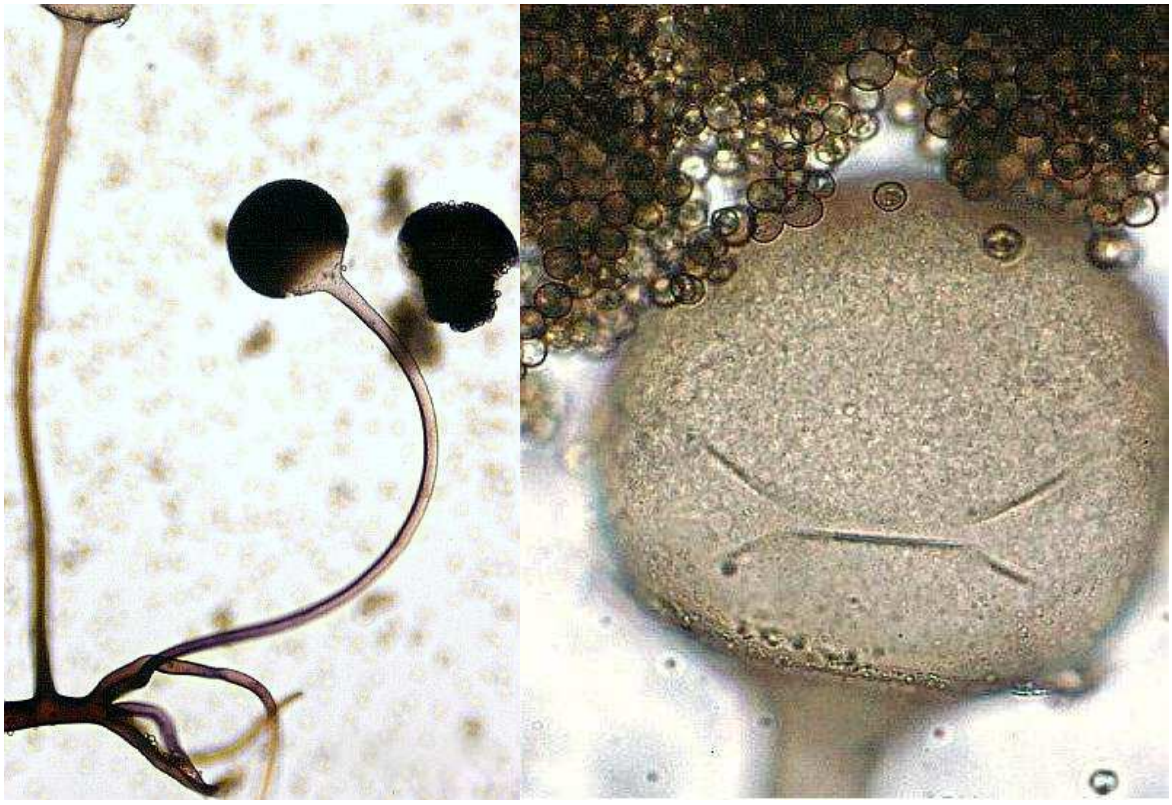


Figure 2 *Typical Rhizopus arrhizus structure under microscope (125 magnification)* [6]

2.2 Lipolytic enzymes

Generally, lipases (EC 3.1.1.3) and esterases or carboxylesterases (EC 3.1.1.1) are enzymes belonging into a group of hydrolases. Esterases are active in aqueous solutions but "true" lipases (triacylglycerol (acyl) hydrolases) catalyze hydrolysis of triacylglycerols into di- and monoacylglycerols, fatty acids and glycerol at an oil-water interface [9]. Lipolytic enzymes produced by microorganisms are of great interests due to their biotechnological potential.

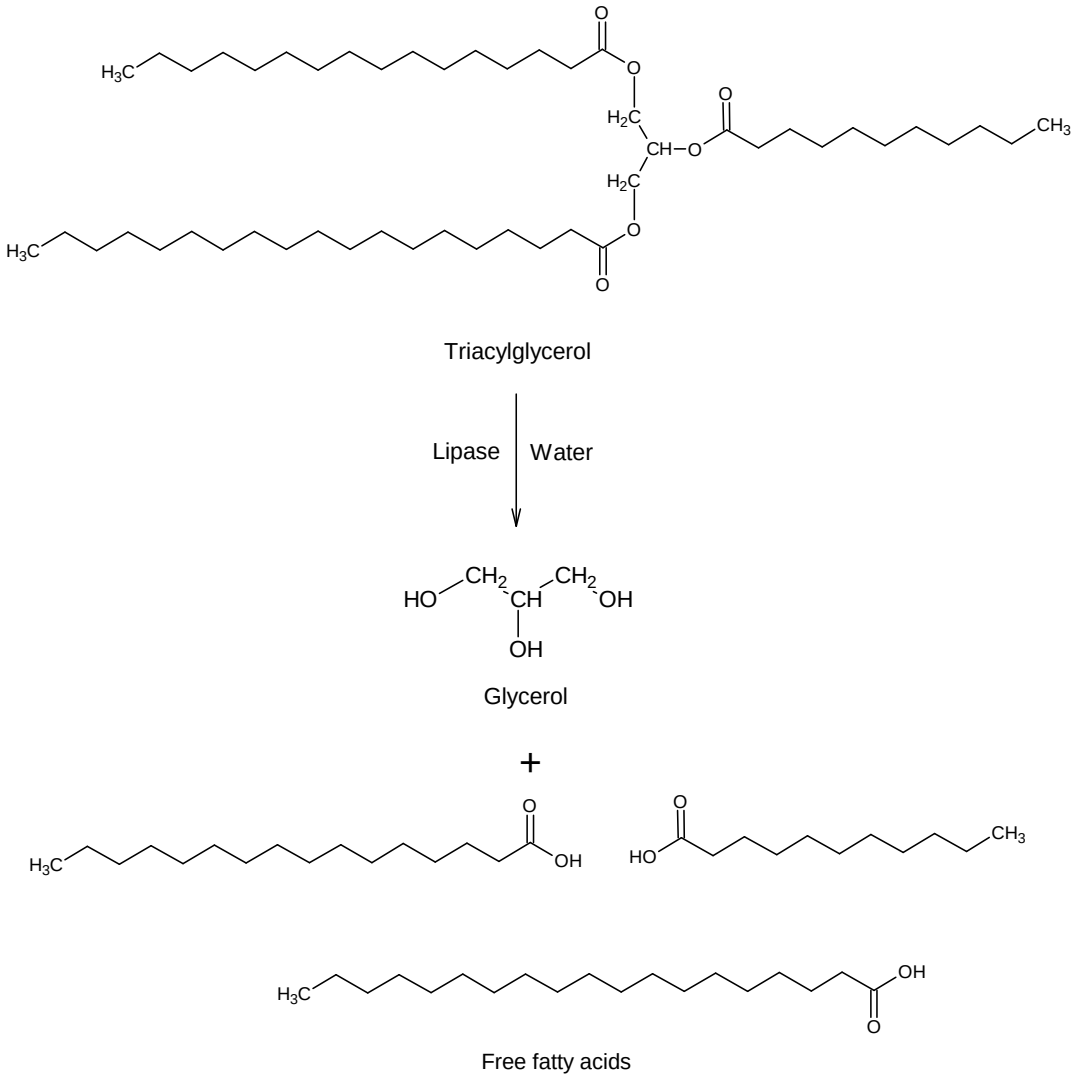


Figure 3 *Reaction scheme of lipases catalytic action: the hydrolytic cleavage of triacylglycerols into glycerol and free fatty acids.*

2.2.1 Classification of substrate specificity of lipolytic enzymes

Specificity of lipolytic enzyme is controlled by:

- molecular properties of enzymes
- structure of a substrate
- factors affecting the interaction (for example covalent bond) between an enzyme and a substrate [9]

Substrate specificity of lipases is related to the structure of the molecule of triacylglycerol.

Molecule of glycerole (propane-1,2,3-triol) as a main building component contains two primaries and one secondary carbon atom. Although the molecule is planar symmetric, primary carbon atoms are sterically different only if the –OH groups are substituted by different substituents. In this case the molecule becomes to be optically active [10]. According to substrate specificity lipases are divided into three basic groups [11]:

- The first group of lipases is denoted as "non specific". Enzymes of this group cleave all three ester bonds of acylglycerol to release fatty acids and completely hydrolyze triacylglycerols to fatty acids and glycerol.
- The second group of lipases is called "1,3-specific". These lipases catalyze hydrolysis of ester bonds releasing fatty acids from the external positions of triacylglycerol and giving rise to 1,2-diacylglycerol, 2,3-diacylglycerol and 2-monoacylglycerol. This group also includes lipases isolated from *Rhizopus* microorganisms. Due to their 1,3-(regio)-specific, lipases of the genus *Rhizopus* are especially suited for the conversion of triglycerides to the monoglycerides and interesterification reactions of fats and oils which have pharmaceutical and food applications [12].
- The third group includes enzymes which prefer certain fatty acids. They are for example enzymes belonging to the groups of carboxylesterases of type B [11].

2.2.2 Chemical and spatial structure of lipolytic enzymes

In all classified lipases there was determined the characteristic spatial conformation referred as α/β-hydrolase bend. Molecule of lipase consists of eight β-chains which can bind up to six α-spirals [13]. It was found out that active site of enzymes is protected by polypeptide chain in the form of lid-like, which prevents the formation of activated complex. The contact of lipase with the lipid phase causes conformational changes to move polypetide chain allowing the access of lipid to active site of enzyme. Hydrophobic part of lid-like ensures hydrophobic interaction with lipid phase and consequently the bond enzyme-substrate becomes stronger. This fact explains the activation phenomena of lipases on water-oil interface [10].

2.2.3 Mechanism of action

Active site of lipases consists of three amino acids: serine, aspartic acid or glutamic acid and histidine. Nucleophilic residue of serine is located on C-end of β_5-chain as GlyXSerXGly pentapeptide. They form characteristic β-bend-α sequence denoted as nucleophilic elbow. Hydrolysis of substrate begins by nucleophilic reaction between Ser oxygen of enzyme and carbonyl carbon of triacylglycerol ester bond. The nucleophilic attack leads to the formation of intermediate product that is stabilized by formation of hydrogen bonds between nitrogen atoms of the rest of enzyme molecule and hydrogen atoms of triacylglycerol. The process continues by releasing of glycerol. Reversible transition-state complex acyl-enzyme subsequently decomposes to free fatty acid and active enzyme [13].

2.2.4 Catalytic activity of enzymes

Catalytic activity of enzyme is defined as the ability of enzyme to transform a substrate into a product. It is expressed as the amount of substrate that is transformed to product per unit of time. Specific catalytic activity is related to the weight of protein. Generally, the rate

of reaction increases with increasing temperature but the increase in temperature also causes the inactivation of enzyme by denaturation of its protein part. According to the intersection of these two opposite processes the dependence called as temperature optimum represents characteristic feature of an enzyme. Determination of temperature optimum is of great importance in all practical applications of enzymes [2].

Properties of most enzymes are dependent on pH of their environment. Dependence of catalytic activity on the concentration of hydrogen ions in solution is given by protonatable groups. These groups are the parts of active site of enzymes and also are present in substrate molecules. Thus, the reaction between the enzyme and the substrate is dependent on the degree of protonation. That is the reason why majority of enzymes are catalytically active in specific interval of pH values. If the pH value is higher or lower than pH optimum of certain enzyme, catalytic activity of enzyme decreases. PH optimum is value of pH at which the activity of an enzyme reaches the maximum. Changing of pH values of reaction medium is one way how to regulate the activity of enzyme [2].

Activity can also depends on redox potential of environment, ionic strength and relative permittivity, which influence the power of non-covalent interactions formed between a substrate and an enzyme [2].

2.3 Enzyme immobilization

Enzyme immobilization can be defined as the attachment of “free” or soluble enzymes to different types of support. As a result of this process the mobility of the enzyme is reduced or lost [14]. This process turns soluble biopolymer (enzyme), to its insoluble form by interaction with specific type of support.

Enzyme immobilization is partial process involving several factors that have to be taken into account. Just the right combination of factors leads to successful immobilization with high activity, stability and reusability of enzyme. Both, pure enzyme and carrier have specific properties. For enzyme it is its activity, stability or selectivity, for carrier it is for example its shape, pore size and particle size. It is also important to choose the optimum conditions for immobilization procedure. Inadequate temperature or pH can lead to denaturation or complete loss of the activity of enzyme. After successful immobilization it is also important to maintain suitable conditions for storage and further use of immobilized enzyme.

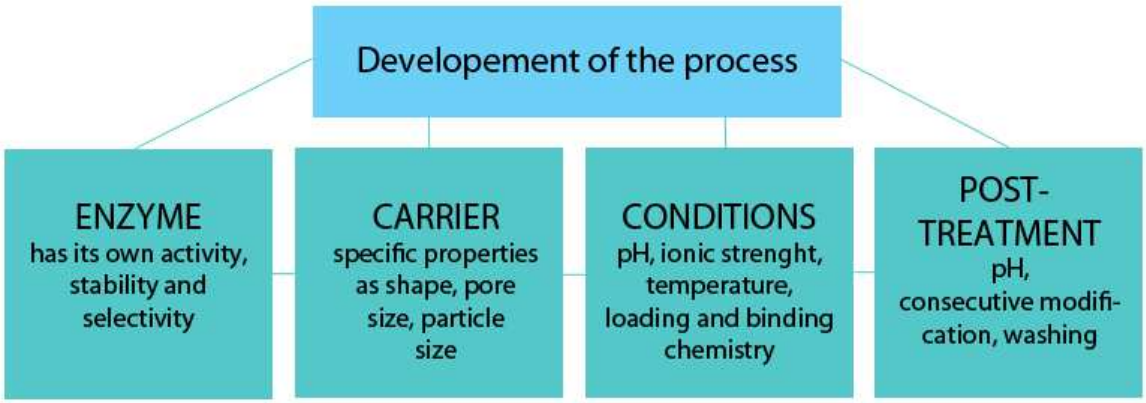


Fig 3 *Development of the enzyme immobilization process*

It is known that enzymes are highly selective catalysts with very different properties and demands on external conditions and treatment. They have different structural moieties which interact with activated support such as nucleophilic residue, carboxylic groups, and hydrophilic pockets. That is also the reason why it is so important to choose the suitable combination of conditions and method of immobilization.

Treatment of immobilized enzymes requires the meeting a number of criteria. It is important to avoid utilization of toxic reagents during and after immobilization process. Design and preparation of enzyme-support system depends also on end-use for different applications of this system, such as biosensors or therapeutic applications [14].

2.3.1 Historical background of enzyme immobilization techniques

In chronological development of enzyme immobilization techniques there are some historical phases that should be mentioned as the important milestones of enzyme immobilization research. The main points of historical development classification are mostly the number of methods developed, the number of different materials used as supports for

immobilization, the understanding of the factors influencing the performance of the immobilized enzymes, and the number of processes utilizing immobilized enzymes [4].

In 1916 Nelson and Griffin bind invertase on $Al(OH)_3$ and charcoal to obtain still catalytically active enzyme species. Unfortunately, the potential of this first immobilization attempt was not recognized in the following forty years [16].

Subsequently, the physical methods of immobilization dominated in 1950s. An example of non-specific physical adsorption of enzymes on solid carriers represents α-amylase adsorbed on activated carbon [4], or chymotrypsine on kaolinite. Further, the method of adsorption developed from simple physical adsorption to specific ionic adsorption, such as chymotrypsin on phosphocellulose [17], lipase and catalase on styrene-polyaminostyrene [4]. Simultaneously covalent immobilization techniques were studied, using lipases and other enzymes which were covalently bound to polyaminostyrene [4] or diazotized cellulose [18]. The main disadvantage of these carriers was a poor retention activity (2 – 20 % of the native activity) [4]. The period around 1960s is indicated as the developing phase. The main focus was still on covalent methods, however, non-covalent immobilization *i. e.* adsorption [19] and an entrapment in synthetic polymers (gels) such as polyvinyl alcohol [20] or polyacryleamide gel was studied as well. The encapsulation in semi-permeable membranes was proposed for the first time [4]. Remarkable progress was done in a research of influence of chemical and physical nature of the carriers on the catalytic characteristics of the enzyme [4], such as activity, retention of activity, and stability [23]. At the end of 1960s the first industrial application of an immobilized enzyme had been developed by Japanese company [4]. This was production of 1-amino acids from racemic amino acids derivatives [19].

In 1970s the main methods (covalent binding, adsorption, entrapment, and encapsulation) were transformed and improved. It was found out that enzyme-immobilization does not have to be performed strictly in aqueous media but also in solution of organic solvent [25]. In this time the potential of enzyme-immobilization in commercial and industrial processes has been completely recognized. Immobilized enzymes would be attractive catalysts in manufacturing of pharmaceuticals or agrochemicals yielding enantiomerically pure compounds, namely because of their selectivity and specificity. Further, biocatalytic processes might meet strict environmental regulations, because of their mild reaction conditions and lower energy consumption [4].

Since 1990s, the scientists started to concentrate their research on production of robust enzymes which are more stable and selective in organic solvent. So there was the development of enzymes, which would be able to work under certain conditions including special organic non-aqueous solvent [4]. Attention was also paid to design new technologies that combine different immobilization techniques to improve properties of immobilized enzymes. It was found that combination of different methods can easily solve some problems occurring when only one method is used.

2.3.2 Methods of immobilization

As were mentioned, during the research of enzyme immobilization in recent years, some different methods have been developed. They can be generally divided into four groups:

- Adsorption of enzyme to water-insoluble support
- Covalent binding by the side chain of enzyme to water-insoluble support
- Entrapment of enzyme into polymer
- Cross – linking of several enzymes molecules between themselves

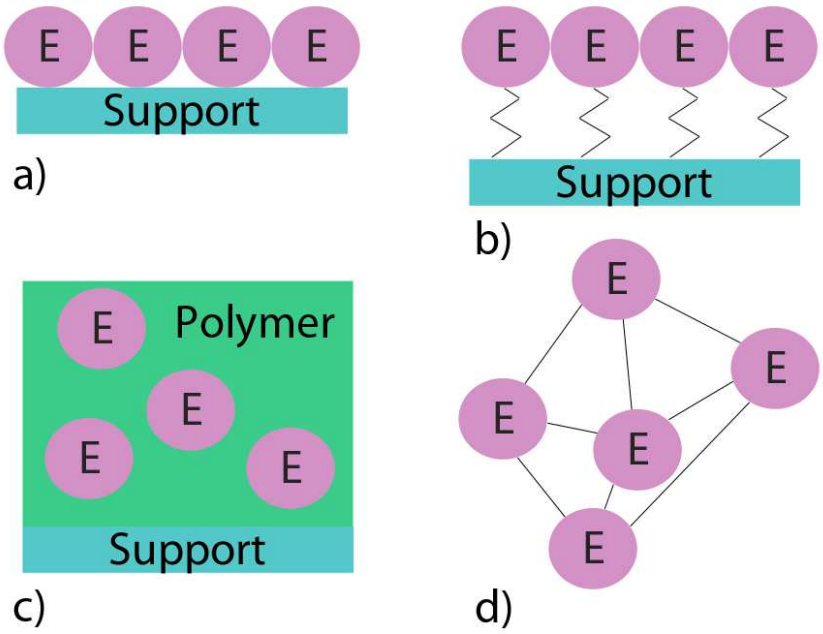


Figure 4 *Scheme of enzyme immobilization techniques: a) Physical adsorption, b) Covalent binding, c) Entrapment, d) Cross - linking*

Thanks to the progress in this research area a lot of problems which caused unsuccessful immobilization have been resolved by combination of these four basic methods. Higher stability of enzyme can be reached by pre-immobilization stabilization strategy or post-immobilization strategy. In the first case, the enzyme is cross linked to form stabilized enzyme preparations. Subsequently entrapment gives suitable particle size and high mechanical stability. High stability can be also achieved by chemical modification of enzyme with hydrophobic polymer [4]. Entrapped enzyme can be moreover cross linked. For example β-amylase from *Bacillus megaterium* immobilized in BSA gel matrix and subsequently covalently cross linked is fourteen times more stable than native enzyme [4]. As a result of these combined methods is a production of robust immobilized enzymes, active in inhospitable conditions as in organic solvent and so forth [4].

Some other parameters like pH optimum, oxidation-reduction potential working range and the apparent Michaelis constant (K_M) for appropriate substrate, can also be affected by immobilization [27].

Table 1 *Comparison of different immobilization methods* [28]

	Adsorption	*Entrapment*	*Cross - linking*	*Covalent binding*
Immobilized process	Easy	Difficult	Difficult	Difficult
Interaction	Weak	Strong	Strong	Strong
Recovery of lipase activity	Low	High	Moderate	Low
Regeneration of immobilized lipase	Possible	Impossible	Impossible	Impossible
Cost	Low	Low	Moderate	High

2.3.2.1 Adsorption

Adsorption was the first ever method developed (1916) and also industrially used. The first immobilized enzyme used in industry was prepared by adsorption of amino acid acylase of DEAE-cellulose [4]. Basic definition of this method is the connection between enzyme and support without any chemical modification. However, non-covalent binding can be classified into many other different groups such as non-specific physical adsorption, ionic bonding, coordination (metal chelating) and affinity adsorption [4]. Non-covalent carrier-bound enzyme immobilization can be classified into these categories:

- Non specific – enzyme is adsorbed by non-specific forces. It means Van der Waals, hydrogen bounds, or hydrophilic interaction.
- Biospecific adsorption – molecular ligands are used for adsorption.
- Affinity adsorption – adsorption to immobilized dyes or immobilized metals.
- Electrostatic interaction – based on charge-charge interaction between the enzyme and the carrier.
- Hydrophobic interaction – based on hydrophobic regions of the enzyme and carrier [4].

If we compare adsorption with other methods, it is the most used method for immobilization due to some unquestionable advantages. They are mild and easy preparation conditions, low cost (depending on type of support and its cost), possibility of regeneration of the carriers for its reuse, no chemical additives required and high activity [28]. However, there are some negatives. It is mainly the possibility of releasing of the enzyme from the carrier during the catalytic reaction because of the weak interactions between them. Immobilized lipase is also sensitive to pH, ionic strength and temperature.

2.3.2.2 Entrapment

Entrapment of an enzyme is process of capture of the enzyme into the inner hollows of some specific matrix or into microencapsules of polymer. What is really important, enzyme inside of the matrix is not attached to the polymer thus its free diffusion is only restrained [28].

Entrapment method can be divided into two special groups:

- Gel entrapment – molecule of enzyme is entrapped into the pores of polymer (with specific size)
- Microencapsulation – molecules of enzymes are entrapped into the pore of polymer

This method is fast, cheap, and usually proceeds at mild conditions. On the other hand, there is mass transfer limitation. It means that enzyme is effective catalyst only for low molecular weight substrates transformations [28].

Lipase (NS44035) from *Novozymes* North America Inc. was entrapped in celite as supported sol-gel. After immobilization, the lipase was used for methanolysis of triolein with efficiency ~ 60 % and possesses the activity comparable to that of its soluble form [30]. Lipase from *P. cepacia* was immobilized in hydrophobic sol-gel. It was used for catalyzing the biodiesel production from soybean oil. The final conversion of about 67 % was higher than that of free lipase [31].

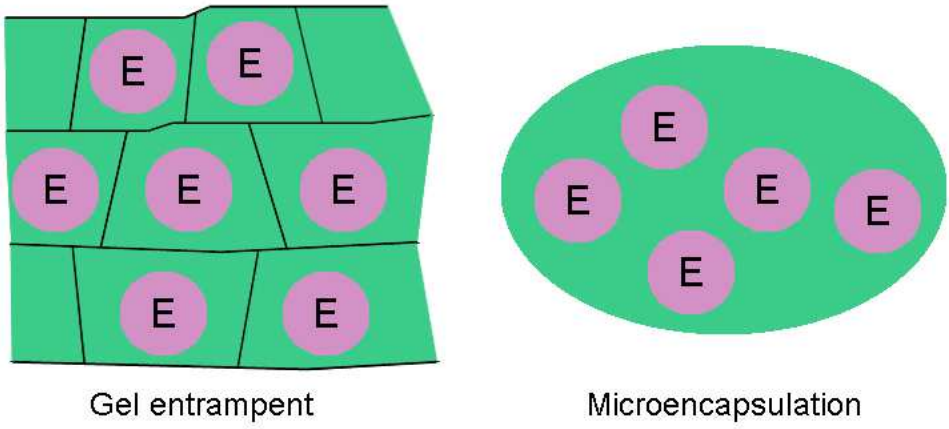


Figure 5 *Scheme of entrapment: a) Gel entrapment, b) Microencapsulation*

2.3.2.3 Covalent binding

This method of immobilization is based on covalent bound between enzyme and carrier. During the catalyst action the interaction between enzyme and support is very strong which makes the enzyme very stable. But covalent binding has some disadvantages as well. Reaction conditions are complicated, there is a high risk of loose of enzyme activity during the process, and some coupling reagents are toxic [28].

2.3.2.4 Cross – linking

In the process of cross – linking the three – dimensional network structure through the interaction between enzyme, coupling reagent, and carrier is formed. An advantage of this method is a strong interaction between the protein and the carrier, but activity of the immobilized enzyme is low. That is the reason why cross – linking is often combined with adsorption to achieve higher immobilization efficiency [28].

2.3.3 Types of carriers

The support which is used for immobilization has a major impact on the stability of the immobilized enzyme and mainly on the success of immobilization. The most important requirements for a carrier are insolubility in water, high capacity for binding of enzyme and chemical inertness, and stability. Binding capacity is determined by the surface area available (pore size, bead size or tube diameter) and density of enzyme binding sites. Inertness can be understood as a degree of non-specific adsorption and the pH, pressure and temperature

stability [27]. It is known that it is not simple to assume the appropriateness of using particular carrier for the immobilization. At present there is not universally recommended support. Types of support can be classified into these categories:

- Hydrophobic biopolymers based on natural polysacharides (cellulose, agarose)
- Organic polymers (polyacrylamide, nylon)
- Inorganic materials (pore glass, iron oxide) [27]

From particular supports used in last few years we can mention a novel material based on graphene and its derivates. Oxidized graphene has become very popular and it is object of intense research thanks to its unique chemical, mechanical and electrical properties. Another advantage of this compound is its low toxicity under physiological conditions. Enzymes can be immobilized on oxidized graphene by covalent binding (with a cross-linker or using the functional groups on the GO surface) or non-covalent binding through weak interactions [32]. However, electrostatic interactions between enzyme and carrier often affect the activity of the enzyme. The interaction between enzyme and GO support is one of the decisive factors for enzyme stability and activity. This restriction is largely successfully solved by reduction of GO. Chemically reduced GO can be effective solution for improving enzyme performance after immobilization [32].

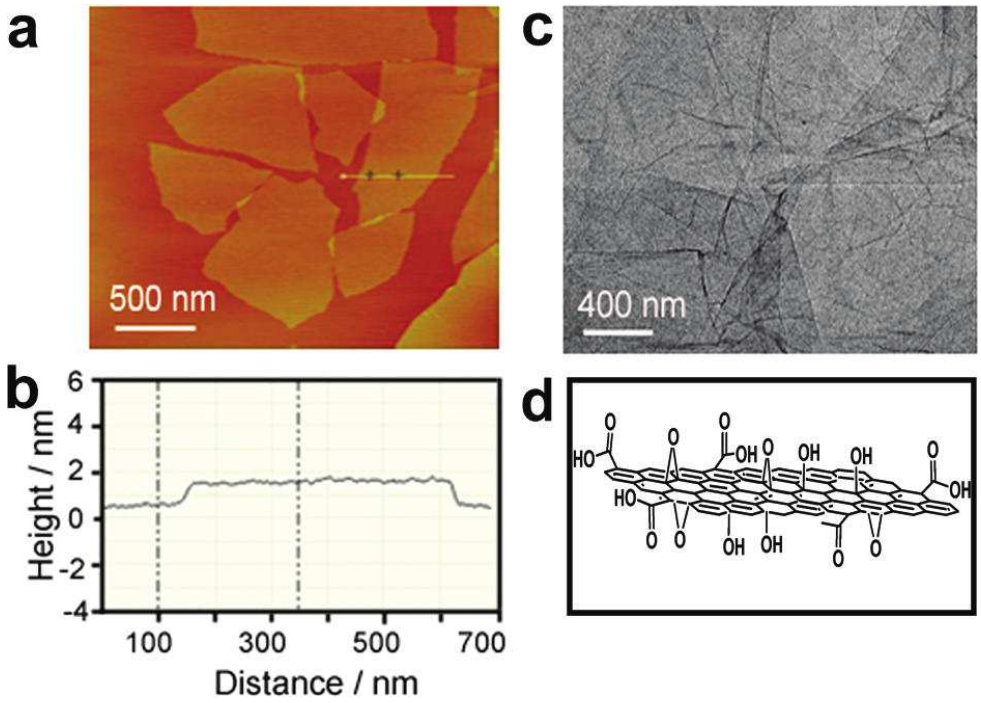


Figure 6 a) *Tapping mode AFM image of oxidized graphene (GO), b) height profile of the AFM image, c) TEM image of the GO, d) schematic model of GO* [33]

2.3.4 Immobilized enzymes used in enzyme engineering

Immobilized enzymes have been used in variety of technology industries. Due to the potential of these technologies, application in praxis is in a significant progress. Immobilized enzymes have found a variety of biomedical and industrial applications and so this area has been developing into an ever-expanding field during last years. In the scheme depicted below the potential of efficient immobilized enzymes from the technological point of view can be

appreciated.

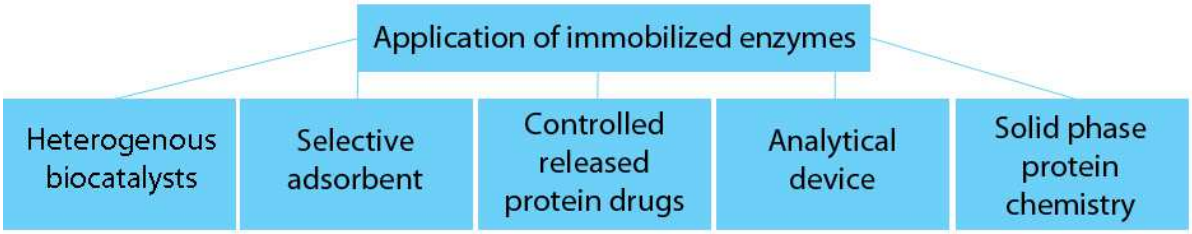


Figure 7 *Application of immobilized enzymes*

Immobilized enzymes and cells are used in antibiotic production, drug metabolism, food industry, biodiesel production, and bioremediation. The success of the extensive usage of immobilized enzymes is going to be effective and expansive also due to the fact, that applications of these methods comparing to the other parallel technologies are easier, cheaper and also environmental friendly [14].

2.3.4.1 Use of immobilized enzymes as biosensors

Biosensor is an analytical device which is used for detection of an analyte by specific detector. Its essential part is the sensitive biological element (enzyme, microorganism, organelle) which interacts with analyte and detector element or transducer. It transforms the signal from analyte interaction to another signal which is measurable. Device for processing, evaluation and display is called as biosensor reader.

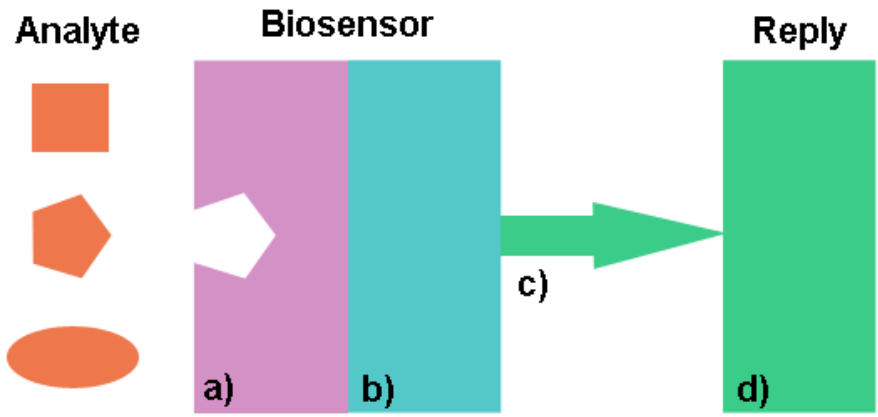


Figure 8 *Scheme of biosensor: a) Receptor, b) Transducer, c) Sensor, d) Reader*

Biosensors are used for medical diagnostic and pathogen and toxin detection in food and water. Analytical technologies based on biosensors are represented also on many major industrial sectors, such as the pharmaceutical, healthcare, food and agricultural industries and environmental monitoring [14]. They are famous analyzers also because they are easy to use, highly sensitive and specific.

Biosensors based on enzyme inhibition are important indicators of organophosphorus and organochlorine pesticides, derivatives of insecticides, heavy metals, and glycoalcaloids. This enzyme – analyte system works on the fact, that detected toxic substances inhibit natural

enzymatic function [14]. In 2005, the application of biosensors based on glucose oxidase immobilized by electro-polymerization for heavy metals detection was described. Another particular case represents urease entrapped in both polyvinylchloride and cellulose triacetate layers on the surface of pH-sensitive iridium oxide electrodes, which were used for determination of mercury [34]. Determination of different neurotoxins was achieved by a combined recognition/determination strategy. It is based on the joint action of acetylcholinesterase and organophosphorus-hydrolase enzymes. It brings the possibility to separate the action of different inhibitors [36].

The development and use of biosensors for detection of various pollutants as pesticides, heavy metals and toxins is a subject of intense research mainly in food and environmental monitoring.

2.3.4.2 Use of immobilized enzymes for antibiotic production

Antibiotic production by immobilized enzymes represents a challenging biotechnological topic of research nowadays. Enzyme-based synthesis of antibiotics such as β-lactam is an environmentally friendly process working at room temperature that avoids the use of organic chlorine solvents.

Among different alternatives, the kinetically controlled synthesis, using immobilized penicillin G acylase in aqueous environment, with the simultaneous crystallization of the product, is the most promising one [14]. The β-lactam acylase is used for the hydrolytic processes in production of penicillin G and cephalosporin C. β-lactam antibiotics are a broad group of antibiotics that contain derivatives of penicillin, cephalosporin, monobactams, carbapens and others. In general, this group includes all antibiotics that contain β-lactam core in the molecular structure.

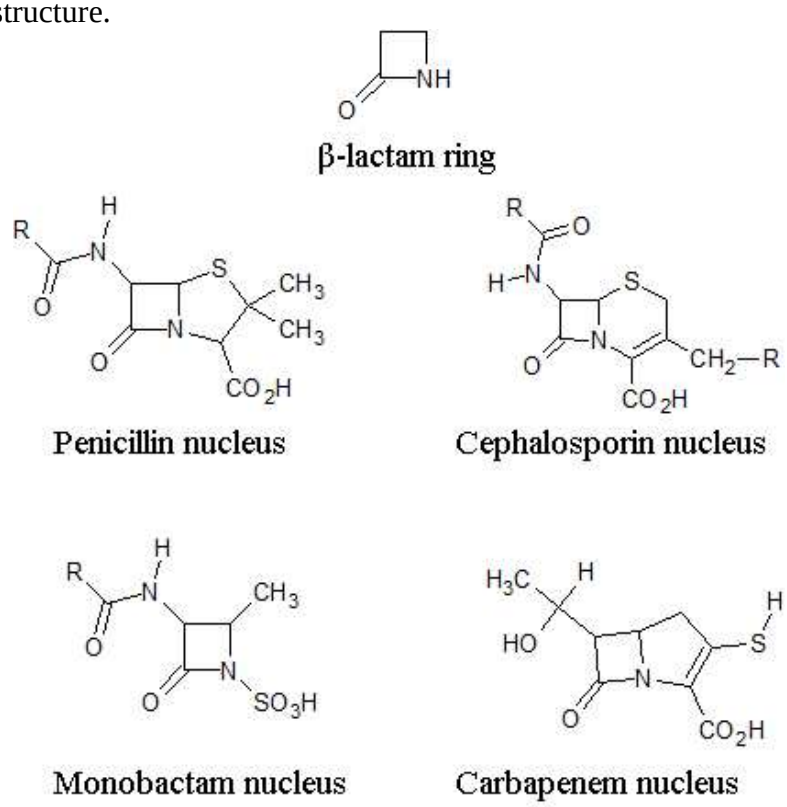


Figure 9 *Chemical structures of β-lactam ring and selected examples of its derivates*

Great progress has been made also in the resolution of racemic mixtures. β-lactam acylases was used for selection of stereoselective acylation/hydrolysis. Conversion of 7-amino-3-deacetoxy-cephalosporin acid to cephalexin by immobilized penicillin G acylase had been designed successfully. Penicillin G acylase reached 85 % conversion of 7-amino-3-deacetoxy-cephalosporin acid to cephalexin and in immobilized form it was possible to reuse it for about ten cycles. The production of cefazoline by immobilized cefazolin syntethase from *E. coli* as a biocatalyst also could be possible [14].

2.3.4.3 Use of immobilized enzymes in medicine and food industry

At present, immobilized enzymes are commonly used for diagnostic and therapy of various diseases. Electrodes based on enzyme technologies represent the main application of immobilized enzymes in medicine. High selectivity and reactivity are the key parameters for biosensor technologies. Numbers of clinical trials showed, that intense activity monitoring of specific enzymes can determine soon a diagnosis of variety physical dysfunctions or diseases. These studies were initiated in order to improve current diagnostics (chromatography, mass spectrometry) and to develop faster and more effective detection of metabolic imbalances signals. Timely diagnosis leads to the effective prevention and therapy of a lot of diseases like diabetes and obesity [37].

Immobilized enzymes have a great application in processing of food samples, processing of whey, production of skimmed milk and many others. Immobilized invertase is used in food industry and distilling, production of glucose – fructose syrupy and D-tagatose. In last few years, D-tagatose was investigated because of its similarities with fructose. It is used like healthier sweetener or ingredient in detergents and pharmaceutical preparations [14].

2.3.4.4 Use of immobilized enzymes for biodiesel production

In recent years biodiesel is becoming more and more attractive as alternative energy source because of its biodegradability, environmental friendliness, and renewability. Biodiesel fuels do not produce sulfur oxide, halogens, carbon monoxide and minimize the soot particulate [13]. From the chemical point of view we can tell, that biodiesel is fatty acid alkyl ester. There are a lot of methods based on chemical catalysis to produce it. Best known is mainly transesterification, with acid or alkaline as catalysts because of its high biodiesel yield (usually less than 96 %) and low catalysts cost. However, the disadvantage of the process is difficulty in the recovery of the catalysts and glycerol, which is formed as a byproduct, potential pollution to the environment and also wastewater treatment that is needed [28]. The biochemical catalysis method using the enzymes offers milder conditions (and it leads to less energy consumption), easy separation of enzyme from the reaction mixture (if the enzyme is immobilized), easier purification and separation of biodiesel, less pollution emission and environmental-friendly process [28]. For example, when the efficiency for the catalysis of transesterification from *C. rugosa* immobilized lipases was studied, the immobilized lipase on Sepabeads EC-OD demonstrated potential as a biocatalyst for further application in biodiesel synthesis using enzyme catalysis [38].

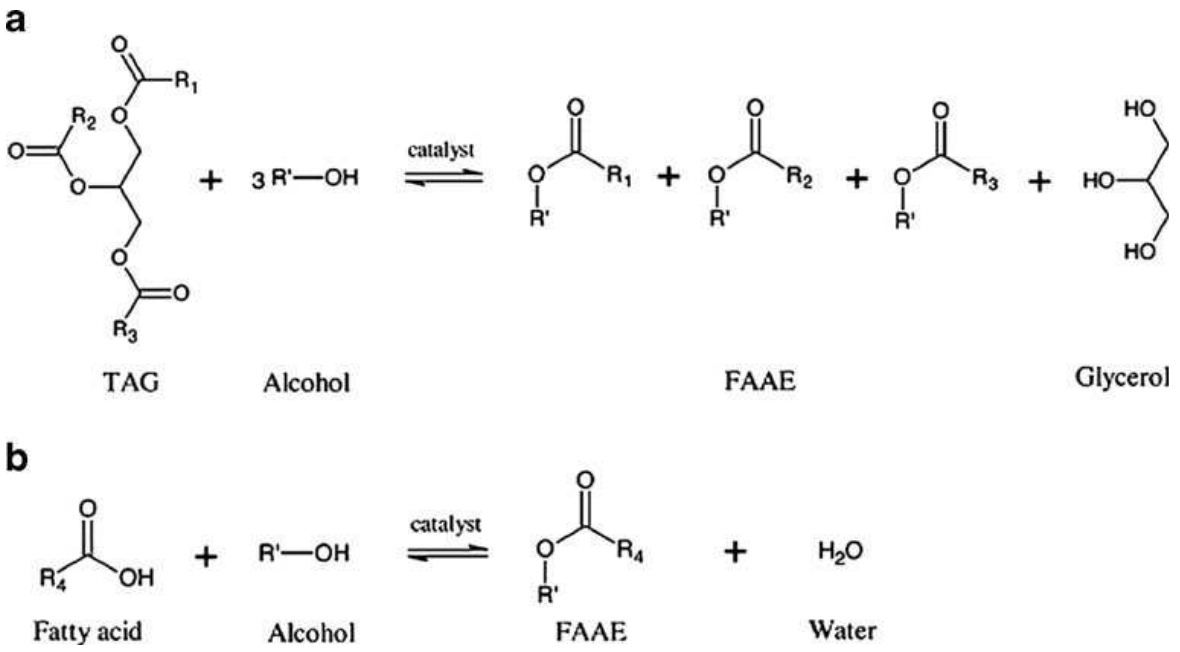


Figure 10 *Synthesis of fatty acid alkyl ester (FAAE) a) Transesterification of triacylglycerides (TAG) and short-chain alcohols leading to FAAE and glycerol. b) Esterification of fatty acid and short-chain alcohol leading to FAAE* [28]

Biodiesel can be produced from vegetable oils, animal fats, microalgal oils and waste products of vegetable oil refinery or used frying oil. Nowadays main attention is given to research of new immobilization supports with enhanced properties over ceramics, kaolinite, silica and zeolites. The main reason is the fact, that enzymatic production of biodiesel is still more expensive than that of the chemical equivalent. Nevertheless, if we take into account also the impacts on the environment, we can tell that the cost is comparable [14].

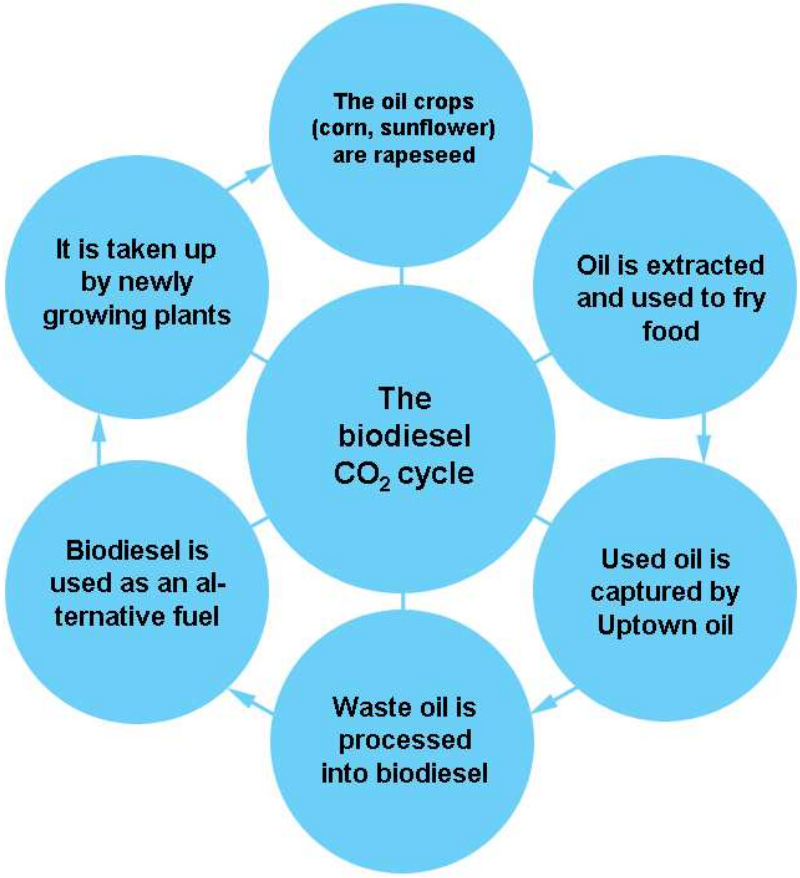


Figure 11 *Cycle of biodiesel in use*

2.3.4.5 Use of immobilized enzymes for bioremediation

Bioremediation is any process where living organisms, cells or enzymes are used to transform toxic substances into nontoxic form. Wide range of enzymes can be used for bioremediation, depending on which contaminant needs to be degraded.

For example, the large number of different pollutants can be degraded by enzymes of white rot fungi which have ability to cleave the carbon-carbon bonds in contaminants [28].There are more than 100,000 dyes with over 7×10^5 ton of dyestuff produced annually and used in textile, dyeing and printing industry. By estimate, 10 – 15 % of the dyes are lost in industrial effluents. Physico-chemical methods for refinement of dyes-polluted water are outdated and little effective. Biodegradation in the presence of microorganisms was considered to be an effective method but the analysis of contaminated soil and water shown that was still toxic. Also the conditions of the environment were not as favorable as required by microorganisms to perform degradation. Due to the recent studies of these processes, enzymatic removal of phenolic pollutants from aqueous solutions has attracted much interest as an alternative to the chemical and microbiological degradation [14].

3 EXPERIMENTAL PART

3.1 Materials and methods

3.1.1 Facilities

spectrophotometer, UV/VIS HELIOS DELTA 2073
drying oven, Memmert 100-800 Schwabach
balance, Scaltec
analytical balance
automatic shaker, Ika Werke-KS 130 basic
automatic shaker, Heidolph REAX top
magnetic stirrer, Lavat
pH meter, Merci s.r.o. WTW series
fridge, Samsung Callex
water bath, HUBER A11

3.1.2 Chemicals

sodium carbonate, Lachema ČSR
sodium phosphate monobasic dodekahydrate, pure, ČSR
potassium phosphate dibasic, Lachema ČSR
sodium hydroxide, pure, Lach:ner. s.r.o. ČR
phosphoric acid, Lachema ČSR
citric acid
glycine
p-nitrophenol, Lach:ner. s.r.o. ČR
p-nitrophenyl laurate, FLUKA (Sigma Aldrich)
polyethylene terephthalate (commercial name Sorsilen)
Rhizopus Arrhizus lipase commercial preparation, FLUKA (Sigma Aldrich)

3.2 Procedures

3.2.1 Spectrophotometric determination of lipolytic activity by p-nitrophenyl laurate

3.2.1.1 The composition of solutions prepared for the determination of RA lipase activity

a) Solution of p-nitrophenyl laurate (50 mM) – 0.02 g of p-nitrophenyl laurate was dissolved in 25 ml of ethanol
b) Solution of p-nitrophenol (1.0 mM) – 13.9 mg of p-nitrophenol was dissolved in 100 ml of distilled water
c) Solution of sodium carbonate (0.1 mM) – 1.06 g Na_2CO_3 was dissolved in 100 ml of distilled water
d) Solution of *Rhizopus arrhizus* lipase commercial preparation (1 mg/ml) – 10 mg of *Rhizopus arrhizus* lipase powder was dissolved in 10 ml of distilled water
e) Buffers:

1. Citric - phosphate buffer pH 5 – 24.3 ml of citric acid (2.101 g of citric acid dissolved in 100 ml of distilled water) was mixed with 25.7 ml of water solution of

$Na_2HPO_4.12H_2O$ (14.328 g of $Na_2HPO_4.12H_2O$ dissolved in 200 ml of distilled water). Final pH was adjusted by solution of NaOH.

2. Citric - phosphate buffer pH 6 – 17,9 ml of citric acid (2.101 g of citric acid dissolved in 100 ml of distilled water) was mixed with 32,1 ml of water solution of $Na_2HPO_4.12H_2O$ (14.328 g of $Na_2HPO_4.12H_2O$ dissolved in 200 ml of distilled water). Final pH was adjusted by solution of NaOH.
3. Citric - phosphate buffer pH 6.5 – 15.4 ml of citric acid (2.101 g of citric acid dissolved in 100 ml of distilled water) was mixed with 34,6 ml of water solution of $Na_2HPO_4.12H_2O$ (14.328 g of $Na_2HPO_4.12H_2O$ dissolved in 200 ml of distilled water). Final pH was adjusted by solution of NaOH.
4. Phosphate buffer pH 7.2 – 1.431 g of $Na_2HPO_4.12H_2O$ and 0.549 g of KH_2PO_4 were dissolved in 240 ml of distilled water, pH was adjusted to 7.2 by solution of NaOH
5. Phosphate buffer pH 8 – 5.3 ml of water solution of acidic sodium phosphate (3.56 g of g of acidic sodium phosphate dissolved in 100 ml of distilled water) was mixed with 94.7 ml of water solution of $Na_2HPO_4.12H_2O$ (14.328 g of $Na_2HPO_4.12H_2O$ dissolved in 200 ml of distilled water). Final pH was adjusted by solution of NaOH.
6. Glycine – NaOH buffer pH 9 – 50 ml of water solution of glycine (1.501 g of glycine dissolved in 100 ml of distilled water) was mixed with 8.8 ml of NaOH (1.6 g of NaOH dissolved in 200 ml of distilled water). Final pH was adjusted by solution of NaOH.
7. Glycine – NaOH buffer pH 10 – 50 ml of water solution of glycine (1.501 g of glycine dissolved in 100 ml of distilled water) was mixed with 32 ml of NaOH (1.6 g of NaOH dissolved in 200 ml of distilled water). Final pH was adjusted by solution of NaOH.
8. Britton – Robinson's buffer pH 11 – 100 ml of phosphoric acid (concentration of 0.04 M) was mixed with 82,5 ml of NaOH (1.6 g of NaOH dissolved in 200 ml of distilled water). Final pH was adjusted by solution of NaOH.

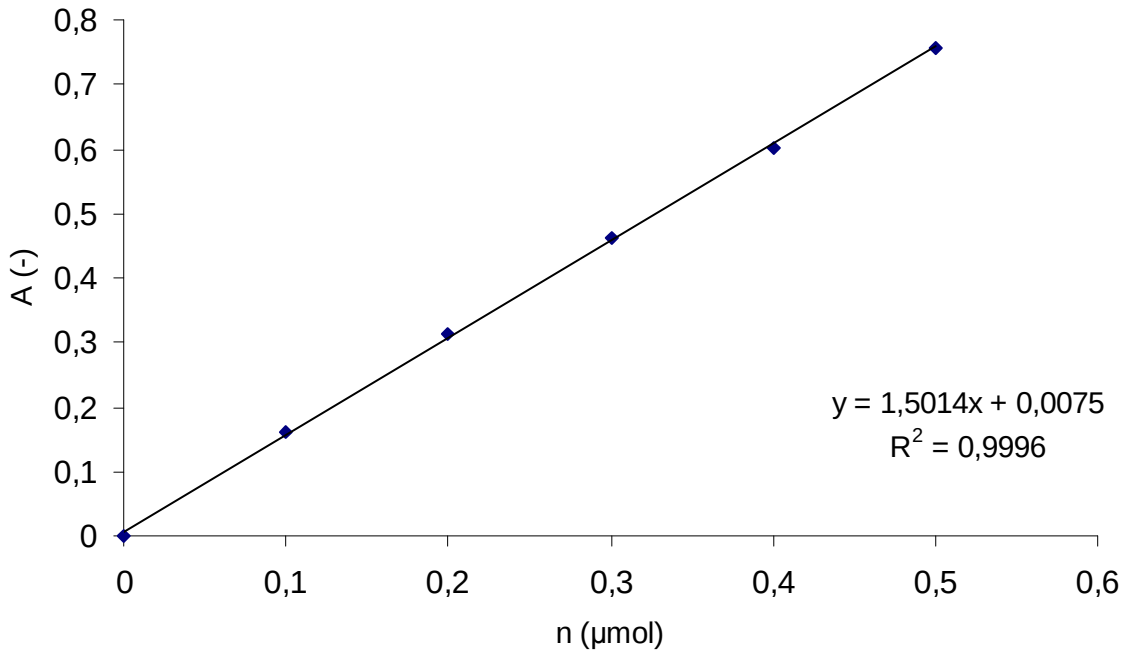
3.2.1.2 Calibration curve

Solution of p-nitrophenol (concentration of 1.0 mM) was used for construction of calibration curve. Five tubes with different concentration of p-nitrophenol were prepared by the following table. Sixth tube was used as blank.

tube	1	2	3	4	5	B
p-nitrophenol (ml)	0.1	0.2	0.3	0.4	0.5	0
phosphate buffer pH 7.2 (ml)	3.25	3.25	3.25	3.25	3.25	3.25
distilled water (ml)	0.4	0.3	0.2	0.1	0	0.5
Na_2CO_3 (ml)	0.5	0.5	0.5	0.5	0.5	0.5
n of p-NP (μmol in reaction mixture)	0.1	0.2	0.3	0.4	0.5	0

Calibration board was prepared from the solution of p-nitrophenol (concentrations of 0.1; 0.2; 0.3; 0.4; 0.5 mM). 3.25 ml of phosphate buffer pH 7.2 was added to this solution. The amount of tubes was stirred and left to incubate for 30 minutes at 37 °C in a water bath. Then 0.5 ml of Na_2CO_3 was added. Subsequently, absorbance of these solutions was measured at

a wavelength of 420 nm.



Graph 1*Calibration curve of p-nitrophenol*

3.2.1.3 Determination of lipolytic activity

3.25 ml of phosphate buffer was added to 0.25 ml of enzyme (concentration of 1.01 mg/ml). Then 0.25 ml of substrate (*p*-nitrophenyl laurate) dissolved in ethanol was added. Reaction mixture was stirred and left to incubate for 3, 6 and 9 minutes at room temperature. Then 0.5 ml of Na_2CO_3 was added to stop the reaction. Subsequently, the absorbance of the solution was measured at a wavelength of 420 nm. For blank, the substrate was replaced with distilled water.

3.2.1.4 Determination of temperature optimum

3.25 ml of phosphate buffer was added to 0.25 ml of enzyme (concentration of 1.01 mg/ml). Then 0.25 ml of substrate (p-nitrophenyl laurate) dissolved in ethanol was added. Reaction mixture was stirred and left to incubate for 30 minutes gradually at 30; 35; 37; 40; 45, 50 and 55 °C in a water bath. Then 0.5 ml of Na_2CO_3 was added. Subsequently, absorbance of this solution was measured at a wavelength of 420 nm. For blank, the substrate was replaced with distilled water.

3.2.1.5 Determination of pH optimum

3.25 ml of buffer was added to 0.25 ml of enzyme (concentration of 1.02 mg/ml). Then 0.25 ml of substrate (p-nitrophenyl laurate) dissolved in ethanol was added. Reaction mixture was stirred and left to incubate for 30 minutes at 37 °C in a water bath. Then 0.5 ml of Na_2CO_3 was added. Subsequently, absorbance of this solution was measured at a wavelength of 420 nm. For blank, the substrate was replaced with distilled water. The pH optimum was measured gradually at pH of 5, 6, 6.5, 7.2, 8, 9, 10, and 11.

3.2.1.6 Determination of thermal stability

0.25 ml of water solution of enzyme (concentration of 1 mg/ml) was left to incubate for 30 minutes gradually at 30; 40; 50; 60 and 70 °C in a water bath. Then 3.25 ml of phosphate buffer pH 7.2 and 0.25 ml of substrate (p-nitrophenyl laurate) dissolved in ethanol were added. Reaction mixture was left to incubate for 30 minutes at 37 °C. Then 0.5 ml of Na_2CO_3 was added. Subsequently, absorbance of this solution was measured at a wavelength of 420 nm. For blank, the substrate was replaced with distilled water.

3.2.2 Immobilization of *RA* lipase by sorption with polyethylene terephthalate (commercial name Sorsilen) used as a carrier

3.2.2.1 The composition of solutions prepared for enzyme immobilization

a) Solution of p-nitrophenyl laurate (2.5 mM) – 0.02 g of p-nitrophenyl laurate was dissolved in 25 ml of ethanol.
b) Solution of sodium carbonate (0.1 mM) – 1.06 g Na_2CO_3 was dissolved in 100 ml of distilled water
c) Solution of *Rhizopus arrhizus* lipase commercial preparation (1 mg/ml) – 10 mg of *Rhizopus arrhizus* lipase powder was dissolved in 10 ml of distilled water
d) Buffers:

1. Citric - phosphate buffer pH 6 – 17,9 ml of citric acid (2.101 g of citric acid dissolved in 100 ml of distilled water) was mixed with 32,1 ml of water solution of $Na_2HPO_4.12H_2O$ (14.328 g of $Na_2HPO_4.12H_2O$ dissolved in 200 ml of distilled water). Final pH was adjusted by solution of NaOH.
2. Citric - phosphate buffer pH 6.5 – 15.4 ml of citric acid (2.101 g of citric acid dissolved in 100 ml of distilled water) was mixed with 34,6 ml of water solution of $Na_2HPO_4.12H_2O$ (14.328 g of $Na_2HPO_4.12H_2O$ dissolved in 200 ml of distilled water). Final pH was adjusted by solution of NaOH.
3. Phosphate buffer pH 7.2 – 1.431 g of $Na_2HPO_4.12H_2O$ and 0.549 g of KH_2PO_4 were dissolved in 240 ml of distilled water, pH was adjusted to 7.2 by solution of NaOH
4. Phosphate buffer pH 8 – 5.3 ml of water solution of acidic sodium phosphate (3.56 g of g of acidic sodium phosphate dissolved in 100 ml of distilled water) was mixed with 94.7 ml of water solution of $Na_2HPO_4.12H_2O$ (14.328 g of $Na_2HPO_4.12H_2O$ dissolved in 200 ml of distilled water). Final pH was adjusted by solution of NaOH.
5. Glycine – NaOH buffer pH 9 – 50 ml of water solution of glycine (1.501 g of glycine dissolved in 100 ml of distilled water) was mixed with 8.8 ml of NaOH (1.6 g of NaOH dissolved in 200 ml of distilled water). Final pH was adjusted by solution of NaOH.

3.2.2.2 Procedure of immobilization

0.5 g of carrier was mixed with 13 ml of phosphate buffer and 1 ml of enzyme solution (concentration of 1 mg/ml). Reaction mixture prepared this way was mixed in automatic shaker for 4 hours. Mixture was placed in a refrigerator.

3.2.2.3 Determination of lipolytic activity of wash solution

The suspension was filtrated. 3.5 ml of filtrate were put into the test tube with 0.25 ml of substrate. The reaction mixture was let to react and after 30 minutes, 0.5 ml of sodium carbonate was added. Subsequently, absorbance of this solution was measured at a wavelength of 420 nm. For blank, the substrate was replaced with distilled water. The suspension was washed until zero lipolytic activity in the filtrate.

3.2.2.4 Determination of lipolytic activity of immobilized enzyme

3.5 ml of suspension was replaced from the Erlenmeyer flask into the beaker with 0.25 ml of substrate solution. The reaction mixture was mixed in magnetic stirrer and it was let to react for 60 minutes. After that, the suspension was filtered and 0.5 ml of sodium carbonate was added into the filtrate. Subsequently, absorbance of this solution was measured at a wavelength of 420 nm. For blank, the substrate was replaced with distilled water.

3.2.2.5 Determination of pH optimum of immobilized enzyme

3.5 ml of suspension was replaced from the Erlenmeyer flask into the beaker with 0.25 ml of substrate solution. Suspension was prepared gradually by replacing buffers with different pH (6, 6.5, 7.2, 8, and 9). The reaction mixture was mixed in magnetic stirrer and it was let to react for 60 minutes. After that, the suspension was filtered and 0.5 ml of sodium carbonate was added to stop the reaction. Suspension was filtered. Subsequently, absorbance of this solution was measured at a wavelength of 420 nm. For blank, the corresponding buffer was used.

3.2.2.6 Determination of thermal stability of immobilized enzyme

3.5 ml of suspension was left to incubate for 30 minutes gradually at 30; 40; 50; 60 and 70 °C in a water bath. Then 0.25 ml of substrate (p-nitrophenyl laurate) dissolved in ethanol was added. The reaction mixture was mixed in magnetic stirrer and it was let to react for 30 minutes. Then 0.5 ml of Na_2CO_3 was added to stop the reaction. Suspension was filtered. Subsequently, absorbance of this solution was measured at a wavelength of 420 nm. For blank, phosphate buffer pH 7.2 was used.

3.3 Determination of lipolytic activity of free soluble enzyme compared with immobilized enzyme

The calculation of lipolytic activity was based on the linear regression equation obtained by evaluation of p-nitrophenol calibration curve:

$$y = 1.5014x + 0.0075$$

$$1 = 1.5014x + 0.0075$$

$$x = \frac{1 - 0.0075}{1.5014} = 0.661$$

Calculation was based on the basic formula of determination of lipolytic activity:

$$a = k \cdot \frac{f}{c_E} \cdot f_{dilution}$$

a – Enzyme activity

f – Conversion factor (directive which was obtained from linear regression equation of calibration curve)

$f_{dilution}$ – Dilution factor

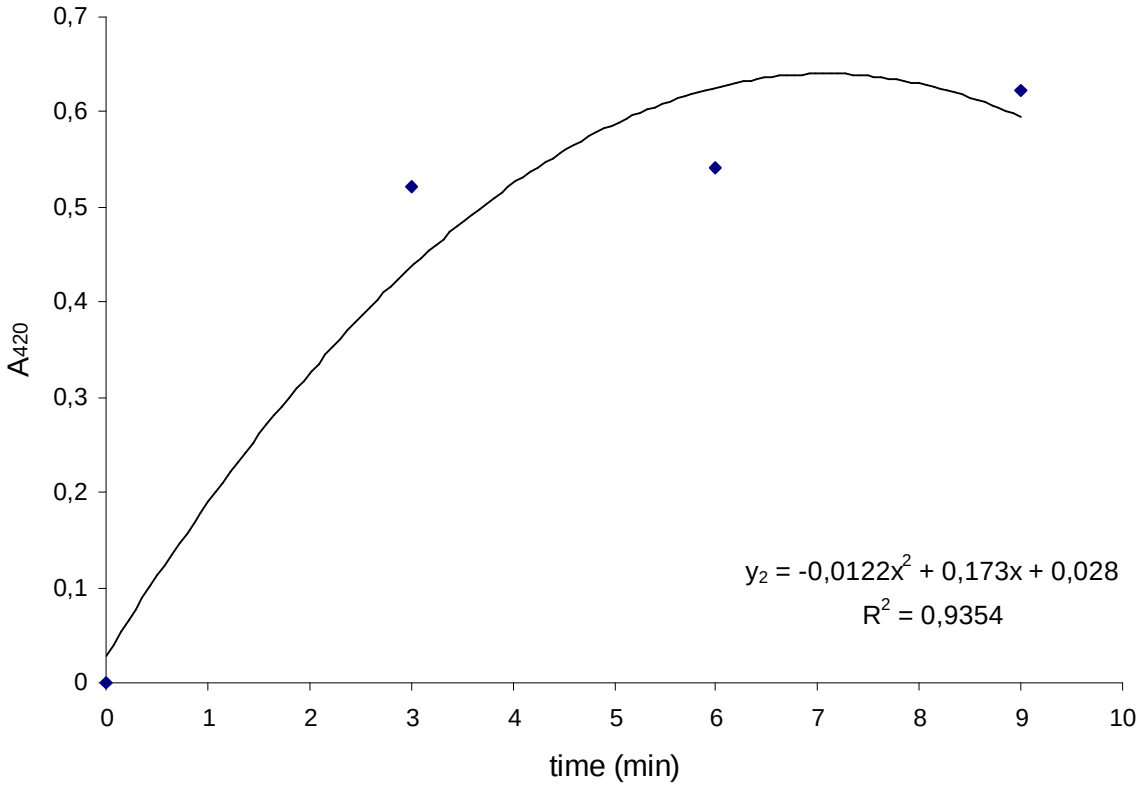
c_E – Characterization of enzyme (units depend on the way of calculation)

$$k = \frac{A_{420}}{t}$$

A_{420} – Absorbance

t – Time

Enzyme solution, which was prepared for determination, contained 10 mg of enzyme dissolved in 10 ml of distilled water. For reaction, 0.25 ml of this solution was taken. This means, that one reaction tube contained 0.25 mg of enzyme. Absorbance of solution prepared as was described in chapter ***3.2.1.3*** was measured in three intervals: 3, 6 and 9 minutes. Results of measurement were used to create the dependence of absorbance on time:



Graph 2 *Dependence of the absorbance on time*

Enzyme activity was calculated by the method of extrapolation to zero time.

$$y = -0.0122x^2 + 0.173x + 0.028$$

$$x' = -0.0244x + 0.173$$

For equation of the tangent in zero time:

$y - y_0 = f'(x_0)(x - x_0)$

x_0, y_0 - Coordinates of the point at which the tangent is constructed, in this case [0, 0]

$f'(x_0)$ - Value of derived functions after substituting of the coordinates of point x for which we make tangent

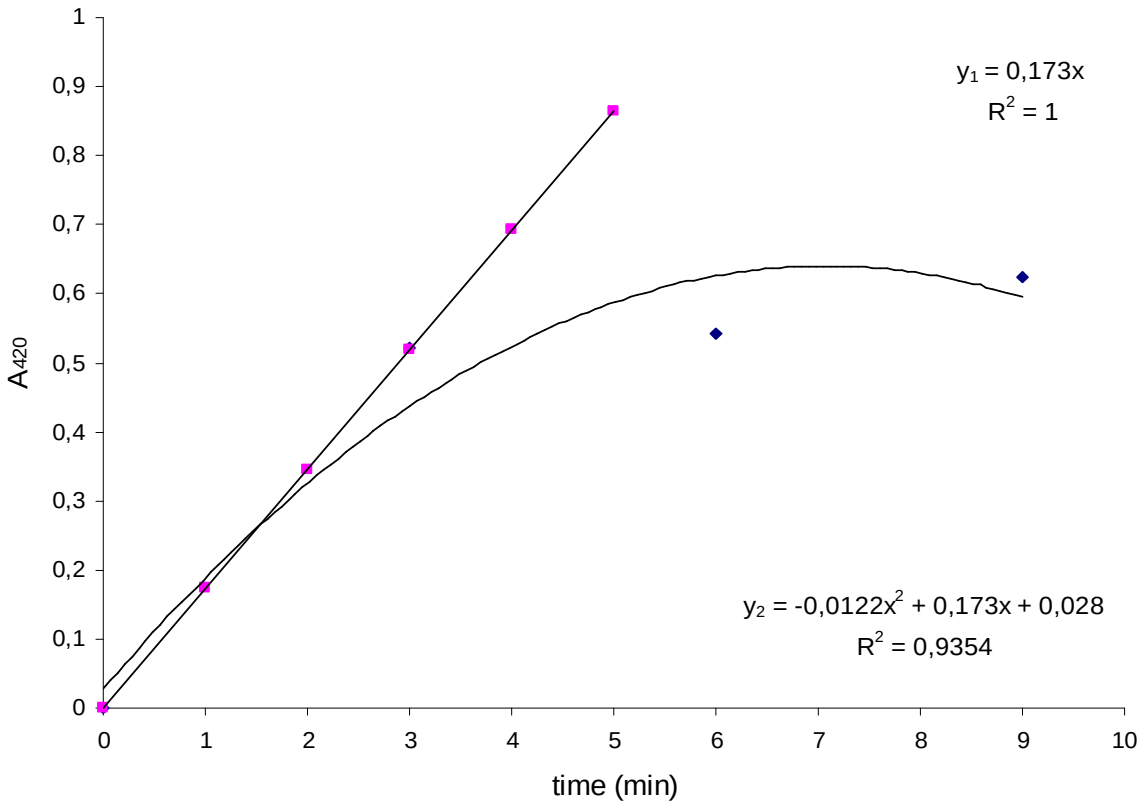
$f'(x_0) = -0.0244x + 0.173$

$f'(x_0) = 0.173$

Equation of the tangent is:

$y - 0 = 0.173(x - 0)$

$y = 0.173x$



Graph 3 *Dependence of the increasing absorbance on time with tangent in zero time*

$$a_1 = k \cdot \frac{f}{c_E} \cdot f_{dilution} = 0.173 \cdot \frac{0.661}{0.25} \cdot 1 = 0.457 \mu mol \cdot min^{-1} \cdot mg^{-1}$$

a_1 - Activity of native enzyme

Activity in wash solution was determined similarly:

First, lipolytic activity in the wash solution (phosphate buffer pH 7.2) was determined in every single wash fraction. Then, the resultant activity was determined by the sum of partial results.

$$a_n = \frac{A_{420}}{t} \cdot \frac{f}{c_E} \cdot f_{dilution} = \frac{0.048}{30} \cdot \frac{0.661}{3.5} \cdot 10 = 3.022 \cdot 10^{-3}\ \mu mol \cdot min^{-1} \cdot ml^{-1}$$

$a_2 = \sum a_n = 0.371\ \mu mol \cdot min^{-1} \cdot ml^{-1}$

a_2 - Activity in wash solution

Amount of enzyme, which binds to the carrier, was determined by following calculation:

1 mg.....0.457 $\mu mol \cdot min^{-1}$

x mg.....0.371 $\mu mol \cdot min^{-1}$

$$x = \frac{0.371}{0.457} \cdot 1 = 0.811\ \mu mol \cdot min^{-1} \cdot mg^{-1}$$

0.811 mg of enzyme was dispersed in the solution, which means that 0.189 mg of enzyme was bound. In the total volume there was 10 ml of solution and 0.5 g of the carrier. For reaction 3.5 ml of suspension was taken:

10 ml.....0.5 g

3.5ml.....x g

$$x = \frac{3.5}{10} \cdot 0.5 = 0.175\ g$$

0.189 g of the enzyme was bound into 0.5 g of the carrier. For calculation it is important to know the amount of enzyme that binds to substrate during one reaction:

0.5 g.....0.189 mg

0.175 g.....x mg

$$x = \frac{0.175}{0.5} \cdot 0.189 = 0.066\ mg$$

Activity of immobilized enzyme was calculated by the formula:

$$a_3 = \frac{A_{420}}{t} \cdot \frac{f}{c_E} \cdot f_{dilution} = \frac{0.102}{60} \cdot \frac{0.661}{0.066} = 0.017\ \mu mol \cdot min^{-1} \cdot ml^{-1}$$

Activity of immobilized enzyme in comparison with its soluble form:

0.457 g.....100%

0.017 g.....x %

$$x = \frac{0.0386}{1.0389} \cdot 100 = 3.72\ \%$$

4 RESULTS AND DISCUSSION

Immobilization was performed at room temperature in the phosphate buffer with initial pH 7.2 for 4 hours. Polyethylene terephthalate was used as a carrier. Concentration of enzyme used for immobilization was 1 mg/ml and 1 ml of this solution was added to the mixture for immobilization.

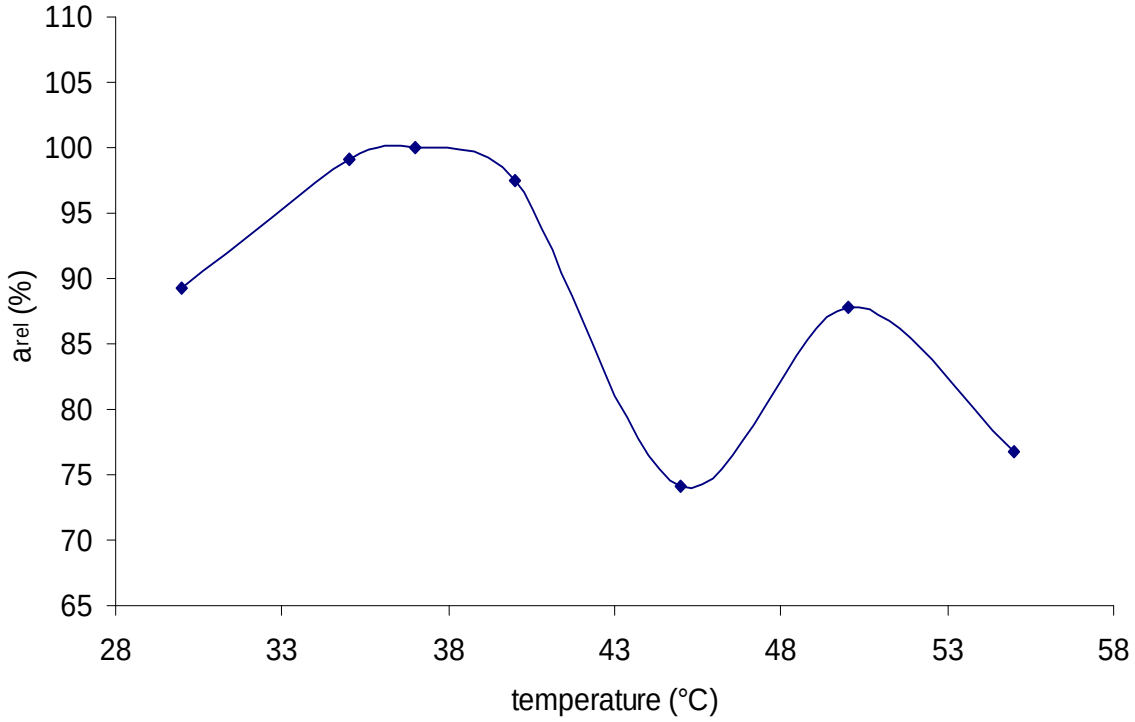
Based on the calculations mentioned in experimental part, lipolytic activity of immobilized enzyme was significantly lowered to about 3.72 % in comparison to the soluble form. The extent of immobilization was about 19 %, since 1 mg of enzyme was added into the reaction mixture, but only 0.189 g was bind on the carrier.

Determination of temperature optimum of soluble *RA* lipase

Temperature optimum was measured in the range from 30 to 55 °C. At temperatures from 30 °C to 37 °C an increase of activity was observed with reaching the maximum value at 37 °C. During further increase of temperature, the enzyme activity decreased by up to 26 % at 45 °C.

Interestingly enough, an increase of activity to the value about 88 % was detected again at 50 °C The presence of two temperature optima is not in accordance with previously reported work. Preetha Sasi *et al*. investigated esterification reactions catalyzed by surfactant-coated *Rhizopus arrhizus* in 2006. The temperature optimum of soluble lipase dissolved in isooctane was observed around 30 °C [39]. On the other hand, two points of temperature optimum may be indicative of the presence of isoenzymes as in the case of fungal lipase isolated from *Fusarium solani* FS1 [40].

Determination of temperature optimum of immobilized *RA* lipase was not carried out because the experiment was performed in a small scale. Consequently very small aliquots taken from the reaction mixture were not possible to be shaken and thermostated at the certain temperature in the same time.

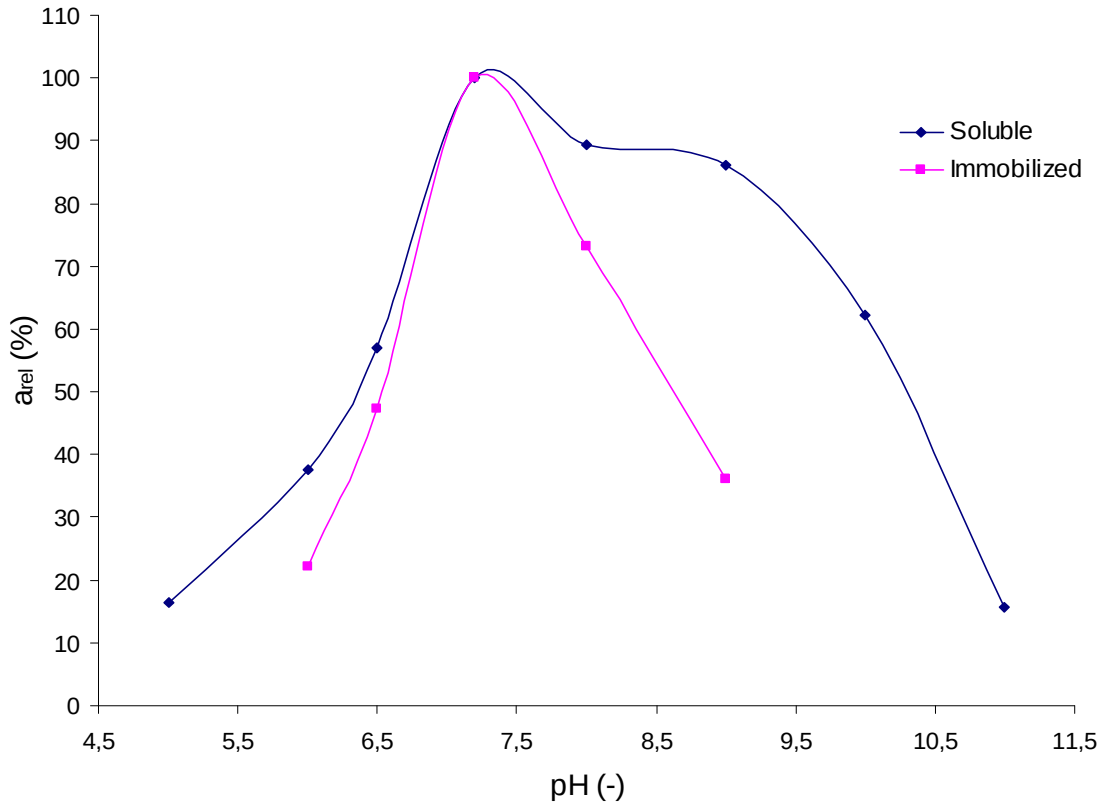


Graph 4 *Dependence of relative lipolytic activity of soluble RA lipase on the temperature (average value of three repeated measurements)*

Thermal stability and pH optimum of "free" soluble enzyme compared with its immobilized form

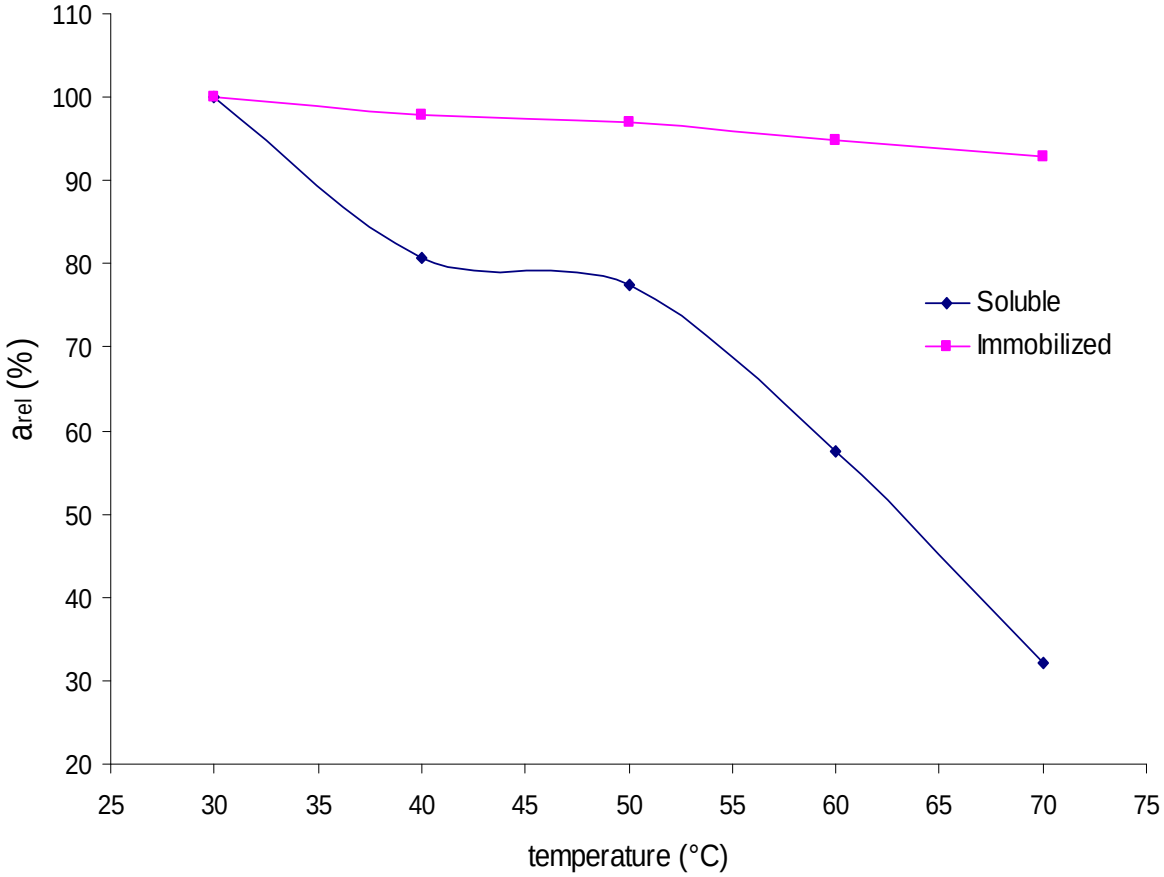
PH optimum was measured in the pH range from 5 to 11 for soluble *RA* lipase, and from 6 to 9 for the immobilized one. The highest lipolytic activity was observed at pH of 7.2 for both enzyme forms. The decrease in activity value to 15 % was seen both in basic and acidic conditions. The decrease of relative activity was more pronounced in the case of immobilized lipase. The optimal value of pH for the highest enzyme activity found out in the neutral range of pH, which correlates well with reported pH optimum of about 6.5 of *RA* lipase in isooctane [39].

Relative activity of immobilized enzyme at pH 6 was approximately equal to that of soluble enzyme at pH 5. Similar correlation was found out in basic conditions. Relative activity of immobilized *RA* enzyme at pH 9 was about 50 % lower than activity of soluble form. Based on the results obtained, one can suggested pronounced dependency of immobilized *RA* lipase activity on pH conditions of the reaction medium in comparison with the soluble enzyme.



Graph *5 pH optimum of soluble enzyme (average value of three repeated measurements) compared with its immobilized form*

Thermal stability of both forms of enzyme was measured at the same range of temperatures *i.e.* from 30 to 70 °C. At mild temperatures from 30 °C to 45 °C soluble enzyme shows relatively high activity. As the temperature was rising, the relative activity decreased rapidly. We could say that at 70 °C, most of enzyme was inactive, or denatured by exposure to high temperature. The relative activity was only about 30 % at 70 °C. By exposure to higher temperature, relative activity of immobilized enzyme also decreased, but this decrease was not as rapid as in the case of the soluble form of enzyme. Relative activity dropped by only 7 % from the maximum value. This result shows that immobilized form of enzyme is thermally more stable than its soluble form.



Graph 6 *Thermal stability of soluble enzyme (average value of three repeated measurements) compared with its immobilized form*

5 CONCLUSION

In theoretical part of this work the attention was paid on the practical applications of immobilized enzymes in last years, types of carriers that are used for immobilization, methods of immobilization and historical background of enzyme immobilization research. Triacylglycerol ester hydrolases (called lipases) as important biocatalysts were discussed. Substrate specificity of lipolytic enzymes, chemical and spatial structure, mechanism of action and catalytic activity of lipases was classified and briefly discussed. The attention was paid to microscopic fungi *Rhizopus arrhizus*, especially its microscopic view, inclusion and occurrence in nature.

In the experimental part, a procedure of immobilization of lipase isolated from microscopic fungi *Rhizopus arrhizus* on polyethylene terephthalate (commercial name Sorsilen) was developed. For the experiment, commercial preparation of this lipase was used. Measurements of lipolytic activity were based on the quantity of p-nitrophenol formed by enzymatic reaction of p-nitrophenyl laurate used as a substrate. Quantity of p-nitrophenol formed was determined from the absorbance value that was measured spectrophotometrically at a wavelength of 420 nm. Determination of lipolytic activity was based on the method of extrapolation to zero time using tangent in zero time. For determination of p-nitrophenol amount, linear regression equation obtained from calibration curve of p-nitrophenol was used in calculations. In subsequent experiments, enzyme characterization as lipolytic activity, temperature optimum, pH optimum and thermal stability was determined for soluble enzyme. PH optimum and thermal stability were also determined for immobilized form of enzyme. Based on the results it was found out, that immobilized enzyme had activity only of about 3.7 % comparing to its native form. This fact could be the consequence of too high amount of enzyme used for immobilization. Large amount of enzyme could cause high sterical barriers in the system of carrier/enzyme/substrate and it may prevent the access of substrate to active site of an enzyme. The activity of immobilized enzyme was found out to be strongly dependent on pH of the reaction environment. On the other hand, its thermal stability was revealed to be much greater than that of enzyme in native form.

The following research will be focused on the determination of the optimal amount of enzyme used for immobilization from the viewpoint of activity maintenance. The catalytic efficiency of selected lipase in the immobilized form on an appropriate carrier will be evaluated as well.

6 LIST OF ABBREVIATIONS AND SYMBOLS

Gly……glycine
Ser…….serine
FAAE …...fatty acid alkyl ester
RA…….*Rhizopus arrhizus*
GO…….graphene oxide
AFM……..atomic force microscopy
TEM……...transmission electron microscopy
pNPL…….p-nitrophenyl laurate

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