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ÚSTAV CHEMIE POTRAVIN A BIOTECHNOLOGIÍ

FACULTY OF CHEMISTRY
INSTITUTE OF FOOD SCIENCE AND BIOTECHNOLOGY

EFFECTS OF DETERGENTS ON ACTIVITY, THERMOSTABILITY AND AGGREGATION OF IMMOBILIZED LIPASES

VLIV DETERGENTŮ NA AKTIVITU, TEPELNOU STABILITU A AGREGACI IMOBILIZOVANÝCH LIPÁZ

DIPLOMOVÁ PRÁCE
MASTER'S THESIS

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Vedoucí práce	**Mgr. Soňa Hermanová, Ph.D.**		
Konzultanti:			

Název diplomové práce:

Vliv detergentů na aktivitu, tepelnou stabilitu a agregaci imobilizovaných lipáz

Zadání diplomové práce:

1. Vypracujte literární přehled k dané problematice
2. Popište použité metody hodnocení
3. Zpracujte naměřené výsledky z experimentů
4. Zhodnoťte získané výsledky formou diskuze

Termín odevzdání diplomové práce: 9.5.2014

Diplomová práce se odevzdává v děkanem stanoveném počtu exemplářů na sekretariát ústavu a v elektronické formě vedoucímu diplomové práce. Toto zadání je přílohou diplomové práce.

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

V Brně, dne 31.1.2014

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1 ABSTRACT

The diploma thesis deals with the issue of the effect of tweens on enzymatic activity of model hydrolase both free and immobilized on carbon-based carrier. In theoretical part, structural features, mechanism of action, and specialty applications of microbial lipases are reviewed along with detergent chemistry, with emphasize on tween family of detergents belonging into non-ionic surfactant group.

In experimental part, effect of tweens on soluble as well as immobilized hydrolase was examined. Immobilization of commercial preparation of lipase was performed by non-covalent adsorption on graphene oxide as a carrier treated with different tweens (tween 20, 60, 80). The activity was determined spectrophotometrically by *p*-nitrophenyl laurate assay. Enhancement of soluble *Rhizopus arrhizus* lipase activity (activity coupling of 104 %) was observed at tween 20 concentration of 10 $mmol \cdot dm^{-3}$, which is highly above critical micelle concentration of this detergent.

On the base of screening study, immobilization protocol comprised the incubation of soluble enzyme at concentration of 0.1 $mg \cdot ml^{-1}$ in phosphate buffer (pH 7.2) with tween 20 (10.8 $mmol \cdot dm^{-3}$) and the carrier for one hour. Both soluble and immobilized lipase exhibited maximum activity at approx. 35 °C. Optimal pH of immobilized lipase shifted to 8 compared to soluble form for which pH optimum at 9 was determined. Thermal stability profile follows almost same trend for both soluble and immobilized enzyme samples. The interactions between carrier and enzyme are suggested to be mainly non–covalent (adsorption, electrostatic interactions). No protein leaching was observed under studied conditions, and significant improvement of storage stability of immobilized lipase was achieved (activity retention of 41 % after 110 days) in comparison with soluble lipase (activity retention of 16 % after 42 days).

KEY WORDS

Lipase, immobilization, *Rhizopus arrhizus*, graphene oxide, detergent

ABSTRAKT

Predmetom tejto diplomovej práce bolo štúdium vplyvu detergentov na aktivitu, termostabilitu a agregáciu voľnej a imobilizovanej formy komerčného preparátu lipázy izolovanej z mikroskopickej huby *Rhizopus arrhizus*. Teoretická časť obsahuje ucelenú rešerš popisujúcu štruktúru, mechanizmus účinku a priemyselný význam spomínanej hydrolázy spolu s popisom chemických účinkov detergentov, pričom dôraz bol kladený predovšetkým na skupinu neionogénnych detergentov s názvom tweeny.

V experimentálnej časti bol študovaný efekt tweenov na rozpustnej a imobilizovanej forme RA lipázy. Imobilizácia spočívala v priamej adsorpcii enzýmu na neupravený nosič. Ako nosič bol použitý oxidovaný grafén ošetrený tweenom (tween 20, 60, 80). Aktivita enzýmu bola stanovená spektrofotometricky za pomoci substrátu *p*-nitrofenyl laurátu. Zvýšenie aktivity voľnej lipázy (104 % oproti maximálnej hodnote) bolo zaznamenané pri použití tweenu 20 o koncentrácii vysoko nad hodnotou kritickej micelárnej koncentrácie (10 $mmol \cdot dm^{-3}$).

Na základe štúdie imobilizačných podmienok, boli nastavené ideálne parametre pre dosiahnutie účinnej imobilizácie v spojení s čo najvyššou lipolytickou aktivitou (koncentrácia enzýmu 0,1 $mg \cdot ml^{-1}$, fosfátový tlmivý roztok pH 7,2, koncentrácia tweenu 10,8 $mmol \cdot dm^{-3}$, čas imobilizácie 1 hodina). Obe formy lipázy vykazovali maximálnu aktivitu pri 35 °C. Optimálne pH sa u imobilizovanej lipázy posunulo na hodnotu 8, v porovnaní s voľnou formou, ktorej pH optimum bolo stanovené na 9. Tepelná stabilita vykazovala približne rovnaký priebeh u oboch foriem skúmanej hydrolázy. Avšak v prípade štúdia stability enzýmu pri dlhodobej úschove bolo po imobilizácii zistené výrazné zlepšenie tohto parametru.

KĽÚČOVÉ SLOVÁ

Lipáza, imobilizácia, *Rhizopus arrhizus*, grafén oxid, detergent

BANČÁKOVÁ, A. *Effects of detergents on activity, thermostability and aggregation of immobilized lipases*. Brno: Brno University of Technology, Faculty of chemistry, 2014. p. ***51*** 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

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CONTENT

2 INTRODUCTION

Lipase industrial applications are rapidly growing research field since these biocatalysts have been discovered. They catalyse the hydrolysis as well as the synthesis of esters from glycerol and free fatty acids. Their great industrial potential results from their high enantioselectivity, stability in organic solvents, board substrate specificity, and no cofactor requirements [1]. Their benefits are widely used in food processing, detergent chemistry, organic synthesis, pharmaceuticals or cosmetics. Most lipases are activated at the water – lipid interface (interfacial activation). This phenomenon can be briefly explained by the moving of polypeptide chain which covers enzyme active site and thus prevents the formation of activated complex due to conformational changes. Hydrophobic part of lid ensures interaction with lipid phase and consequently the bond enzyme-substrate becomes stronger [2].

One of the biggest problems when biological substance is used in application technologies (such as biosensors, protein chips, surgical instruments, drug delivery *etc.*) is a random adsorption of biocomponents on noncomplementary materials. This phenomenon called nonspecific binding has been shown to be successfully solved by application of specific detergents into reaction systems. Especially, application of tweens as non ionic surfactant has been studied in systems of graphene composites. It was found that aliphatic chain of tween molecule can prevent nonspecific binding of biomolecules [3].

Effects of detergents on lipase conformational changes, to enhance their activity were studied as well. It was found that detergents when applied into enzymatic systems increase substrate concentration at the interface [4] and reduce hydration shell around the ester bonds [5] and thus increase catalytic activity of the protein.

In this work, detailed study of the influence of non ionic detergents on activity of soluble and immobilized lipase isolated from microscopic fungi *Rhizopus arrhizus* (in this work denoted as RA lipase) has been presented. This enzyme was selected from the practical point of view as the catalyst with more specialized properties. RA lipase have become more interesting due to its strong 1, 3 – position specificity [6]. Based on studied literature it can be concluded that the detergent – lipase interaction is very specific to the detergent used and thus detailed study of as much parameters as possible is necessary.

3 THEORETICAL PART

3.1 Lipolytic enzymes

Generally, lipases (EC 3.1.1.3) and esterases or carboxylesterases (EC 3.1.1.1) represent members of a group of hydrolases. Esterases are reported to be active catalyst in aqueous solutions and so-called true lipases (triacylglycerol (acyl) hydrolases) catalyze hydrolysis of triacylglycerols into di- and monoacylglycerols, fatty acids, and glycerol at an oil-water interface [7] (Fig. 1).

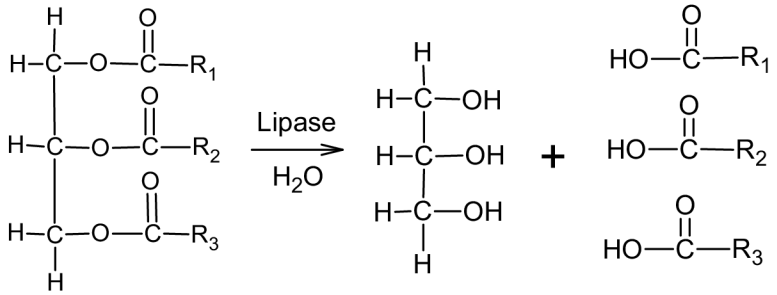

Figure 1 *Simplified way of lipases-catalyzed hydrolysis*

3.1.1 Conserved structural feature of lipolytic enzymes

Considering the 3D structure, characteristic conserved conformation classified as α/β-hydrolase bend was determined for most of classified lipases. Macromolecules of lipase consist of central β sheet (lipase core) being formed of up to eight different β strands connected with up to six α-spirals [1]. It was found out that active site of enzymes is protected by polypeptide chain in the form of lid, 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 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 [2].

The phenomenon of lipase activation is a result of conformational changes induced upon binding to a lipid interface. X – ray crystallographic studies have shown buried catalytic sites. α – helical lid in water conditions covers the enzyme active site and thus block the access to the substrate. Subsequently, after contact with hydrophobic surface α – helical lid rolls back and the active site becomes completely accessible. Furthermore, the hydrophobicity around the active site is substantially enhanced and enzyme takes over the active conformation [8].

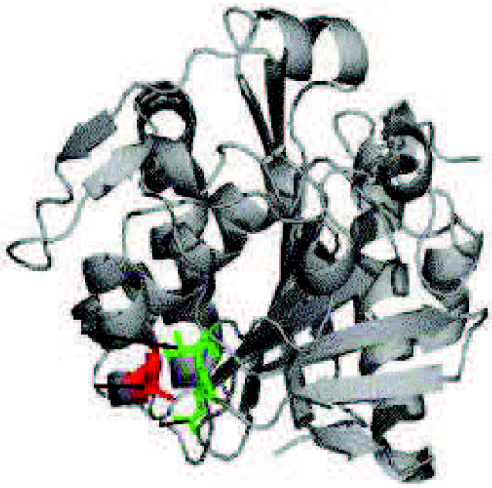

Figure 2 *Model structure of Rhizopus arrhizus lipase [9]*

3.1.2 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 motif denoted as nucleophilic elbow. Hydrolysis of substrate begins by nucleophilic reaction between Ser oxygen of enzyme and carbonyl carbon of triacylglycerol ester bond (Fig. 3). 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 [1].

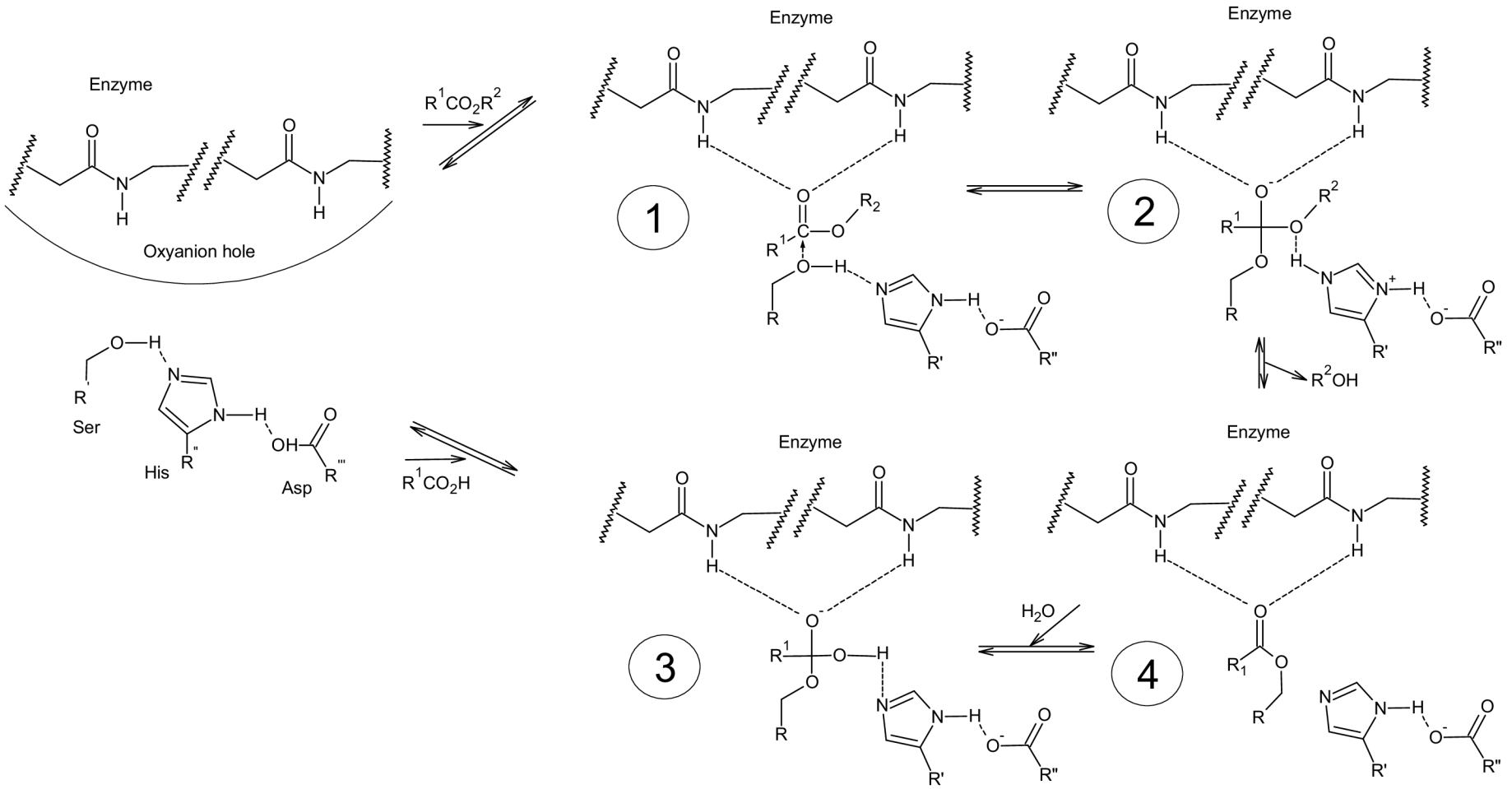

Figure 3 *The catalytic mechanism of lipase – catalyzed hydrolysis: 1.) Binding to the substrate, 2.) Tetrahedral intermediate 1, 3.) Tetrahedral intermediate 2, 4.) The covalent acyl-enzyme R^1CO-E' intermediate* [10]

3.1.3 Specialty applications of microbial lipases

Microbial lipases play part in wide range of industrial segments and their applications are favoured by the increasing availability of lipases in soluble (free) or immobilized form. They are specifically used in the processing of fats and oils and detergents, food processing, the synthesis of fine chemicals and pharmaceuticals or as the accelerators of fatty waste degradation [11]. Lipases are used as catalysts for both enantioselective hydrolysis of racemic esters of primary or secondary alcohols in aqueous media (Fig. 4) and enantioselective esterification of racemic primary or secondary alcohols in organic solvents. Lipase catalysis results in the production of enantiopure compounds that are important intermediates for pharmaceutical products, fine chemicals and agrochemicals [10]. The examples of concrete industrial applications are summarized in the table below.

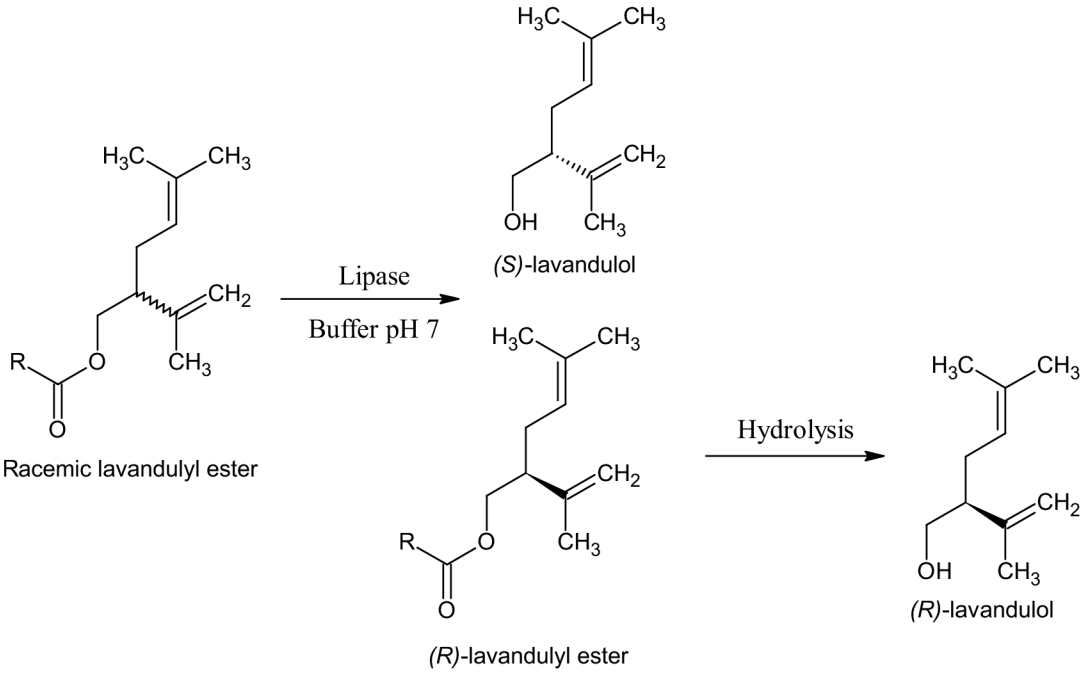

Figure 4 *Example of enantioselective hydrolysis of racemic ester – Lipase mediated hydrolysis of racemic lavandulyl esters in a phosphate buffer* [13]

Table 1 *Industrial applications of microbial lipases* [14]

Industry	***Action***	***Target product or application***
Detergents	Hydrolysis of fats	Laundry and household detergents
Food processing	Altering and replacing of one or more fatty acids	Bakery products, cheese flavour development, beverages, removing fats from meat and fish products [15]
Paper industry	Hydrolysis of wood triglycerides	Pulp and paper with improved quality
Organic synthesis	Catalysis of chemo-, regio- and stereoselective transformations [16]	Ester synthesis, regioselective acylation, resolution of racemic acids and alcohols
Pharmaceuticals	Transesterification, hydrolysis	Specialty lipids, digestive aids
Cosmetics	Synthesis	Emulsifiers, moisturizers

3.1.4 Lipase isolated from microscopic fungi *Rhizopus arrhizus*

The thesis is focused on lipase from *Rhizopus arrhizus* (oryzae), which was selected from the practical point of view as the catalyst with more specialized properties. Lipases isolated from *Rhizopus Arrhizus* fungi have become more interesting due to its strong 1, 3 – position specificity [6].

Hydrolysis, glycerolysis and esterification reactions of Rhizopus arrhizus lipase

One of the most important factors in chocolate industry is polymorphic behaviour of cocoa butter because it is the major solid fat in chocolate. Polymorphic structure significantly influences final chocolate quality like gross, texture, snap, heat resistance, and fat bloom resistance (Fig.5). Wille and Lutton in 1966 used X – ray diffraction spectroscopy to describe six polymorphic forms of cocoa butter and suspected that polymorphic transformations affecting chocolate quality are influenced by storage temperature and special treatment during chocolate production process (rapid cooling, melting and recrystallization) [17]. Polymorphic behaviour of fats and oils can be inhibited or controlled by some emulsifiers. It was found that by selective enzymatic hydrolysis of oils it is possible to synthesize specific glycerolipids that could act as emulsifiers and dynamic controllers of the polymorphic transformation of cocoa butter. Nakae *et al*. in 2000 achieved significant retardation of polymorphic transformation of cocoa butter by adding microbial hydrolyzed glycerolipids into the system. They extracted mono-, di-, and trigalactosyldiacylglycerol from pumpkin. The intermediates were then hydrolyzed to the corresponding galactosylmonoacylglycerols by catalysis of 1,3-specific lipase from *Rhizopus arrhizus*. Monogalactosyldiacylglycerol was then hydrolyzed to diacylglycerol by galactosidase from *Aspergillus oryzae*. [18].

Figure 5 *Example of chocolate fat blooms after refrigeration* [19]

Another interesting application of RA lipase consists in controlled hydrolysis of aliphatic polyesters, which are one of the most promising biodegradable materials nowadays. Thus they can be used in agricultural, sanitary fields as well as in packaging applications for production of cosmetic and beverage bottles, trash bags etc. In 2004 study on selective hydrolysis of aliphatic co-polyesters induced by lipases from *Rhizopus arrhizus* and *Mucor miehei* was reported by Rizzarelli and Impallomeni [8].

Important key factor for RA lipase lipase industrial application is its strong 1,3 – position specifity already mentioned. During last decades lipases were used for synthesis of optically active compounds because of their high enantioselectivities and stability in organic solvents. This particular lipase, together with lipases from *Candida antarctica* and *Candida cylindracea* has shown to be effective in the process of enantioselective synthesis of (±)-trans-2-(4-hydroxy-3-methoxyphenyl)-3-hydroxymethyl-7-methoxy-2,3-dihydroxybenzo[*b*]furan (Fig.6) that can be used as a starting material for the complete synthesis of its natural biologically active derivatives [20].

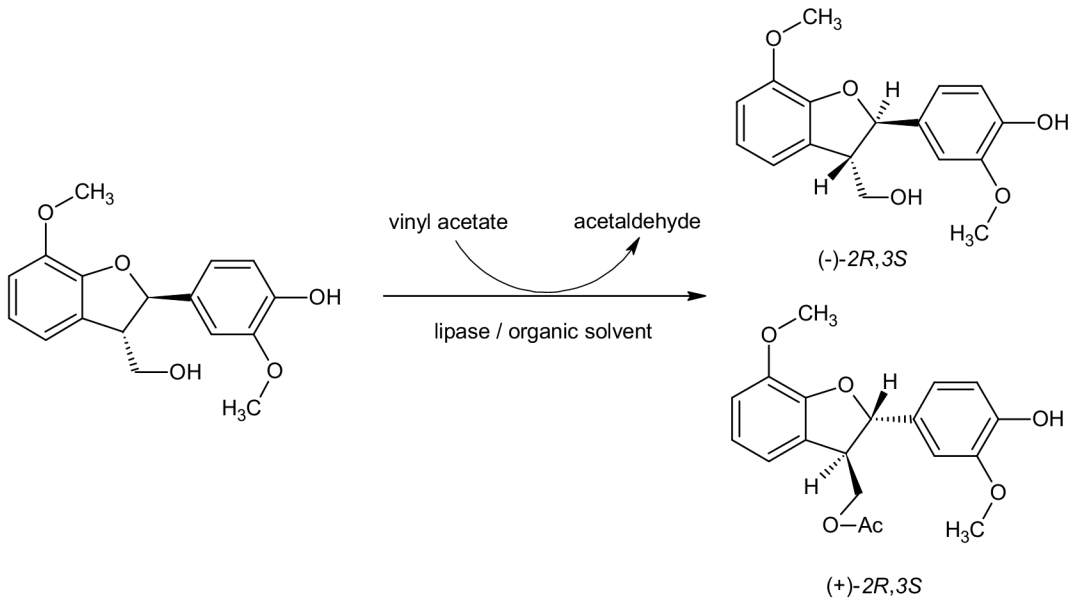

Figure 6 *Lipase catalysed acylation of (±)-trans-2-(4-hydroxy-3-methoxyphenyl)-3-hydroxymethyl-7-methoxy-2,3-dihydroxybenzo[b]furan [20].*

Production of 2-monoglycerid by glycerolysis of triacylglycerols with 1,3 – position specific RA lipase with conversion of 92 % was proved by Millqvist *et al* in 1994 [21]. Suggestion of the glycerolysis mechanism by 1,3 – position specific lipase was provided in 2005 [22]. Nowadays, glycerolysis reactions have been classified into four groups. The knowledge of this mechanism was used to predict the results of glycerolysis at different molar ratio of 1,3-palmitin-2-olein to glycerol [22].

- Group I – hydrolysis of triacylglycerol (Fig.7)
- Group II – hydrolysis of diglyceride and esterification of monoglyceride (Fig.8)
- Group III – hydrolysis of monoglycerol and esterification of glycerol and fatty acids (Fig.9)
- Group IV – acyl migration and isomerization of monoglycerides and diglycerides (Fig.10) [22].

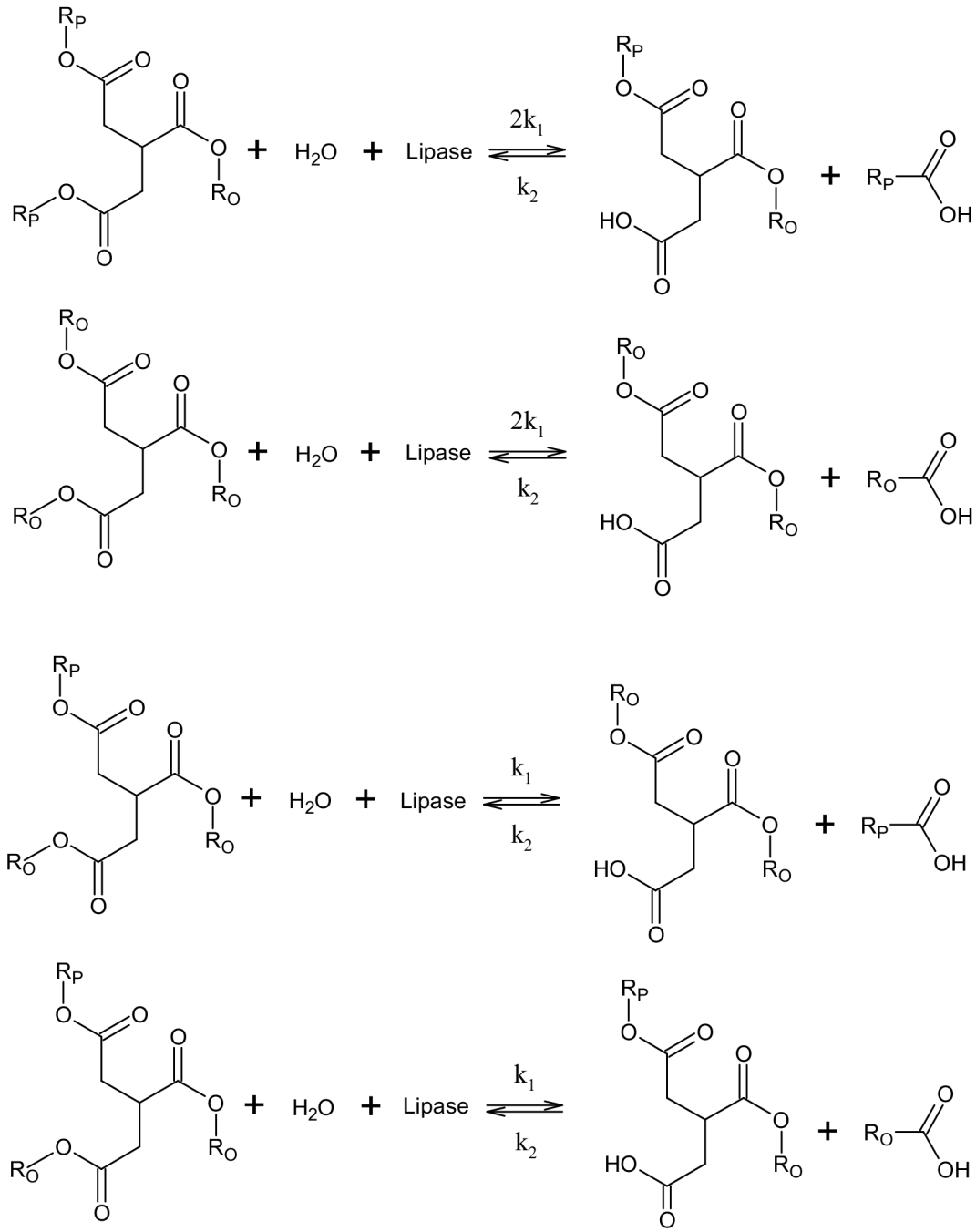

Figure 7 *Example of triacylglycerol hydrolysis on 1-palmitin-2-olein (k_1 – hydrolysis rate constant of lipase a single fatty acid in triacyl-glycerol, k_2 – esterification rate constant for a single hydroxyl group in diacylglycerol)* [22]

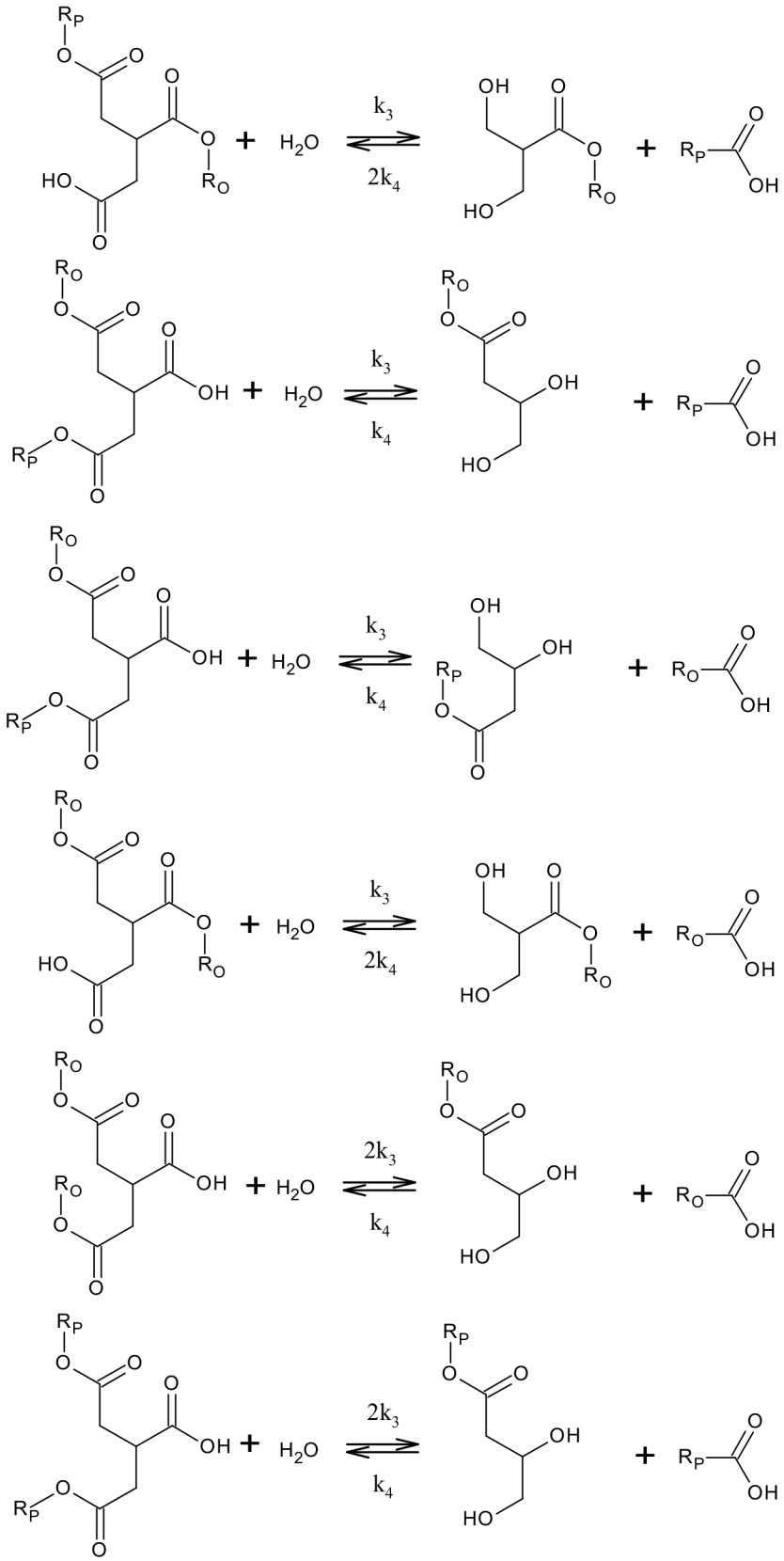

Figure 8 *Hydrolysis of diglyceride and esterification of monoglyceride (k_3 – hydrolysis rate constant of lipase for a single fatty acid in diglyceride, k_4 – esterification rate constant of lipase for a single hydroxyl group in monoglyceride* [22].

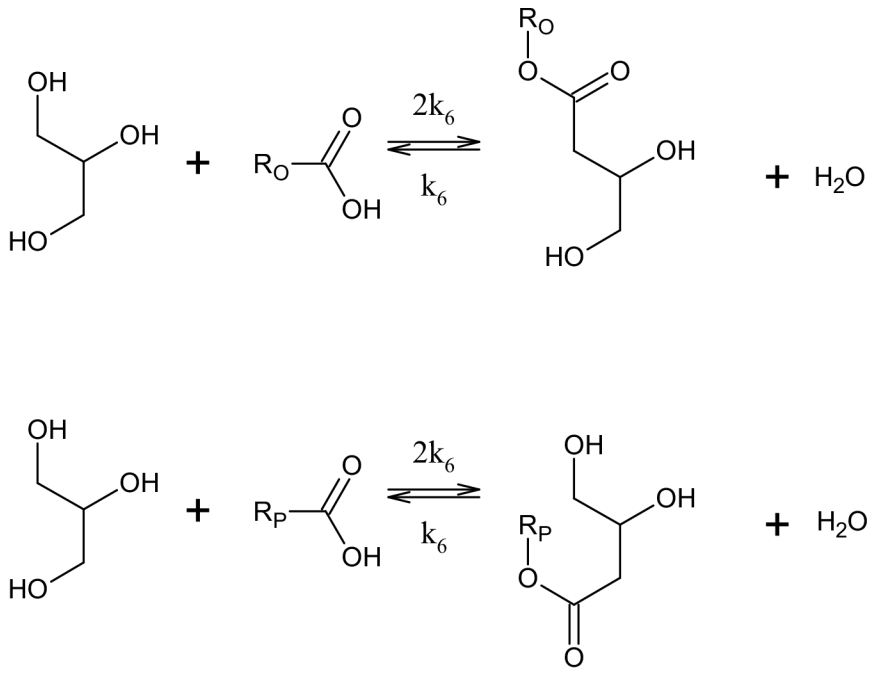

Figure 9 *Hydrolysis of monoglycerol and esterification of glycerol and fatty acids (k_6 – esterification rate constant of lipase between a fatty acid and glycerol)* [22].

- Group IV – acyl migration and isomerization of monoglycerides and diglycerides [22].

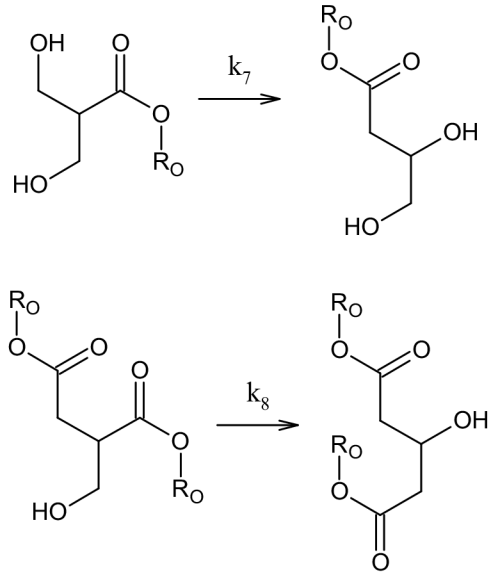

Figure 10 *Acyl migration and isomerization of monoglycerides and diglycerides (k_7 and k_8 are isomerization rates)* [22].

The knowledge of this mechanism was used to predict the results of glycerolysis at different molar ratio of 1,3-palmitin-2-olein to glycerol [22].

3.2 Tween chemistry

3.2.1 Introduction to chemistry of detergents

Detergents belong to group of surfactants, which are chemical substances with the ability to lower interfacial tension between two phases. Generally, detergents are surfactants with cleaning properties. By lowering surface tension, they help to dissolve hydrophobic components (dirt and grease) and they are widely used as a washing agents and emulsifiers. Their function is connected with amphipathic character of molecules, which are usually consisted of polar headgroup and nonpolar hydrophobic hydrocarbon chain (Fig.11)

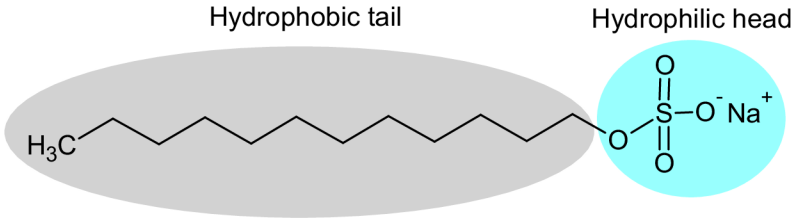

Figure 11 *Detergent molecule with hydrophilic and hydrophobic parts*

At low concentration they are in the form of water soluble monomeric molecules. After the concentration of detergents in reaction mixtures reaches the point so-called critical micelle concentration (CMC), the formation of micelles occurs (Fig.12). The size of micelles depends on the type of detergent. In addition CMC and micelle size is also given by ionic strength, temperature of the solution pressure, pH and the presence of impurities. For instance whereas CMC of ionic detergents is almost not affected by temperature and decreases with growing salt concentration, those of non-ionic detergents are significantly affected by salt concentration and increase with increasing temperature [23].

Relationship between lipase activity and critical micelle concentration has been investigated. For instance, Salameh and Wiegel reported influence of CMC on lipase activity enhancement depending on type of detergent used in lipase activation. The activity profiles of non-ionic detergents (Tween 20, Tween 80, Triton X-100) and ionic detergents (SDS) are similarly based on the fact that the activation starts at low concentrations below CMC values and thus does not involve micelles. On the other hand maximal activation as well as inhibition occurs in the presence of micelles [24].

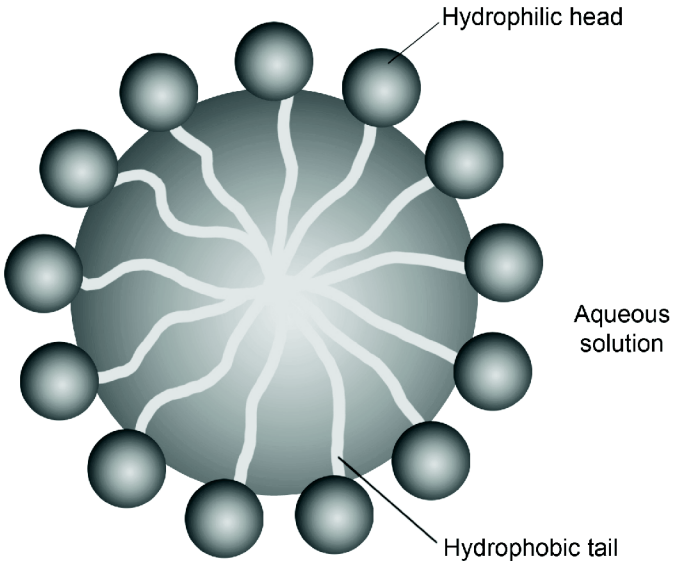

Figure 12 *A schematic of a spherical micelle of surfactant molecules in aqueous solution* [25].

Classification of detergents

According to the whole electrical charge, detergents are classified into three basic groups (Fig.13). Ionic detergents are compounds that contain a net charge head group. They can be either cationic (cetyl trimethyl – ammonium bromide with positively charged trimethylammonium group) or anionic (sodium dodecyl sulphate with negatively charged sulphate group) [26].

Detergents solubilise biological cell membranes (cell lysis) and afford release of intracellular materials in soluble form. Detergents break the protein-protein, protein-lipid and lipid-lipid associations, denature proteins and other macromolecules. Non – ionic detergents, which contain uncharged hydrophilic head groups, are generally better suited for breaking lipid – lipid and lipid – protein interactions than protein –protein interactions. Alkyl glycosides are the most popular in the isolation of membrane proteins because they are homogenous with respect to their structure and composition and the polar sugar group can be easily synthesized in pure forms [26].

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Amphoteric / Zwitterionic detergents are unique in a way they are able to combine properties of ionic and non – ionic detergents. They can be anionic, cationic or non – ionic in dependence on the acidity or pH of the water or contain two charged groups of different sign. [27].

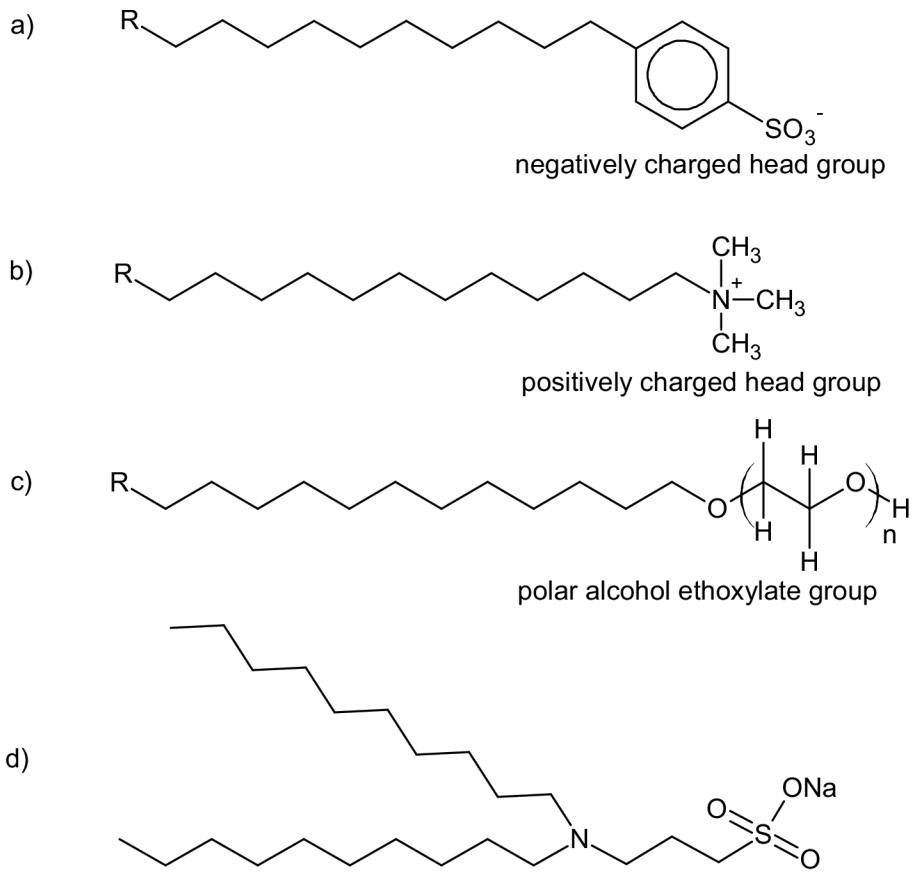

Figure 13 *Example of each types of detergents – a) anionic detergent (alkyl benzensulfonate detergent), b) cationic detergent (quarternary ammonium detergent), c) non-ionic detergent (alcohol ethoxylate detergent, d) Zwitterionic detergent (N-Decyl-N,N-dimethyl-3-ammonio-1-propanesulfonate)* [28]

Effect of tween on conformational changes of enzymes

Activation of lipase comprises characteristically the opening of a surface loop (lid like structure) and residing directly over the enzyme active site. Besides the phenomenon of interfacial activation, three other factors were considered to be contributing: an increase of substrate concentration at the interface [4], better orientation of the scissile ester bond [5], and reduction in the hydration shell around the ester bond. Conformational rearrangement induced by detergent activity is thought to lead to optimized active site geometry with a result of enhanced catalytic activity of the protein [5].

Simons *et al.* reported a conformational change occurring in *Staphylococcus aureus* lipase, which were induced by Triton X-100 [30]. Micelles of both non-ionic and ionic detergents induced the conversion of the pancreatic lipase from closed to open form in the study performed by Hermoso *et al.* [31]. Jutilla *et al.* examined conformational changes of *Humicola lanuginosa* lipase (HLL) induced by pentaoxyethylene octyl ether (C_8E_5) (Fig.14), which resulted in enhanced lipolytic activity under defined conditions. In their study, the model of influence of detergent different amount in reaction mixture on the hydrophobic active site of enzyme was suggested. The interactions between HLL and C_8E_5 were measured by means of fluorescence spectroscopic analysis. It was found out that the low concentration

of detergent increased the activity of enzyme towards the substrate. Decrease in the activity was ascribed to raising detergent concentration. However, above the CMC, catalytic activity was highly influenced by detergent and reached its maximum. Intercalation of detergent into the active site hole causes a steric block to the access of the substrate. At a threshold concentration (close to CMC), more detergent molecules participate in the formation of aggregates on the hydrophobic surface of the lipase. These aggregates have lifetime long enough to stabilize the active open-lid conformation [5].

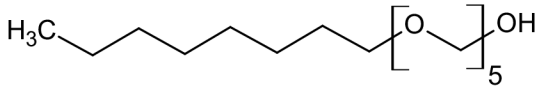

Figure 14 *Non-ionic detergent: pentaoxyethylene octyl ether (C_8E_5)* [32]

3.2.2 Tween family of detergents

Tweens are classified as a group of polysorbate (polyoxyethylene sorbitan esters) based non-ionic emulsifiers. Polysorbates are formed by the reaction of ethylene oxide molecules with sorbitan ester with equivalent molecular weight (Fig.15). Formed long polyoxyethylene chain acts as hydrophilic agent and thus polysorbates are the most hydrophilic emulsifiers among the non- ionic types [33].

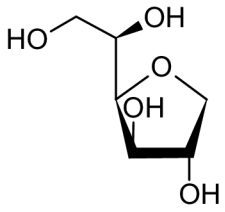

Figure 15 *Sorbitan; (3S)-2-(1,2-dihydroxyethyl)tetrahydrofuran-3,4-diol*

Tween molecule consists of oligooxyethylene chains and fatty acid ester moiety. Three terminal hydrophilic hydroxyl groups can be modified for further applications and an aliphatic chain acts during adsorption on a hydrophobic surface [3].

Tween representatives differ from each other by character of aliphatic chain and thus they have different ability of absorption on a hydrophobic surface. For example, Tween 20 and Tween 80 are presented at fig.16 and fig.17. Whereas Tween 20 is based on lauric acid (polyoxyethylene (20) sorbitan monolaurate), Tween 80 contains oleic acid (polyoxyethylene (20) sorbitan monooleate) [34].

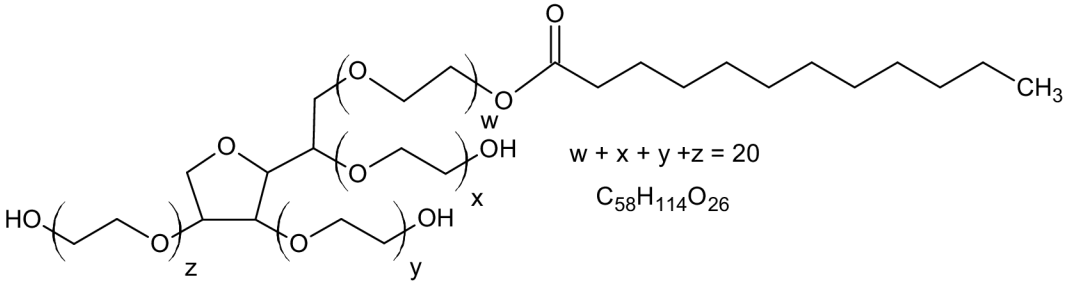

Figure 16 *Tween 20 – polyoxyethylene (20) sorbitan monolaurate*

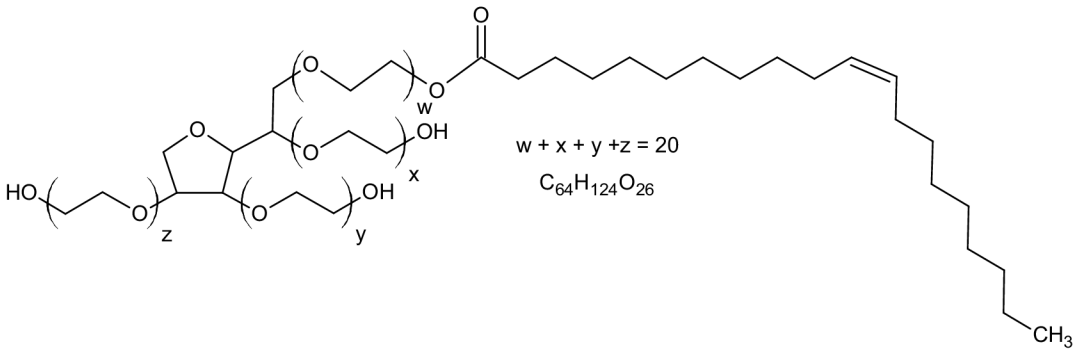

Figure 17 *Tween 80 - polyoxyethylene (20) sorbitan monooleate*

In the literature, HLB (hydrophilic – lipophilic balance) number, which is a measure of how much a surfactant will move into the water and oil phase, is given for particular tween. A high HLB number (more than 10) like 16.7 (Tween 20) or 13.4 (Tween 80) indicates that the surfactant will travel into the water phase *i.e.* it is water soluble [35].

Tweens as lipase inducers

Increasing market for compounds produced by lipase catalysed transformation has resulted in growing demand to identify lipases of specific properties (substrate specificity, stability, pH and temperature optimum, *etc.*). One of the most important aspects is effective microbial productivity of lipases under interest and facile isolation of the product. Lipases are reported frequently in the literature to be inducible enzymes with oils as the inducers. However, several other inducers like hydrocarbons and surfactants have also been reported as agents with the same effect [36].

Kumar and Gupta in 2008 studied a series of modified cultivation media with three different inducers (hydrocarbon, oil, and surfactant). Among inducers tested, Tween 80 was shown to be the most effective one in enhancement of lipase production [36], [37].

Tween 80 was also studied as a carbon source in lipase production of *Serratia marcences* and its presence in the mineral cultivation media induced enhanced lipase production [38].

Effect of tween on lipase catalytic behaviour and enantioselectivity

The effect of different surfactants on catalytic activity and enantioselectivity (ability to synthesise enantiomeric or diastereoisomeric products) of lipase has been investigated because of its simplicity for practical use. Most of the surfactants had inhibitory influence on lipases. However, Tween 60, Tween 80 and nonyl phenol polyethyleneoxy ether was found to have positive effect on lipolytic activity. Moreover, Tween 80 was proved to play positive role in enantioselectivity of lipase [1].

Liu *et al.* studied the enhancing effect of Tween 80 on lipase performance in enantioselective hydrolysis of ketoprofen ester (Fig.18) [1]. Ketoprofen (2-(3-benzoylphenyl)propionic acid) belongs to the group of 2-arylpropionic acids (profens). *(S)*-enantiomer is therapeutically active as an inflammation reducer and relieves pain, whereas *(R)*-enantiomer prevents periodontal disease and thus can be used as a toothpaste additive [1]. Lipase from *Candida rugosa* preferentially produces *(S)*-enantiomer, however from practical point of view, enantioselectivity of pure form of this molecule is not satisfactory for industrial

application. Further studies and experiments showed significant improvement of enantioselectivity resulting in production of optically pure *(S)*-enantiomer (purity of 96.4%) [1].

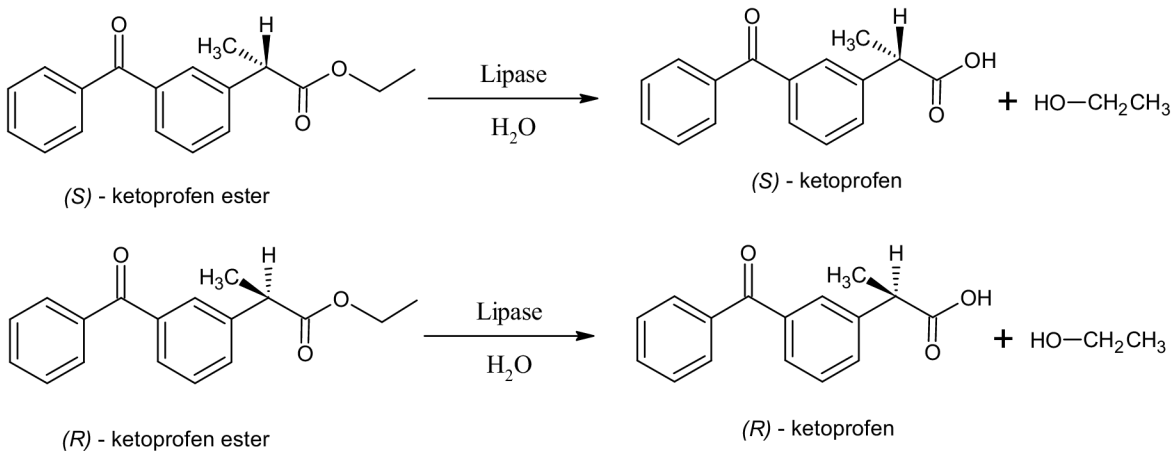

Figure 18 *Enantiospecific enzymatic hydrolysis of ketoprofen ethylester (2-(3-benzoylphenyl)propionic acid ethylester) [41].*

Nowadays, proteins immobilized on nanoparticles are subject of an intense research. A wide range of nanomaterials with various shapes, sizes, and properties were studied and proved as carriers. The main advantage is high surface area which leads to higher enzyme loading, forming of the nanoscale dispersion, and more facile surface functionalization [1].

Enhancement of efficiency of enzyme immobilization on hexamethylenediamine functionalized graphene oxide in the presence of Tween 20 was reported by *Pavlidis et al.* (defined in their previous work: Functionalized Multi-Wall Carbon Nanotubes for Lipase Immobilization [43]). Tween 20 was used as a complementary agent in covalent immobilization to cover the hydrophobic regions of the carrier making it more hydrophilic and preventing the formation of non-specific interactions [1].

Effects of tween on GO itself (Fig.19) has been studied as well. Park *et al.* declared that tween helps to disperse GO sheets in water. Moreover, hydrophobic graphene sheets (produced by reduction of graphene oxide) were found to have an ability of non covalent adsorption (Fig.20) by surfactant which results in the formation of homogenous colloidal suspensions [45].

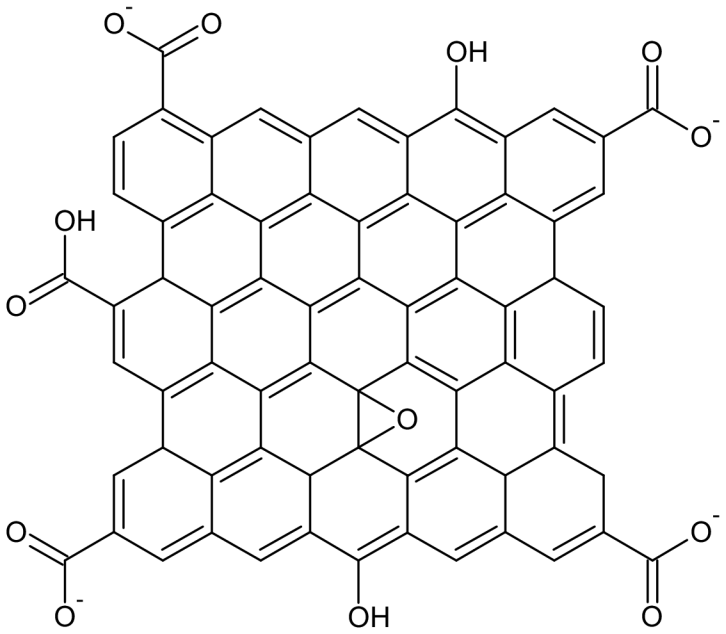

Figure 19 *Scheme of graphene oxide sheet. During the oxidation process, functional groups (mainly the epoxy group on the basal plane and hydroxyl and carboxylic groups at the edge side) are introduced to the graphene oxide sheets [46]*

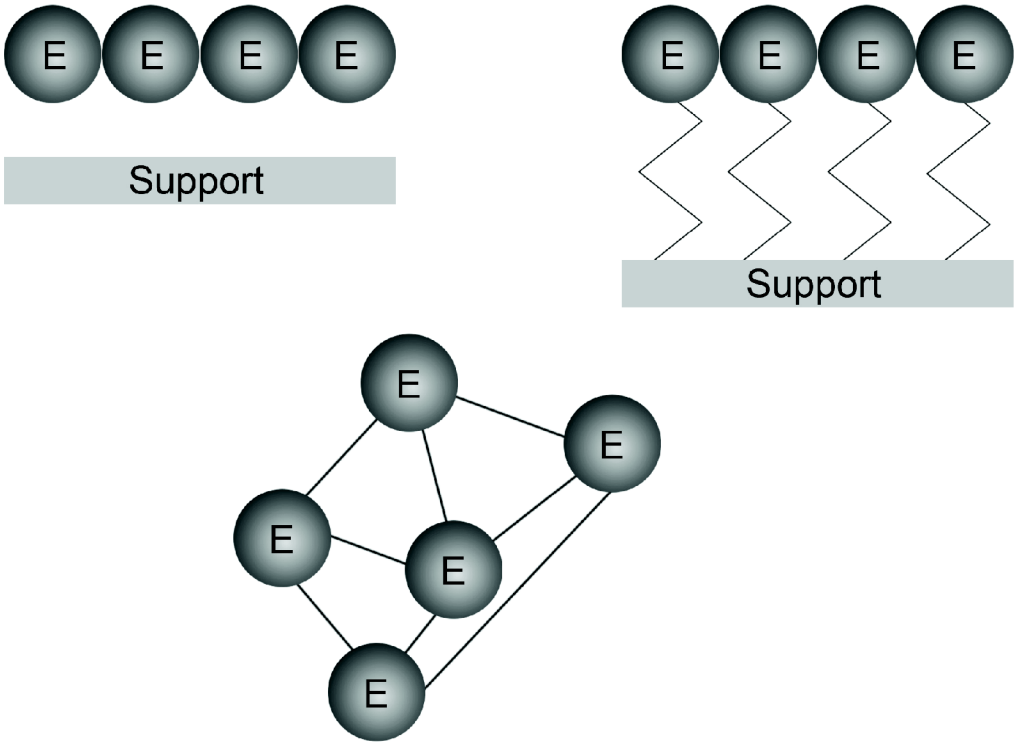

Figure 20 *Scheme of enzyme immobilization techniques: Physical adsorptions, covalent bonding, cross - linking*

Commercial applications of Tween 20, Tween 60 and Tween 80

Generally, polysorbates are gentle surfactants that do not affect protein activity and have very low critical micelle concentration. Polysorbates are commonly used as washing agents in immunoblotting and ELISA [34]. Thanks to their biodegradability (in living organisms they are digestible into component molecules that can be degraded in usual ways), non-toxicity and non-irritability to the skin they are considered as human- and environmental- friendly and thus they are increasingly used in food, pharmaceutical and cosmetic industry [47]. The most common applications of Tweens 20, 60, and 80 are summarized at the table 2.

Table 2 *Industrial applications of Tweens 20, 60, and 80.*

Tween	***Possible synonymes***	***Target product or application***
Tween 20	polysorbate 20, polyoxyethylene sorbitan monolaurate, PEG (20) sorbitan monolaurate	blocking agent in PBS* or TBS** wash buffer for ELISA, Western blotting and other immunoassay methods, lysing of mammalian cells, solubilisation of membrane proteins [34]
Tween 60	polysorbate 60, polyoxyethylene sorbitan monostearate, PEG (60) sorbitan monolaurate	emulsifier and stabilizer in food, cosmetics, and other industry [48]
Tween 80	polysorbate 80, polyoxyethylene sorbitan monooleate, PEG (80) sorbitan monooleate	stabilizing agent for proteins, tests for the identification of some mycobacteria phenotype [34]

*PBS – phosphate buffered solution
**TBS – tris-buffered solution

4 EXPERIMENTAL PART

4.1 Materials and methods

4.1.1 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
magnetic stirrer, Jeio Tech
pH meter, Merci s.r.o. WTW series
fridge, Samsung Callex
water bath, HUBER A11
spectrophotometer, SPEKOL 1300
spectrophotometer SPEKOL Analytik jena AG
thermo block heater

4.1.2 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
acetic acid, Lach:ner. s.r.o. ČR
tris(hydroxymethyl)aminomethane, Lach:ner. s.r.o. ČR
p-nitrophenol, Lach:ner. s.r.o. ČR
p-nitrophenyl laurate, FLUKA (Sigma Aldrich)
Rhizopus Arrhizus lipase commercial preparation, FLUKA (Sigma Aldrich), denoted as RA lipase
tween 20 – polysorbatum 20, Lach:ner. s.r.o. ČR
tween 60 – polysorbatum 60, Lach:ner. s.r.o. ČR
tween 80 – polysorbatum 80, Lach:ner. s.r.o. ČR

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

a) Solution of *p*-nitrophenyl laurate (2.5 $mmol \cdot dm^{-3}$) – 0.01 g of *p*-nitrophenyl laurate was dissolved in 25 ml of ethanol.
b) Solution of *p*-nitrophenol (1.0 $mmol \cdot dm^{-3}$) – 13.9 mg of *p*-nitrophenol was dissolved in 100 ml of distilled water.
c) Solution of sodium carbonate (1 $mmol \cdot dm^{-3}$) – 10.6 g of Na_2CO_3 was dissolved in 100 ml of distilled water.

d) Solution of *Rhizopus arrhizus* lipase commercial preparation (1 $mg \cdot ml^{-1}$) – 10 mg of *Rhizopus arrhizus* lipase powder was dissolved in 10 ml of distilled water.

e) Buffers:

1. Sodium acetate buffer pH 5 – Acetic acid water solution (23.9 ml, 0.2 $mol \cdot dm^{-3}$) was mixed with 34.2 ml of sodium acetate water solution (0.2 $mol \cdot dm^{-3}$). Finally pH value was adjusted by solution of NaOH (1 $mol \cdot dm^{-3}$) and solution was diluted to 100 ml in a volumetric flask.

2. Phosphate buffer pH 6 – 87.7 ml of acidic sodium phosphate water solution (0.2 $mol \cdot dm^{-3}$) was mixed with 12.3 ml of water solution of $Na_2HPO_4.12H_2O$ (0.2 $mol \cdot dm^{-3}$). Finally pH value was adjusted by NaOH (1 $mol \cdot dm^{-3}$) and the solution was diluted to 200 ml in a volumetric flask..
3. Phosphate buffer pH 7 – 39 ml of acidic sodium phosphate water solution (0.2 $mol \cdot dm^{-3}$) was mixed with 61 ml of water solution of $Na_2HPO_4.12H_2O$ (0.2 $mol \cdot dm^{-3}$). Finally pH value was adjusted by NaOH (1 $mol \cdot dm^{-3}$) and volume was replenished to 200 ml.
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 value was adjusted to 7.2 by NaOH (1 $mol \cdot dm^{-3}$).
5. Phosphate buffer pH 8 – 5.3 ml of acidic sodium phosphate water solution (0.2 $mol \cdot dm^{-3}$) was mixed with 94.7 ml of water solution of $Na_2HPO_4.12H_2O$ (0.2 $mol \cdot dm^{-3}$). Finally pH value was adjusted by solution of NaOH (1 $mol \cdot dm^{-3}$) and volume was replenished to 200 ml.
6. Tris buffer pH 9 – 5 ml of hydrochloric acid (0.2 $mol \cdot dm^{-3}$) was mixed with 50 ml of tris(hydroxymethyl)aminomethane (1.6 g of NaOH dissolved in 100 ml of distilled water). Finally pH value was adjusted by NaOH (1 $mol \cdot dm^{-3}$).

4.2 Procedures

4.2.1 Spectrophotometric determination of lipolytic activity by *p*-nitrophenyl laurate

Calibration curve

Solution of *p*-nitrophenol (1.0 $mmol \cdot dm^{-3}$) was used for construction of calibration curve. Five solutions with different concentration of *p*-nitrophenol were prepared and their composition is as follows (Table 3).

Table 3 *Composition of solutions used for calibration curve measurement*

Tube	*1*	*2*	*3*	*4*	*5*	*B*
p-nitrophenol (ml)	0.10	0.20	0.30	0.40	0.50	0
phosphate buffer pH 7.2 (ml)	3.25	3.25	3.25	3.25	3.25	3.25
distilled water (ml)	0.40	0.30	0.20	0.10	0	0.50
Na_2CO_3 (ml)	0.50	0.50	0.50	0.50	0.50	0.50
n of p-NP (μmol in reaction mixture)	0.10	0.20	0.30	0.40	0.50	0

Calibration board was prepared from the solution of *p*-nitrophenol (concentrations of 0.1; 0.2; 0.3; 0.4; 0.5 mmol·dm^{-3}) diluted in ethanol. Before measurement 3.25 ml of phosphate buffer (pH 7.2) was added to this solution. The tube was stirred and solution was left to incubate for 30 min 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.

Determination of lipolytic activity

RA enzyme aqueous solution (0.125 ml, 0.1 mg·ml^{-1}) was placed into the reaction flask with 1.625 ml of phosphate buffer (pH 7.2). After that 0.125 ml of substrate was added. The reaction mixture was stirred and left to react for 30 min. Enzymatic hydrolytic reaction was stopped by adding 0.250 ml of sodium carbonate solution (1 mol·dm^{-3}). Subsequently, absorbance of this solution was measured at a wavelength of 420 nm. For blank, the phosphate buffer (pH 7.2) was used.

As the substrate a solution of *p*-nitrophenyl laurate diluted in ethanol was standardly used for all following measurements.

Determination of temperature optimum

Phosphate buffer (1.625 ml) was added to 0.125 ml of enzyme solution (0.1 mg·ml^{-1}). Then 0.125 ml of ethanol solution of substrate was added. Reaction mixture was stirred and left to incubate for 30 min at 30; 35; 40; 45, 50 and 55 °C in a water bath. Enzymatic hydrolysis reaction was stopped by adding 0.250 ml of sodium carbonate solution (1 mol·dm^{-3}). Subsequently, absorbance of this solution was measured at a wavelength of 420 nm. For blank, the phosphate buffer (pH 7.2) was used.

Determination of pH optimum

Respective buffer (1.625 ml) was added to 0.125 ml of enzyme water solution (0.1 mg·ml^{-1}). Then 0.125 ml of substrate dissolved in ethanol was added. Reaction mixture was stirred and left to react for 30 min at laboratory temperature. Enzymatic hydrolysis reaction was stopped by adding 0.250 ml of sodium carbonate solution (1 mol·dm^{-3}). Subsequently, absorbance of this solution was measured at a wavelength of 420 nm. For blank, the phosphate buffer pH 7.2 was used. The pH optimum was measured at pH of 5, 6, 7, 8, and 9.

Determination of thermal stability

Enzyme water solution (0.125 ml, 0.1 mg·ml^{-1}) was left to incubate for 30 min at 30; 40; 50; 60 and 70 °C in a water bath. Then 1.625 ml of phosphate buffer (pH 7.2) and 0.125 ml of substrate dissolved in ethanol were added. Reaction mixture was stirred and left to incubate for 30 min at laboratory temperature. Enzymatic hydrolysis reaction was stopped by adding 0.250 ml of sodium carbonate solution (1 mol·dm^{-3}). Subsequently, absorbance of this solution was measured at a wavelength of 420 nm. For blank, the phosphate buffer (pH 7.2) was used.

4.2.2 Immobilization procedure

Process of carrier homogenization

Five mg of carrier was placed into the reaction flask with 9 ml of buffer and stirred for 15 min at magnetic stirrer. After that the mixture was placed into ultrasonic bath and left to incubate for 1 hour.

For the purpose of enzyme immobilization 0.110 ml of tween was added into the reaction mixture before the ultrasonic treatment. Resulting mixture was washed with respective buffer for three times.

Immobilization protocol

Enzyme solution (1 ml) was added into homogenized carrier mixture and left to incubate for certain time period (30, 60, 90, 120, and 240 min) at an automatic shaker.

Determination of coupling yield of immobilized enzyme

Immobilized enzyme suspension (0.125 ml) was placed into the reaction flask with 1.625 ml of phosphate buffer (pH 7.2). After that 0.125 ml of substrate was added. The reaction suspension was mixed by using magnetic stirrer. Then it was left to react for 30 min at laboratory temperature. Enzymatic hydrolysis reaction was stopped by adding 0.25 ml of sodium carbonate solution (1 $mol \cdot dm^{-3}$). The suspension was placed into Eppendorf tube and left to centrifuge (16, 000 rpm) at 4 °C for 10 min. Subsequently, absorbance of supernatant solution was measured at a wavelength of 420 nm. For blank, the phosphate buffer (pH 7.2) was used.

Determination of immobilization yield

Supernatant (0.5 ml) was placed into the cuvette. Protein concentration was measured spectrophotometrically using default program which counts amount of protein per volume unit. For blank, the phosphate buffer (pH 7.2) was used.

For determination of total protein amount not adsorbed on carrier surface, the immobilized enzyme suspension was washed by phosphate buffer (pH 7.2) until no protein concentration was measured in supernatant.

Calculation of immobilization yield

Amount of enzyme proteins immobilized on the carrier was determined spectrophotometrically. The calculation was based on the determination of enzyme proteins remained in the supernatant after immobilization by following formulas.

- Amount of enzyme immobilized on the carrier: $m = m_0 - m_1$
- Calculation of the immobilization yield: $\mathrm{IY}(\%) = \frac{m}{m_0} \cdot 100$

m_0 – initial amount of protein
m_1 – amount of protein remained in the reaction mixture
m – amount of protein immobilized on the carrier

Determination of pH optimum of immobilized enzyme

Respective buffer (1.625 ml) was added into 0.125 ml of immobilized enzyme suspension (0.1 $mg \cdot ml^{-1}$). Then 0.125 ml of substrate dissolved in ethanol was added. The reaction mixture was mixed by using magnetic stirrer. Then it was left to react for 30 min. Enzymatic hydrolysis reaction was stopped by adding 0.25 ml of sodium carbonate solution (1 $mol \cdot dm^{-3}$). The suspension was placed into Eppendorf tube and centrifugate (16, 000 rpm) at 4 °C for 10 min. Subsequently, absorbance of supernatant solution was measured at a wavelength of 420 nm. For blank, the phosphate buffer (pH 7.2) was used. The activity was measured at pH of 5, 6, 7, 8, and 9. Activity of soluble enzyme measured in phosphate buffer (pH 7.2) was used as 100 % for data evaluation.

Determination of temperature optimum of immobilized enzyme

Phosphate buffer (1.625 ml) was added to 0.125 ml of immobilized enzyme suspension. Then 0.125 ml of substrate dissolved in ethanol was added. Reaction mixture was mixed by using magnetic stirrer and left to incubate for 30 min at 30; 35; 40; 45, 50 and 55 °C in a thermo block heater. Enzymatic hydrolysis reaction was stopped by adding 0.25 ml of sodium carbonate solution (1 $mol \cdot dm^{-3}$). The suspension was placed into Eppendorf tube and centrifuged (16, 000 rpm) at 4 °C for 10 min. Subsequently, absorbance of supernatant solution was measured at a wavelength of 420 nm. For blank, the phosphate buffer (pH 7.2) was used. Activity of soluble enzyme measured in phosphate buffer (pH 7.2) at 35 °C was used as 100 % for data evaluation.

Determination of thermal stability of immobilized enzyme

Immobilized enzyme suspension (0.125 ml) was left to incubate for 30 min at 30; 40; 50; 60 and 70 °C in a water bath. Then 1.625 ml of phosphate buffer (pH 7.2) and 0.125 ml of substrate dissolved in ethanol were added. Reaction mixture was mixed by using magnetic stirrer and left to react for 30 min. Enzymatic hydrolytic reaction was stopped by adding 0.25 ml of sodium carbonate solution (1 $mol \cdot dm^{-3}$). The suspension was placed into Eppendorf tube and centrifuged (16, 000 rpm) at 4 °C for 10 min. Subsequently, absorbance of supernatant solution was measured at a wavelength of 420 nm. For blank, the phosphate buffer (pH 7.2) was used.

Calculation of activity coupling yield

The calculation of lipolytic activity was based on the linear regression equation obtained by evaluation of *p*-nitrophenol calibration curve (Fig.21).

Lipolytic activity expressed in units $U \cdot mg^{-1}$ represents the amount of enzyme (protein), which catalyzes one μmol of substrate per one min related to the whole amount of protein.

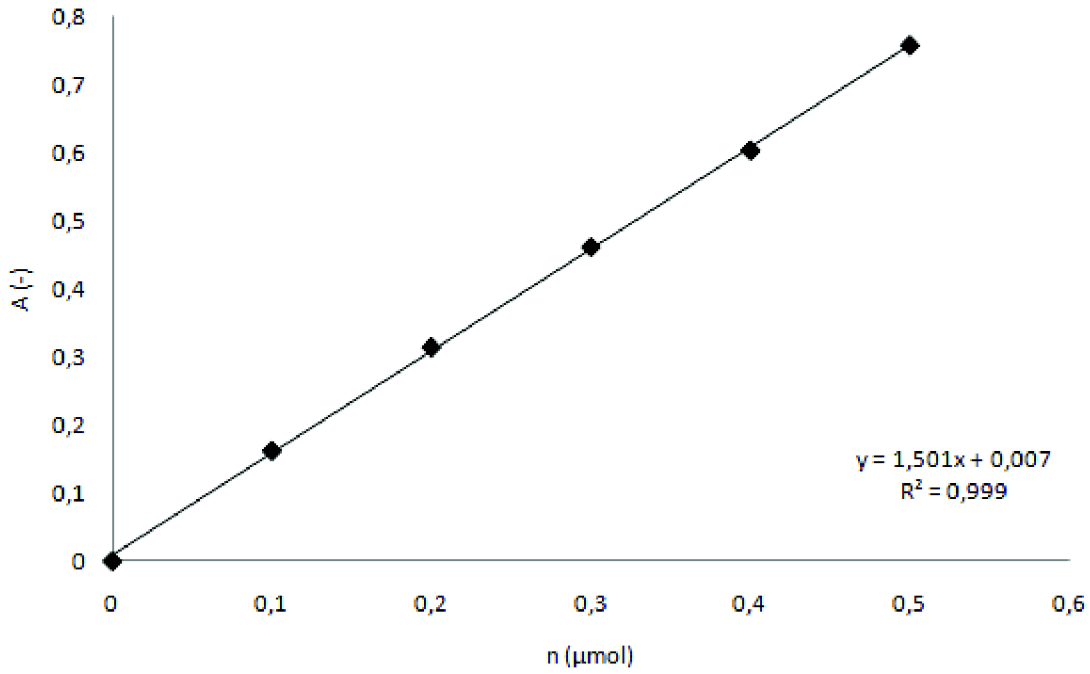

Figure 21 *Calibration curve of p-nitrophenol*

$$y = 1.501x + 0.007$$
$$1 = 1.501x + 0.007$$
$$x = \frac{1 - 0.007}{1.501} = 0.661$$

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

$$a = \frac{A_{420} \cdot x}{t \cdot c_E}$$

a – enzyme activity
c_E – characterization of enzyme (units depend on the way of calculation)
A_{420} – Absorbance
t – time

Determination of coupling yield:

$$\% = \frac{a}{a_0} \cdot 100$$

a – enzyme activity
a_0 – enzyme activity of soluble RA lipase measured in defined conditions (enzyme concentration of 0.1 $mg \cdot ml^{-1}$, substrate *p*-nitrophenyl laurate at concentration of 2.5 $mmol \cdot dm^{-3}$, laboratory temperature)

4.3 Determination of kinetics profile of soluble and immobilized lipase

The determination of kinetics profile of both soluble and immobilized RA lipase was performed by the procedure described in previous chapters and the enzymatic activity was measured at four different concentration of substrate (1.75 mmol·dm^{-3}, 2.5 mmol·dm^{-3}, 3.75 mmol·dm^{-3}, and 5 mmol·dm^{-3}).

Graphical method of K_m and v_{max} calculation

The rate of enzymatic reaction is defined as amount of substrate formed per time unit as follows.

$$\text{reaction rate} = \frac{\Delta P}{\Delta t}$$

P – product concentration
t – time

This rate depends on the substrate concentration (availability of substrate). The reaction rate decreases with time during which the substrate is consumed in the reaction and its concentration lowers. Initial velocity represents the reaction rate in the first minutes of the reaction, when the effect of time is omissible. The relationship between substrate concentration and initial velocity is represented by Michaelis – Menten equation as follows.

$$v = \frac{v_{\max} \cdot S}{K_m + S}$$

v – initial velocity
v_{max} – maximal velocity
S – substrate concentration
K_m – Michaelis constant

The Michaelis constant represents the concentration of substrate required to achieve half the maximum velocity of the enzyme reaction. In general, it represents the affinity between enzyme and substrate and it is also dissociation constant of the complex enzyme – substrate. From the definition, this constant is constant for given couple enzyme – substrate, and it is not affected by substrate concentration [54].

$$\mathrm{E + S \leftrightarrow ES \rightarrow P + E}$$

For the determination of K_m and v_{max} of the immobilized system, the linear form of the Michaelis – Menten equation was used:

$$y = \mathrm{k}x + \mathrm{q}$$

$$\frac{1}{v} = \frac{K_m}{v_{\max}} \cdot \frac{1}{S} + \frac{1}{v_{\max}}$$

The values of K_m and v_{max} were obtained from linear regression where q represents reciprocal value of v_{max}, and K_m is a reciprocal value of q and k division:

$$K_m = \left(\frac{\mathrm{q}}{\mathrm{k}}\right)^{-1}$$

5 RESULTS AND DISCUSSION

Choosing of appropriate emulsifier is one of the key aspects in production of stable nanodispersion system. The difference between various tweens and thus their effect on reaction system depends on aliphatic chain length and carbon number of fatty acid in the emulsifier structure [47]. In general, emulsifiers are used to stabilize nanodispersions, which is very important for system homogenization and thus getting good results and high immobilization and coupling yields. The study is focused on direct (non-covalent) immobilization of lipase from *Rhizopus arrhizus* (RA) on graphene oxide in the presence of tween.

Activity assay was done with *p*-nitrophenyl laurate (pNPL) as a substrate, where retention of initial enzyme activity (activity coupling) was determined by measuring the relative activity of immobilized system. The total amount of enzyme immobilized on the GO carrier treated with tween (0.022 $ml \cdot mg^{-1}$ of carrier) was determined by calculating of the residual protein concentration in the supernatant after the immobilization. For this experiment spectrophotometric determination of protein concentration was chosen as the most suitable because of its high sensitivity and very low interference with other substances (this method does not interfere with surfactants whereas a lot of other ones do). Because of an impairment of the standard deviation of the results, all experiments were carried out in triplicate

5.1 Study of tween 20 impact on activity of soluble enzyme

Delorme *et al.* studied effects of surfactant on lipase structure, activity, and inhibition in 2011 and they declared that these effects are strongly dependent on specific combination of enzyme and surfactant and can be positive as well negative in particular cases [49]. Effect of surfactant on lipolytic activity of soluble RA lipase was measured by using a series of different concentrations of tween 20 as a tween family representative. The experiment was carried out at tween concentrations from 15 to 0.06 $mmol \cdot dm^{-3}$. The critical micelle concentration of tween 20 (0.06 $mmol.dm^{-3}$) was chosen as the lowest one. The experiment was stopped when a significant decrease of lipolytic activity was measured (usually after 180 min). Specific activity of RA lipase before incubation was 9.9 $U \cdot mg^{-1}$. The results are presented at Fig. 22.

When concentrations of tween 20 were higher above CMC (15, 10, and 5 $mmol.dm^{-3}$) the catalytic activity of lipase increases from the very beginning of the experiment. Initial increase of catalytic activity can be explained by positive effect of micelles formation in the system. It is predicted that binding of surfactant micelles to the enzyme promotes its conformational changes. Pignol *et al.* showed that nonionic detergent has ability to bind to the active site pocket and act like some kind of substrate analog [50].

Presence of detergent at low tween concentrations, close to CMC, had different effect on lipase activity (Fig.22). The relative activity rose slowly until two hours of incubation, and then started to increase rapidly.

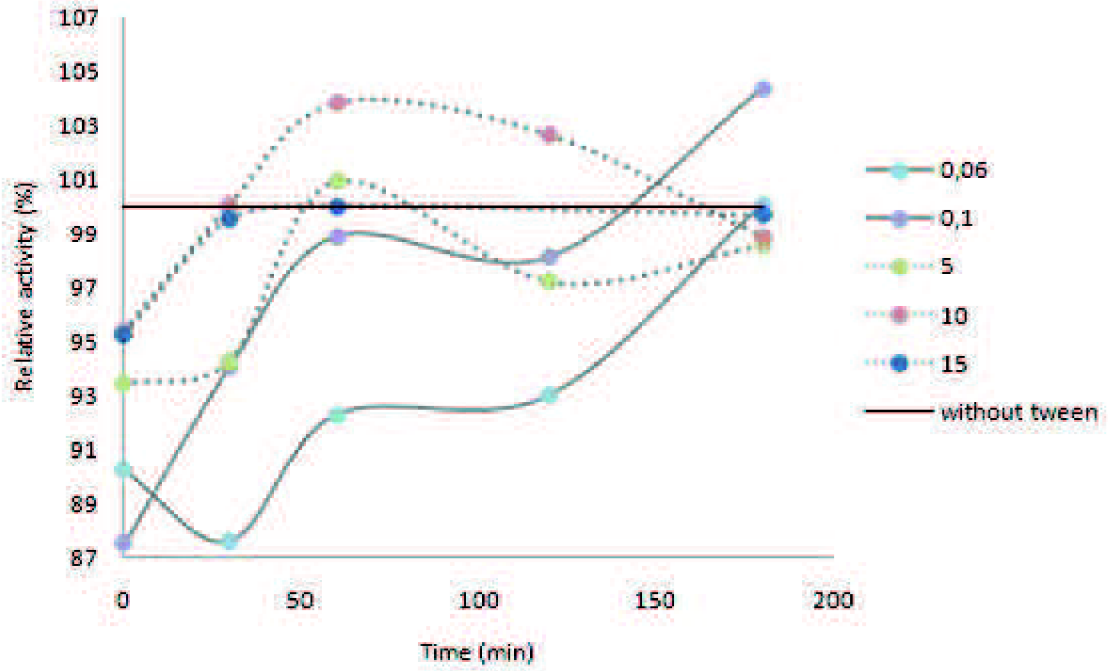

Figure 22 *Incubation of RA in phosphate buffer solution with tween 20, RA lipase specific activity before incubation with tween was 9.9 U·mg^{-1}*

For all following immobilization experiments, the concentration of tween about 10.8 mmol.dm^{-3} was chosen since the positive effect on RA lipase activity was clearly measured (activity retention of 103 %). This result agrees with literature study, where nonionic detergents at the concentrations high above CMC were reported to function as positive agent on lipolytic activity. This concentration value was selected also with regard of work performed by Pavlidis. The study was focused on different hydrolases from *Bacillus subtilis* and *Candida antarctica* immobilization on functionalized carbon – based nanomaterials [51]. Parameters of soluble RA lipase were measured without using tween.

5.2 Effect of different tweens on adsorption of model lipase on graphene oxide

Effect of different representatives of nonionic tweens: tween 20, tween 60, and tween 80, on efficient immobilization of RA lipase on graphene oxide as a support were examined. The direct (non-covalent) immobilization was performed at laboratory temperature in phosphate buffer solution (33.4 mmol.dm^{-3}, pH 7.2) using the detergent concentration of 10.8 mmol.dm^{-3} with immobilization time of 1 h. Reaction conditions were chosen according to Pavlidis [51]. Results are shown at Figure 23. Because of an impairment of the standard deviation of the results, all experiments were carried out in triplicate.

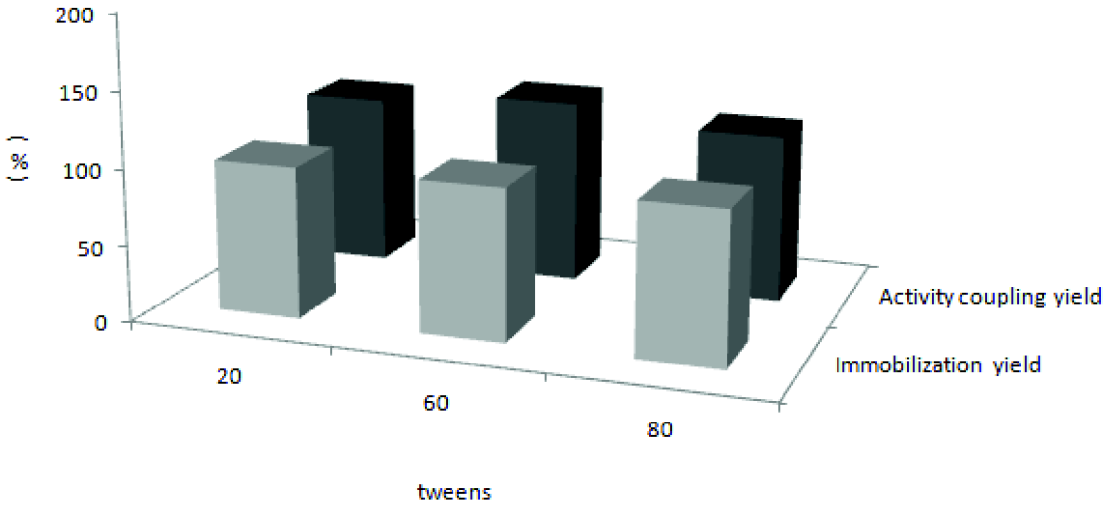

Figure 23 *Efficiency of direct immobilization of RA lipase on graphene oxide in the presence of different tweens (immobilization time of 1 hour).*

Among tested tweens, tween 20 has the shortest aliphatic chain and thus enables formation of the smallest nanoaggregates. In our study the activity coupling was even 113±9%. Pavlidis *et al.* [51] measured only 10 % of activity retention for *Candida antarctica* lipase immobilized on GO in the presence of tween 20. The authors utilized tween due to the minimization of non-specific adsorption of enzyme on the support. High activity coupling achieved in our experiment can be explained by combining the enhancement of carrier apparent solubility by covering its hydrophobic surface, interaction of detergent micelles with active site of lipase, and possibly also interfacial activation phenomenon occurrence. Following experiments were carried out only with tween 20, which was used in Pavlidis *et al*. published study.

Immobilization yield of RA lipase at concentration of 0.1 $mg \cdot ml^{-1}$ on graphene oxide was 100 % in all three tweens used.

5.3 Optimization of immobilization conditions

For determination of the most suitable immobilization conditions, defined as those with the best immobilization efficiency and high activity coupling, parameters like time of immobilization, different concentration of enzyme, and pH of the immobilization environment were studied. Concentration of tween in phosphate buffer solution was 0.012 vol. % for all assays.

Influence of enzyme different concentration on efficiency of immobilization

For non-covalent immobilization a series of different initial concentrations of enzyme: 0.1, 0.25, 0.5, 1, and 2 mg·ml^{-1} was examined (Fig. 24). Immobilization procedure in the presence of tween 20 was set to 1 h and experiment was performed at 25°C.

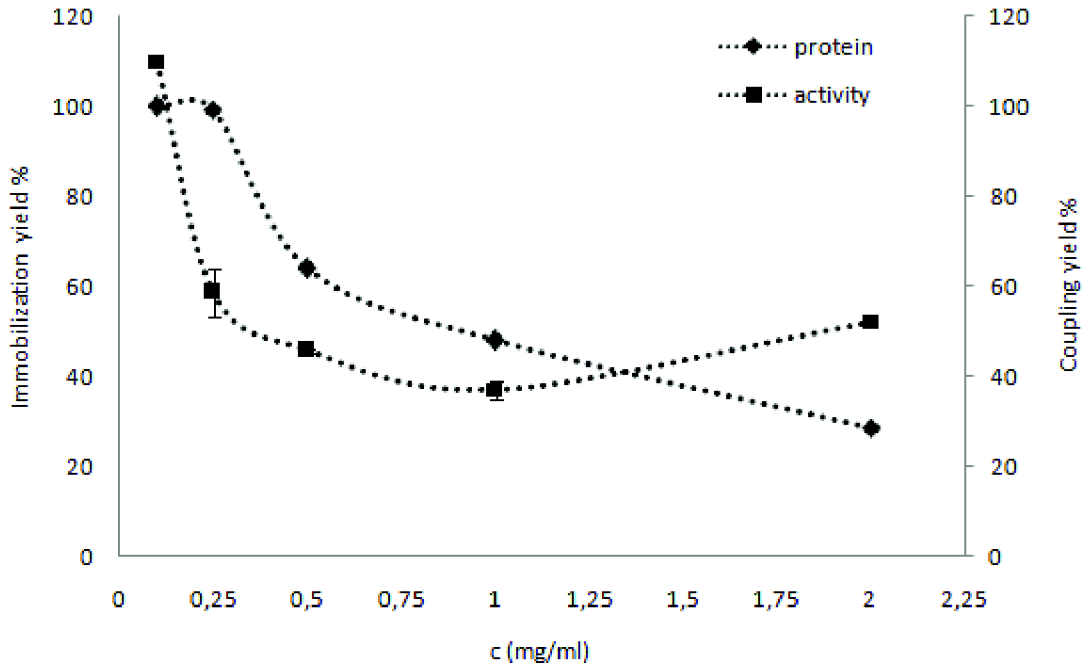

Figure 24 *Coupling and immobilization yield at various enzyme concentrations (average value of three repeated measurements) in the presence of tween 20*

The highest immobilization yield was achieved at enzyme concentration of 0.25 mg·ml^{-1}. Based on the results it can be assumed that at enzyme concentration of 0.1 mg·ml^{-1} (20 μg of enzyme per mg of carrier), the binding capacity of carrier surface reached its maximum. When higher lipase concentration for immobilization was used, enzyme molecules bind to the molecules already adsorbed on the surface and thus the coupling yield is reduced in spite of the fact that enzyme loading is increasing (Table 4).

Table 4 *Protein loading (μg) on carrier at different concentrations of enzyme were used*

Initial enzyme concentration (mg·ml^{-1})	***Amount of proteins (μg)***	***Amount of enzyme per weight unit of carrier (μg·mg^{-1})***
0.1	100	20
0.25	248	50
0.5	320	64
1.0	478	96
2.0	568	114

Moreover, higher enzyme concentration can cause formation of steric barriers near to enzyme active site which causes activity reduction as well.

With respect of the results obtained in this experiment, concentration of 0.1 $mg \cdot ml^{-1}$ has been chosen for all further experiments.

Influence of incubation time on immobilization efficiency

Incubation time of 30, 60, 90, 120, and 240 min for immobilization process efficiency was examined. For the test purpose, enzymatic concentration of 0.1 $mg.ml^{-1}$ was used. Immobilization yield reached 100 % after 60 min of immobilization process. Afterwards, the immobilization yield was measured for 4 hours to detect undesired leaching of protein due to the interaction change between carrier treated by tween and the enzyme.

Immobilization time of one hour was selected according to work of Pavlidis *et al.,*2011 where authors immoblized hydrolases on amine functionalized GO using tween 20 as agent inhibiting non-specific interactions between carrier and protein [43]. Based on the results it could be concluded that immobilization time of one hour is necessary to achieve maximum immobilization yield and the leaching did not occur.

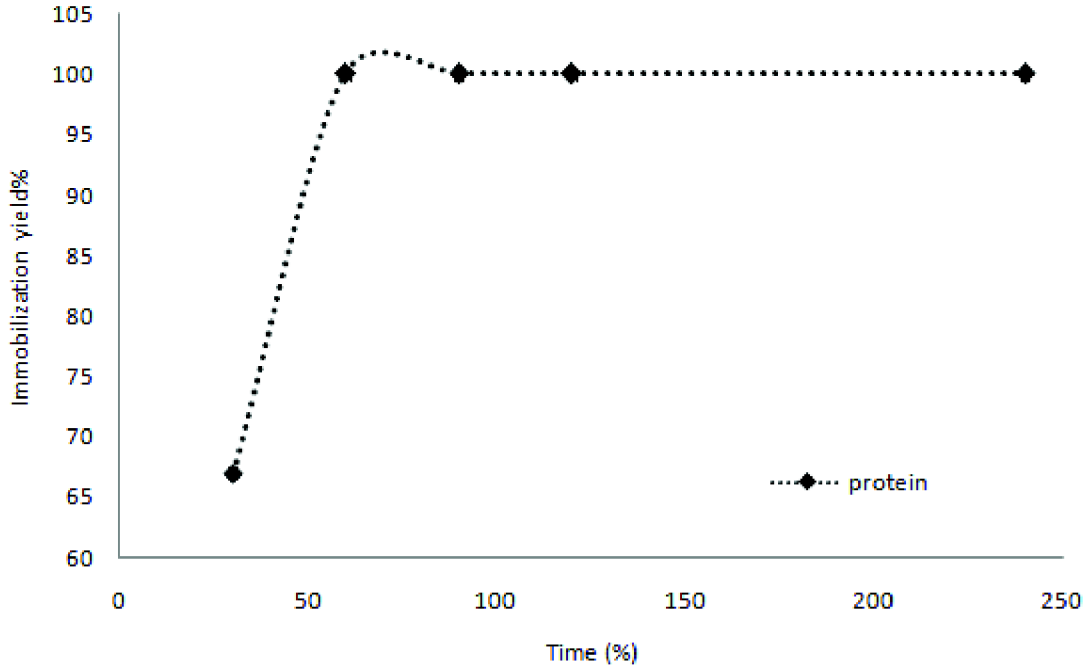

Figure 25 *The influence of incubation time from 30 to 240 min on coupling and immobilization yield of RA lipase immobilized on carbon-based carrier treated with tween 20 (110 µl)*

Influence of pH value of buffer solution on immobilization efficiency

Five different buffer solutions (pH 5, 6, 7, 8, 9) were examined to adjust the best conditions for immobilization process. Enzyme concentration of 0.1 $mg \cdot ml^{-1}$ was chosen with regard to previous testing (test with different concentrations of enzyme). The immobilization

time was 1 hour, and concentration of tween was 10.8; (110 µl). The experiment was performed at laboratory temperature, and following buffer solutions were used: sodium acetate buffer for pH 5, phosphate buffer for pH 6 – 8, and tris - HCl buffer for pH 9.

For understanding the interactions governing the immobilization at different pH value the surface chemistry of GO carrier is briefly discussed. Sheets of oxidized graphene contain three main functional groups: carboxylic, phenolic, and epoxy. Carboxylic groups can deprotonate in pH range from 3 to 11 which causes development of weak negative charges in the solution. Similarly, phenolic groups are source of weak negative charge due to the fact that they act as acids and release proton into the environment. This phenomenon gives hydrophilic character to the GO particles. When deprotonation occurs, the repulsion causes the graphene layer to fold away. Planar shape is renewed when the system is protonated with hydrogen ions. Based on this fact, raising pH (lower concentration of hydrogen ions) helps to change the morphology of GO molecule and thus increases its hydrophilic character [45].

In our study, immobilization efficiency decreases rapidly with raising pH value. Figure 26 shows significant decrease of immobilization yield at higher pH values.

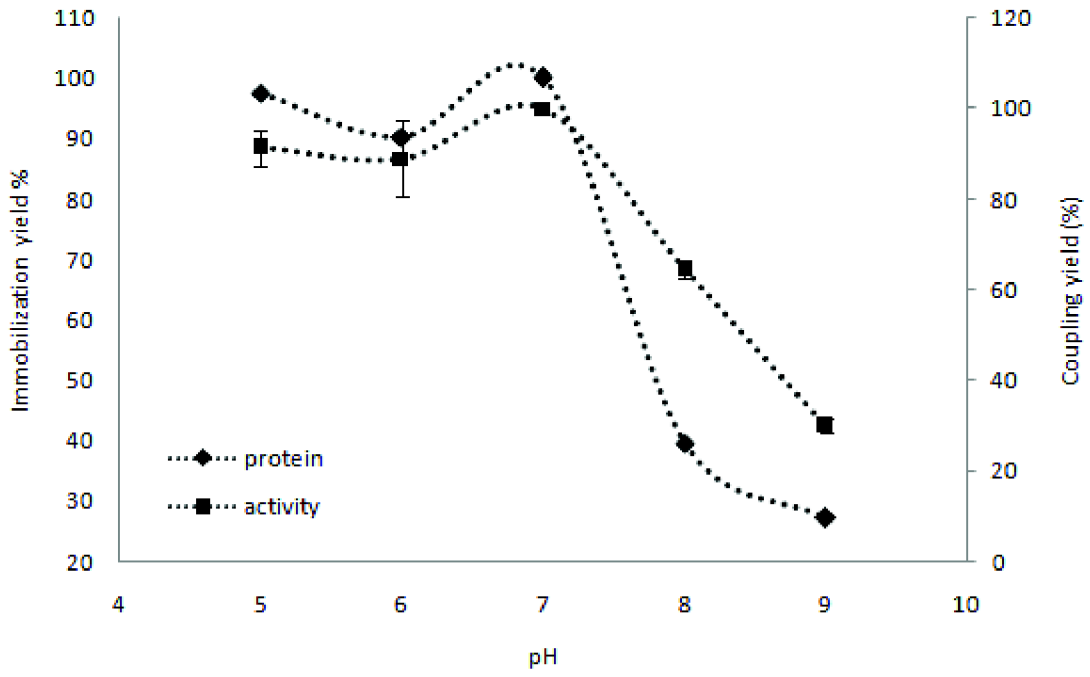

Figure 26 *Coupling and immobilization yield in systems immobilized at different pH (average value of three repeated measurements)*

At pH 8, the immobilization yield was only 64 %, and at pH 9 it reaches its minimum of about 33 %. Immobilization efficiency was only slightly lower in acidic range.

The lipolytic activity followed almost the same trend as immobilization yield. Unfortunately, the discussion of results could not be supported by the ability of lipase for

immobilization itself, which is connected with its isoelectric point since it was not measured. Based on the results, pH value of 7.2 was chosen as the most suitable one in accordance with previously published work [51].

5.4 Temperature optimum, pH optimum and thermal stability of "free" soluble enzyme compared to its immobilized form

On the base of previously discussed results, immobilization procedures were performed at lab temperature in the phosphate buffer with initial pH 7.2 for 1 hour. Graphene oxide was used as a carrier and Tween 20 was added to improve and ameliorate the immobilization conditions by enhancing of carrier solubility by covering its hydrophobic surface.

Concentration of enzyme used for immobilization was 0.1 $mg \cdot ml^{-1}$. Under conditions including appropriate pH value, temperature, and time of immobilization process), the immobilization yield reached 100 % and we observed lipase hyper activation phenomenon with activity coupling even higher than 100% (up to 113 %). Immobilized RA lipase was evaluated and compared with soluble counterpart regarding pH optimum, temperature optimum, and thermal stability.

Optimal pH of immobilized and soluble RA enzyme

Optimal pH was assayed in the pH range from 5 to 10 for soluble *RA* lipase, and from 5 to 9 for the immobilized one. The highest lipolytic activity was observed at pH of 8 for the immobilized form and at pH of 9 for the soluble one (Fig. 27).

It is obvious that low as well as high pH values significantly affect the activity of soluble and also immobilized enzyme. Soluble enzyme shows high sensitivity only in acidic conditions, and stayed relatively stable in alkaline conditions with only small activity decrease. Activity of immobilized RA enzyme decreases by 54 %, when the pH value was decreased from 8 to 9.

The pH optimum of both RA lipase forms was determined at pH 8. Reported study from literature states the optimal pH of lipases isolated from *Rhizopus* at 7.5. Nevertheless, lipases isolated from different strains can vary in this parameter as documented by pH stability range of RA from 7 to 11 reported by Niu *et al. in* 2006 [52]. The results measured leads to the conclusion that RA lipase studied in this work belongs to the alkaline group of lipases with maximum lipolytic activity at pH above neutral range.

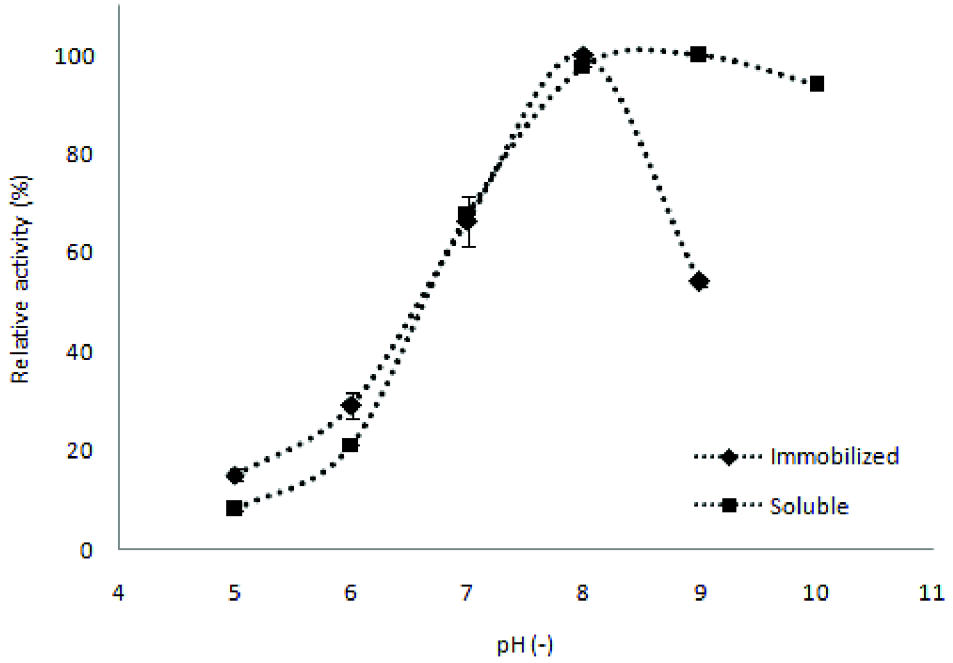

Figure 27 *Optimal pH of immobilized and soluble RA enzyme (average value of three repeated measurements). The maximum activity was regarded as 100 %.*

Thermal stability and temperature optimum of immobilized RA enzyme

The optimum temperature of lipase activity was determined by carrying out the enzyme catalyzed reactions at different temperatures (30 °C, 35 °C, 40°C, 45 °C, 50 °C, and 55 °C) and at pH 7.2.

At temperatures from 30 °C to 35 °C an increase of activity was observed with reaching the maximum value at 35 °C. During further increase of temperature, the enzyme activity decreases by up to 34 % at 40 °C in the case of soluble RA enzyme. Moreover, immobilized RA lipase showed significant change of temperature optimum (Fig. 27). Whereas the activity of immobilized lipase remained relatively high at 40 °C (91 % of the maximum) and only mild decrease was recorded from temperature 45 to 55°C, only 66 % of soluble form remained active at 40 °C. At 55 °C, the immobilized RA displayed retention of 67 % of maximal activity displayed at 35 °C in comparison with soluble enzyme, for which significant decrease more than 50 % of activity was measured.

Interestingly enough, an increase of activity to the value about 75 % was observed at 50 °C The presence of two temperature optima is in accordance with our previous work [53]. Two points of temperature optimum may be indicative factor of the presence of isoenzymes as in the case of fungal lipase isolated from *Fusarium solani* FS1 [56].

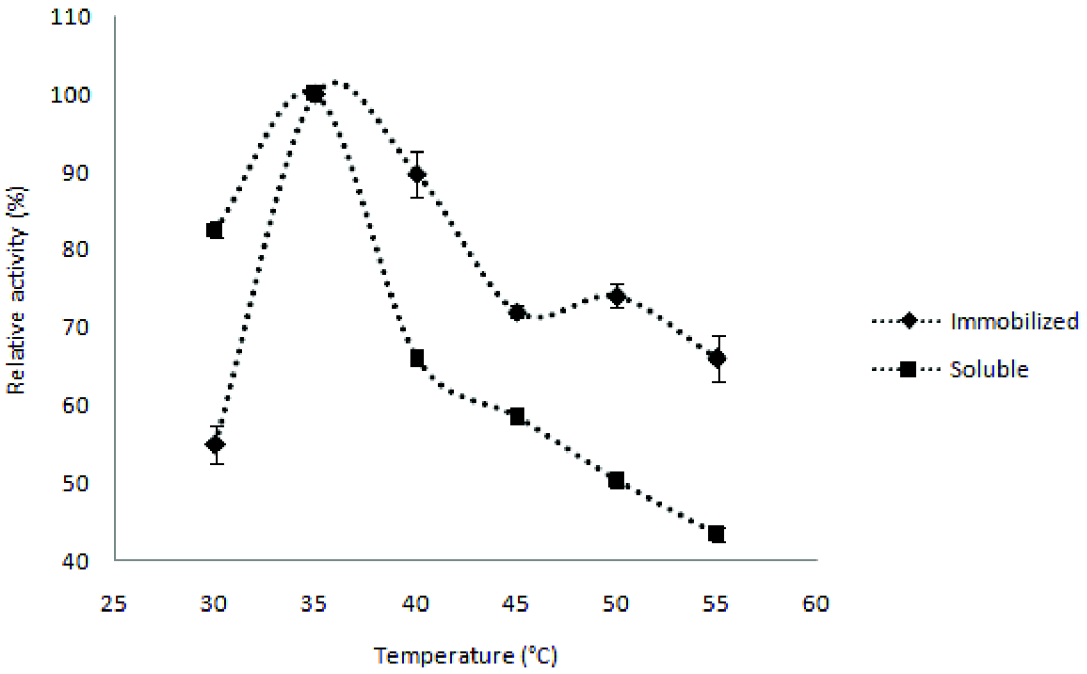

Figure 28 *Temperature optimum of immobilized and soluble RA lipase (average value of three repeated measurements). The maximum activity was regarded as 100 %*

Thermal stability was measured at the range of temperatures from 25 to 70 °C for soluble RA lipase, and from 30 to 70 °C for immobilized one. The experiments were carried out by thermostating of the immobilized enzyme at the required temperature in a water bath for one hour. The enzyme activity at the start of the experiment was taken as 100 % and the residual lipase activity after incubation was determined.

Immobilization itself does not appear to enhance the thermal stability of immobilized enzyme. At mild temperatures from 25 °C to 30 °C soluble as well as immobilized enzyme showed high activity with the maximum at 30 °C for both forms (soluble: 102 %, immobilized: 117 %) . As the temperature was rising, the relative activity decreased rapidly. At 70 °C, most of enzyme was inactive, or denatured by exposure to high temperature and the relative activity dropped by more than 90 % in both forms, compared to maximum value at 30 °C. Half-life of the enzyme is the time at which the enzyme retains 50 % of its initial activity [55]. It was determined at the temperature between 40 – 50 °C.

Based on these results only non-covalent bonds between enzyme and carrier are assumed, which means that any prevention against undesirable conformational changes thanks to covalent bonds cannot be considered. It should be mentioned that commercial untreated lipase containing certain amount of stabilizers was used for experiment. Another explanation of results is that soluble lipase was sufficiently stabilized during duration of experiment.

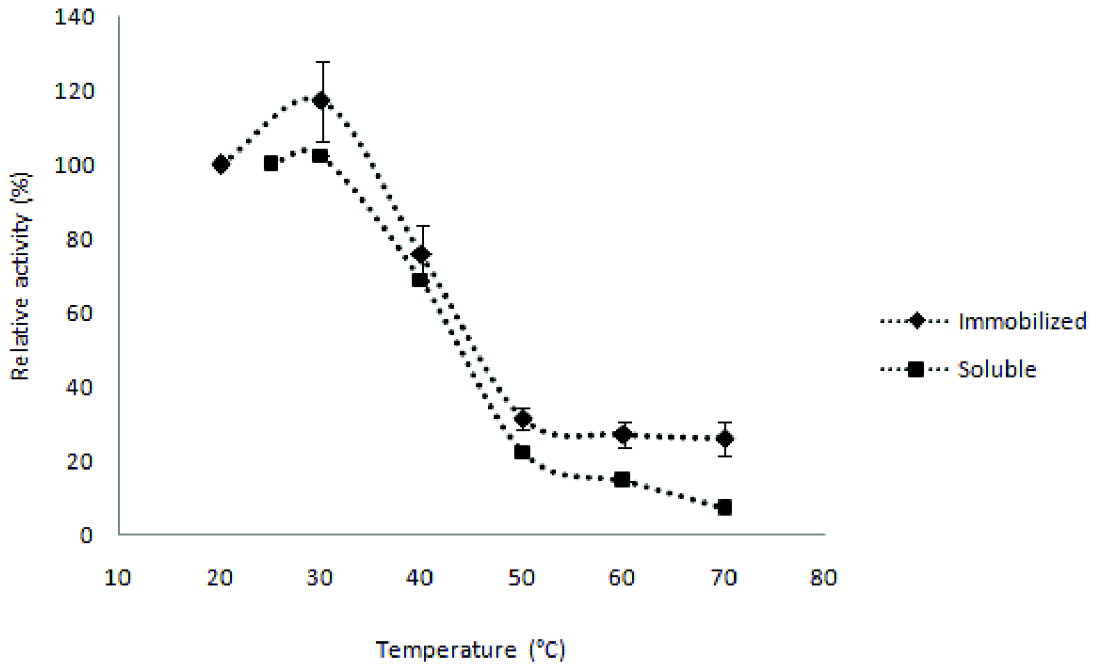

Figure 29 *Thermal stability of soluble RA compared with its immobilized form (average value of three repeated measurements). Lipolytic activity measured initially at laboratory temperature was regarded as 100 %.*

5.5 Kinetics profile of immobilized lipase compared to soluble form

Determination of enzymatic kinetics profile after immobilization belongs to one of the most important parameters for immobilization process evaluation. For example, if the charge on the carrier and substrate are opposite, K_M value decreases in comparison with that measured for soluble enzyme. In any case the lower Michaelis constant means the higher enzyme affinity to the substrate.

To determine apparent Michaelis-Menten constant, the hydrolysis of p-NPL at concentrations of 1.75, 2.5, 3.75, and 5 mmol·dm^{-3} catalysed by soluble and immobilized RA enzyme was studied. Figure 30 shows kinetics parameters determined for two simultaneously performed runs catalysed by soluble enzyme. The same reactions were conducted also for immobilized RA enzyme (Fig. 31).

Michaelis constant measured for reaction catalyzed by immobilized sample is approximately the same. Based on that results, summed up in table 5, it is assumed that affinity towards substrate after adsorption was relatively the same. Considering activity coupling which was 100 % it can be suggested that protein conformational changes did not affect resulting activity. However, in the presence of tween, even higher affinity towards substrate was expected. Lower accessibility of the substrate to the active site was caused by diffusion restriction. On the other hand, due to the porous carrier particles structure of unknown size, enzyme is considered to have worse active site availability to substrate.

Table 5: *Determination of K_M and v_{max} of soluble lipase compared with immobilized form*

Soluble RA lipase		**Immobilized RA lipase**	
∅ K_m (mmol·dm^{-3})	0.40±0.09	***∅ K_m (mmol·dm^{-3})***	0.30±0.18
∅ v_{max} (µmol·min^{-1}·ml^{-1})	42±6	***∅ v_{max} (µmol·min^{-1}·ml^{-1})***	9±3

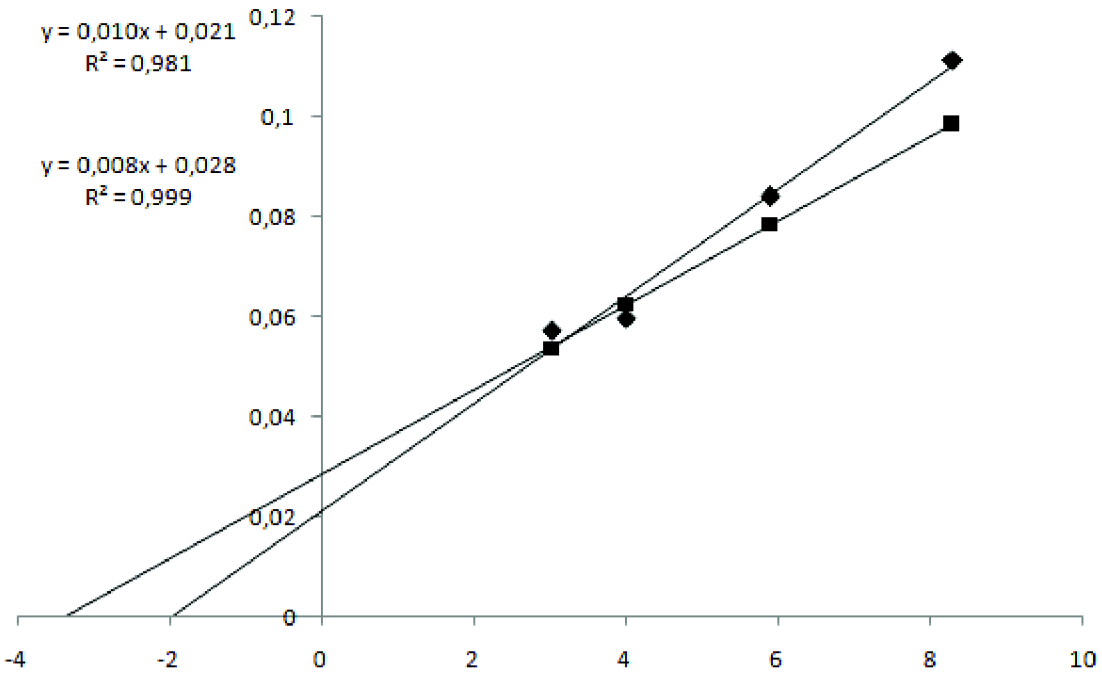

Figure 30 *Determination of K_M and v_{max} of hydrolysis catalysed by soluble lipase*

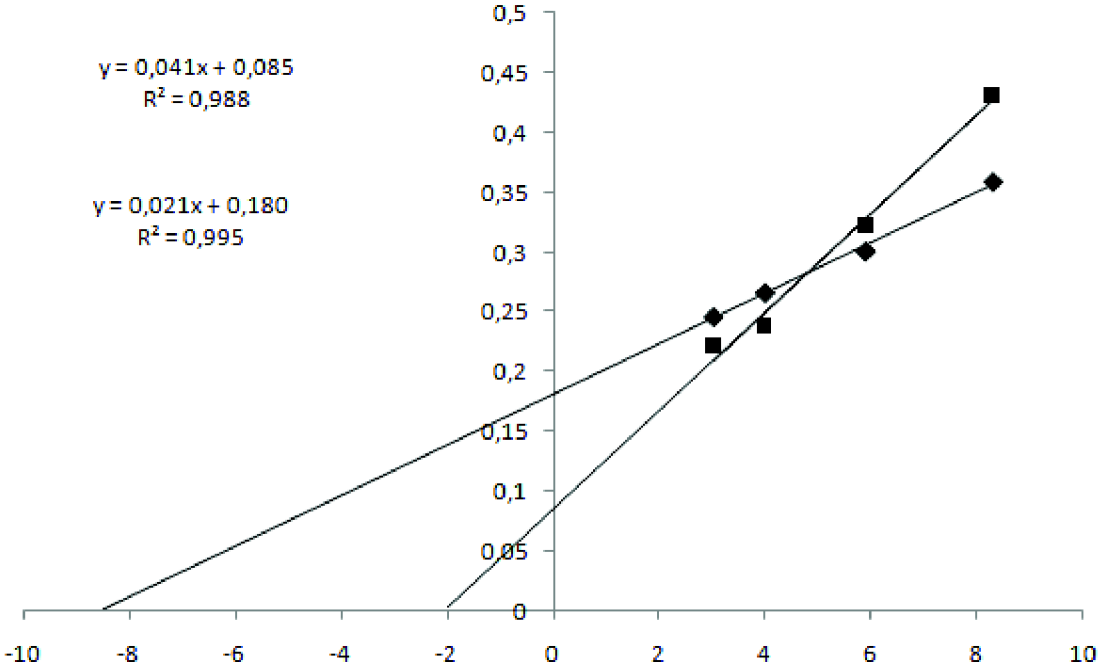

Figure 31 *Determination of K_M and v_{max} of hydrolysis catalysed by immobilized lipase*

5.6 Storage stability of immobilized RA lipase compared with the soluble counterpart

Storage stability of soluble RA lipase and of immobilized one was examined in time period of 110 days for immobilized form, and 40 days for soluble form. Both systems were stored in the phosphate buffer pH 7.2 at the temperature of 6 °C. The experiment was finished when significant activity decrease was observed (40 days for soluble form, 110 days for immobilized form).

For immobilization, the enzyme concentration of 0.1 $mg \cdot ml^{-1}$ was used. Immobilization process was carried out for one hour at laboratory temperature. Concentration of tween 20 of 10.8 $mmol \cdot dm^{-3}$ was used in the immobilization protocol.

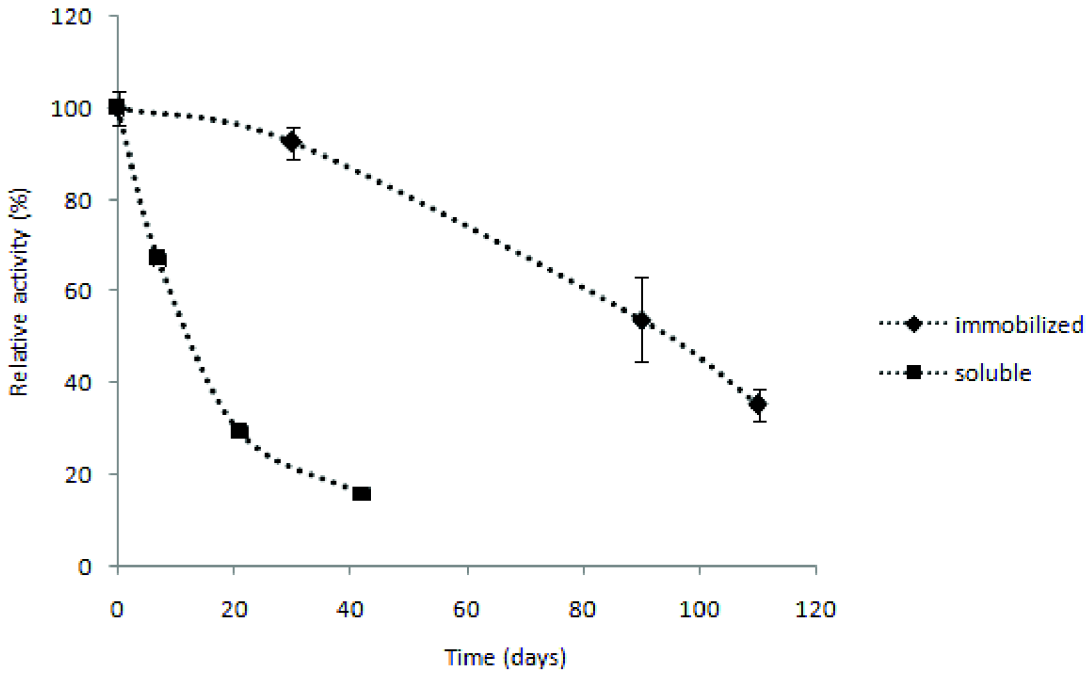

Figure 32 *Storage stability of immobilized RA lipase compared to the soluble one.*

Based on the results obtained, immobilization process improved the shelf life of enzyme solution. Whereas the activity of soluble enzyme was only about 30 % after 20 days of storage, 92 % of immobilized enzymatic form stayed active even after 30 days. First significant activity decrease was observed after 90 days.

6 CONCLUSION

In theoretical part of this thesis, practical applications of microbial lipases, their mechanism of action, structural properties, and studied possibilities of enhancing their enzymatic activity are summarized. Literature concerning detailed study of detergents as chemical agents used in wide range of industrial sectors such as pharmaceutics, cosmetics, and various chemical procedures was reported as well. Information focusing on detergents includes description of their chemical structure, mechanism of action and classification into three groups (ionic, non ionic, and amphoteric). Specialty attention was paid to tween family of detergents, which are classified as non ionic detergents.

Experimental part provides study of impact of different tweens and namely of tween 20 (polyoxyethylene sorbitan monolaurate) on activity, thermostability, and aggregation of free as well as immobilized *Rhizopus arrhizus* lipase. For the immobilization experiments, commercial preparation of lipase from microscopic fungi *Rhizopus arrhizus* (FLUKA) was studied. As the carrier, graphene oxide was studied.

Among tested tweens (tween 20, 60, 80), tween 20 was chosen as the most suitable for further tests. It was found out, that formation of tween micelles occurring in the system enhances the lipolytic activity of RA lipase.

Procedure of immobilization of RA lipase on graphene oxide carrier was performed at enzyme concentration of 0.1 $mg \cdot ml^{-1}$, tween concentration of 10.8 $mmol.dm^{-3}$, and carrier concentration of 1 $mg \cdot ml^{-1}$ at phosphate buffer (pH 7.2). Results were compared with parameters of soluble enzyme without tween.

Soluble enzyme showed pH optimum in range from 8 to 10, whereas immobilized form displayed pH optimum at pH 8. Temperature optimum of immobilized and soluble RA lipase was the same (35 °C). Immobilized sample showed better tolerance to increased temperature as documented by slight decrease of activity in the range of 40 – 55 °C. For soluble lipase significant decrease more than 50 % of activity was measured even at 40 °C. Thermal stability of enzyme does not seem to be improved by immobilization. This fact was ascribed to non-covalent bonds formed between enzyme and carrier. Thus any prevention against undesirable conformational changes thanks to covalent bonds cannot be considered. Significant improvement of storage stability was achieved by immobilization. Immobilized RA lipase stayed relatively high active even after 90 days compared to soluble form which activity was almost lost after 40 days.

Presence of tween leaded to high immobilization yield compared to published literature [51]. On the other hand, it could cause suppression of covalent bonds formation between functional groups and thus thermal stability improvement in immobilized lipase was not observed.

Concerning soluble RA lipase form, presence of tween could suppress lipase aggregation occurring at high concentrations, because more than 100 % activity coupling was achieved. Phenomenon of lipase disaggregating in presence of detergents was observed in work of Salameh *et. al* published in 2010 [24].

From the viewpoint of further research, increased weight ratio of enzyme protein to nanomaterial and covalent modification of carrier represent other aims.

7 LIST OF ABBREVIATIONS AND SYMBOLS

RA lipase…*Rhizopus arrhizus* lipase
CMC…critical micelle concentration
GO…graphene oxide
Gly…glycine
Ser…serine
pNPL…*p*-nitrophenyl laurate
K_M…Michaelis-Menten constant
v_{max}…maximum velocity
U...μmol·min^{-1}
U·mg^{-1}... μmol·min^{-1} per mg of protein
v…initial velocity
v_{max}…maximal velocity
S...substrate concentration
K_m…Michaelis constant
IY…immobilization yield

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