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# (Hydroxypropyl)methyl cellulose-chitosan film as a matrix for lipase immobilization: Operational and morphological study

Evdokia Vassiliadi [a,b], Anastasios Aridas [a,b], Véronique Schmitt [c], Aristotelis Xenakis [a], Maria Zoumpanioti [a,*]

[a] National Hellenic Research Foundation (NHRF), Institute of Chemical Biology, 48 Vassileos Constantinou Ave., Athens 11635, Greece
[b] Department of Biological Applications and Technology, University of Ioannina, Ioannina 45110, Greece
[c] Centre de Recherche Paul Pascal (CRPP) UMR 5031 CNRS, University of Bordeaux, Bordeaux, France

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ABSTRACT

The present work reports on the use of a hybrid blend of biopolymers as a matrix for lipase immobilization. (Hydroxypropyl)methyl cellulose (HPMC) and Chitosan (CS) were combined in order to formulate a film on which *Mucor miehei* lipase was immobilized. The biocatalyst was studied upon the model reaction of propyl laurate synthesis. The system was examined in terms of its capability to provide an appropriate environment where lipase will maintain its activity. The ratio of the polymers used was examined and HPMC:CS=2:1 proved to form the most promising matrix. Increasing the amount of the immobilized enzyme appears to improve the reaction yield indicating, however, mass transfer limitations. Apparent activation energy was calculated and energy input showed that ultra-sonication accelerated the initial rate of the reaction. Different reaction solvents were tested with isooctane being the most effective. The enzyme-containing film showed a remarkable reusability, since it can be used for up to 35 times without loss of activity. Finally, Atomic Force Microscopy (AFM) was performed to observe the morphology of the most promising films. The HPMC/CS film exhibits a nanostructure without a unique characteristic length and a roughness of 42.8 nm while the presence of enzyme smoothens the film as the roughness decreases to 5.5 nm.

## 1. Introduction

Hydrogels are defined as three-dimensional macromolecule networks swollen by large amounts of water. They are divided in different categories according to their ingredients and formation procedure, such as beads, and thin films [1]. Their properties allow their use in various fields, including catalysis, drug delivery and food applications [2]. Their catalytic applications are related to their ability to provide a suitable environment for the immobilization of enzymes, thus offering a unique medium for maximizing their activity and stability [3]. Various hydrogels based on biopolymers have been prepared by using alginate, agarose, starch, gelatin, cellulose, chitosan, and their derivatives, since they have exceptional properties combining efficiency and biocompatibility [4 – 6].

Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) are capable of catalyzing several reactions including esterification, transesterification, hydrolysis, epoxydations [7] and aminolysis [8 – 12]. The immobilization of enzymes on various polymeric carriers has gained interest due to numerus advantages [13]. Among several immobilization methods [14 – 16] entrapment in polymeric hydrogels has been proposed as alternative solution to acquire a biocatalyst with good mechanical properties [14,17]. In most cases, enzyme immobilization is accomplished with the aid of a cross-linker, such as glutaraldehyde [18 – 20].

Thus far, several natural polymers have been used for the design of an efficient support for enzymes. The use of a blend of biopolymers provides enhanced immobilization capability, biodegradability and flexibility [14,21]. What is more, mixtures of biodegradable and biocompatible polymers belonging to well-known families of natural polysaccharides (cellulose, starch, chitin) [22] are already approved for use in food industry.

Cellulose and chitin are the two most abundant natural polymers [5, 23]. Cellulose ethers such as methylcellulose (MC) and (hydroxypropyl) methyl cellulose (HPMC) are water-soluble derivatives of cellulose [24]. Chitosan is a natural polymer derived by deacetylation of chitin.

*Abbreviations:* AFM, atomic force microscopy; CS, chitosan; GLY, glycerol; HPMC, (Hydroxypropyl)methyl cellulose.

* Corresponding author.
*E-mail address:* mariaz@eie.gr (M. Zoumpanioti).

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Cellulose derivatives can interact with chitosan via hydrogen bonds, electronic or hydrophobic associations, chain entanglements, and van der Waals forces, resulting in the formation of hydrogels [24]. One of the biggest advantages of chitosan over chitin and other potential support/protective materials (e.g. silica) is that slightly acidic solutions of chitosan can be readily cast into beads, films or fibres [25]. HPMC, in contrast to chitosan has large hydrophobic segments and consequently exhibits strong surface activity. Therefore, simultaneous use of HPMC and chitosan could expand their potential uses.

Furthermore, plasticizers can be introduced to reduce frictional forces between polymer chains, such as those derived from hydrogen bonds or ionic forces. The incorporation of glycerol (GLY) into the film formulation also has the effect of retaining the film's mechanical properties [26]. In addition, the elongation of blended films increases with plasticizer content, even if a high content also produces a loss in tensile strength [27].

In the present study chitosan and a cellulose derivative (HPMC) were combined in order to formulate a promising enzyme carrier used as a biocatalyst, as tested upon propyl laurate synthesis. The biocatalyst was studied in terms of its capability to provide an appropriate environment where lipase from *Mucor miehei* can maintain its activity and stability in organic media. Several parameters were tested to optimize the biocatalyst, such as the ratio of the two polymers and the enzyme loading. The reusability was also tested with excellent results. Furthermore, the biocatalyst was observed via Atomic Force Microscopy (AFM) to assess the film topology in presence or absence of enzyme and/or plasticizer. The aim of the present study is to develop and optimize a biocatalyst which will subsequently be proposed for the synthesis of high added value products of industrial interest.

## 2. Experimental

### 2.1. Materials

Lipase from *Mucor miehei* (*M. miehei*) was supplied by Fluka, Basel, Switzerland and had a specific activity of 1.19 U/mg of protein (1 U corresponds to the amount of enzyme which liberates 1 μmol butyric acid per min at pH 8.0 and 40°C using tributyrin as substrate). Chitosan (CS) from shrimp and other crustacean shells (viscosity 200 – 600 mPa.s, 0.5% in 0.5% Acetic acid, 20°C; Deacetylation value: 80%, molecular mass 1526.464 g/mol) was purchased from TCI, Belgium. (Hydroxypropyl)methyl cellulose (HPMC) (3600 – 5500 cP) as well as lauric acid were obtained from Sigma, Darmstadt, Germany. Acetic acid and glycerol were obtained from LachNer, Neratovice, Czech Republic. All other materials were at least reagent grade. Millipore Milli-Q water was used for the preparation of gels and buffer solutions.

### 2.2. Methods

#### 2.2.1. Film preparation

Films based on CS/HPMC were prepared by solubilizing CS in acidic water (1% acetic acid) and HPMC in distilled water, resulting in a 2% w/w solution, each. The solutions were left overnight until homogeneous. Then a mixture was prepared at the proper ratio and placed on a petri dish to dry overnight. After drying, approximately 0.3 g of film is produced. It is worth noting that after mixture of the two solutions, no phase separation could be observed whatever the composition, showing the good biopolymers' compatibility.

To prepare films containing glycerol, 0.15 g anhydrous glycerol was added to 3 g of the solution of polymers prior to drying, resulting in producing a film with 0.5 g of glycerol/g.

#### 2.2.2. Lipase-containing film preparation

To formulate the biocatalyst, the appropriate amount of Tris/HCl buffer, pH 7.5 containing lipase was added in the polymers solution, prior to drying. In a typical experiment, 1 g of CS solution 2% w/w was mixed with 2 g HPMC solution 2% w/w, followed by the addition of 30 μL buffer solution containing 0.3 mg of lipase (commercially available) and stirred at room temperature. The mixture was left overnight on a petri dish to dry, thus formulating a dehydrated film where the enzyme is immobilized. This way a biocatalyst is produced with final lipase concentration of 1 mg/g of film. Before use, the film was washed three times with 5 mL of isooctane to remove enzyme molecules that may not be effectively immobilized on the gel network. After enzyme immobilization on the film, it was examined whether the solution with which the biocatalyst was washed, contained any amount of the lipase. Since proteins tend to have a characteristic absorbing peak at about 280 nm, the wash was spectrophotometrically evaluated [3] and no traces of the enzyme were found. These results show that there is no enzyme leakage after the procedure of drying.

#### 2.2.3. Lipase-catalyzed reactions

The esterification reactions took place under ambient conditions, unless otherwise stated. Approximately, 0.3 g of a film containing 0.3 mg of lipase was placed in a screw cap bottle with 10 mL of the appropriate organic solvent containing lauric acid and 1-propanol (100 mM, each). The bottles were preserved at room temperature without stirring, unless stated otherwise. At fixed time intervals samples of 10 μL each were withdrawn and analyzed by GC. Each experiment was performed in duplicate and conversions were calculated by using calibration curves of butyl laurate ester in the presence of dodecane as an external standard. After plotting the amount of produced ester against time, the slope was calculated giving the initial rate in terms of mM/min.

In order to compare and evaluate the performance of the biocatalyst under different conditions, the initial rate was chosen as the most appropriate means to compare given heterogeneous reactions [28]. Therefore, each reaction was monitored until approximately 10% yield.

For the gas chromatography (GC) analysis a DP5 column, Agilent, (30 m x 0.25 mm i.d. x 0.32 μm film thickness) was mounted on a Hewlett-Packard (HP) Model GC-6890C. Injector as well as detector temperature was 270 °C and oven temperature was held constant at 200 °C.

#### 2.2.4. Energy input

To study the effect of energy input on the biocatalytic activity of *M. miehei* lipase immobilized on the films, different methods were tested. In the first case, the reaction took place in controlled temperature by placing the reaction vials in a water bath (Memmert water bath W 200), at 45 °C. In the second case, the reaction took place under mechanical agitation, by placing the reaction vials on an orbital shaker at 200 rpm. In the third case, the reaction vials were placed in a sonicator (Ultrasonic bath UCI-150). As a control, a reaction that took place at room temperature, without stirring or any energy input was used.

#### 2.2.5. Effect of incubation temperature

To study the effect of temperature on the biocatalytic activity of *M. miehei* lipase immobilized on the films, its catalytic activity against propyl laurate synthesis was tested after incubation at different temperatures. For this purpose, a water bath was used under controlled temperature at 30, 40, 50, and 60 °C.

#### 2.2.6. Products diffusion

To study the ability of the products to diffuse in the biocatalyst, films were prepared in the absence of enzyme and placed in vials with isooctane as an external solvent, containing 100 mM butyl laurate. At fixed time intervals, samples were withdrawn and ester concentration in the external solvent was calculated by GC analysis.

#### 2.2.7. Morphological analysis via Atomic Force Microscopy (AFM)

Film morphology was examined by dynamic force mode (tapping mode) AFM in air at 25 °C using a Dimension-Icon, Brüker apparatus equipped with Tap 300 Al-G tips from Budget Sensors. Films were

prepared as described in 2.2.1 in the absence and 2.2.2 in the presence of enzyme. In each case, the mixtures were left to dry on microscope slides instead of petri dishes. Each sample was observed in different scan sizes adapted to the observation (going from 300 × 300 nm2 to 60 × 60 μm2). Height and phase images were recorded, simultaneously. The roughness is estimated through Rq the root-mean-square roughness of the sample over a 100 $\mu m^2$ area.

## 3. Results and discussion

The aim of the present study was to develop a potential enzyme biocatalyst based on the blend of two polymers, CS and HPMC, offering a system with improved properties. In order to optimize the biocatalyst, a model esterification reaction was used and several parameters were examined. More specifically, the effect on the esterification rate of the amount of enzyme immobilized on the films, the use of different reaction solvents, the use of glycerol as a plasticizer and the energy input were tested.

### 3.1. Biocatalyst optimization

To investigate the effect of the polymers' ratio of the film on the biocatalytic activity of *M. miehei* lipase, film hydrogels were prepared with different CS to HPMC ratios. The lipase-containing films were used to catalyze propyl laurate synthesis in isooctane, as described in Materials and Methods section. Fig. 1 presents the effect of HPMC/CS ratio on the esterification rate.

As can be seen from Fig. 1, increasing the CS content in the film initially increases the reaction's initial rate, with the highest efficiency achieved for polymer ratio HPMC:CS 2:1. Further increase of the CS content leads to lower esterification rates. This could be attributed to changes in the surface properties of the carrier, such as hydrophobicity and hydrogen bond forming ability. It has been reported in literature [29] that HPMC forms hydrogen bonding interactions with chitosan. Furthermore, it has been shown that there is an increase in the values of the polar surface free energy and the hydrophilic character of the film, when HPMC ratio is increased in the blend [30]. Thus, change of the polymer ratio could lead to changes of the surface properties, affecting the interaction between the enzyme and the carrier. Surface hydrophilicity is a very important parameter since it affects the interaction between the enzyme and the carrier [31], affecting the activity of the immobilized enzyme and thus the performance of the biocatalyst. Our results are also in agreement with the findings of Badgujar and coworkers [21]. Badgujar et al. reported for films based on chitosan and hydroxyl-methyl cellulose (HMC) containing lipase that the gradual addition of chitosan to the film leads to increased lipase activity; however, after a certain chitosan concentration lipase activity decreases.

### 3.2. Addition of glycerol

It is reported in literature that glycerol, when added to a film, induces changes to its morphology playing the role of a plasticizer [32]. To investigate whether the addition of glycerol can improve the mixed chitosan-HPMC films, glycerol was added to the blend of the two polymers alongside with enzyme addition and the mixture was left to dry overnight on a petri dish to form a film.

As can be seen from Fig. 2, the addition of glycerol to the film results in a slight raise of 7% of the reaction's initial rate. As reported in literature, glycerol increases the intensity of the hydrogen bonds in chitosan films, affecting the water affinity of the films [33]. In another study [34], the addition of glycerol increased the mobility of amylase and amylopectin chains, increasing the film's flexibility. Therefore, in our case the increased lipase activity may be attributed to a more flexible matrix. Moreover, it was observed that when glycerol was used for film preparation, a more fragile film was produced that was easily shred when removed from the dish. The smaller film parts offer increased interface, leading to higher conversion rates. However, the high error bar shown in Fig. 2 for the glycerol-containing biocatalyst reveals that these films give results with low reproducibility. This could be attributed to the fact that glycerol is also a substrate for the immobilized lipase. Therefore, glycerol consumption by the enzyme leads to matrix decomposition, thus affecting the model reaction that is being investigated. It should be mentioned here that all experiments involving films in the absence of glycerol had a high reproducibility (small error bars; Figs. 1 and 2) and as a result, films prepared without glycerol were chosen for further study.

### 3.3. Effect of lipase concentration on the reaction rate

To study the effect of the amount of lipase immobilized in HPMC/CS films on the esterification reaction, several films were prepared with different *M. miehei* lipase concentration. Fig. 3 shows the effect of varying the lipase concentration, calculated in terms of mg of lipase per g of gel, on the esterification rate of 1-propanol (100 mM) with lauric acid (100 mM) in isooctane. As can be seen from Fig. 3, the esterification rate increases with the increase of lipase content. This is in agreement with many studies that show that increasing the amount of enzyme has a positive effect on the reaction rate [35–37]. However, as shown in Fig. 3,

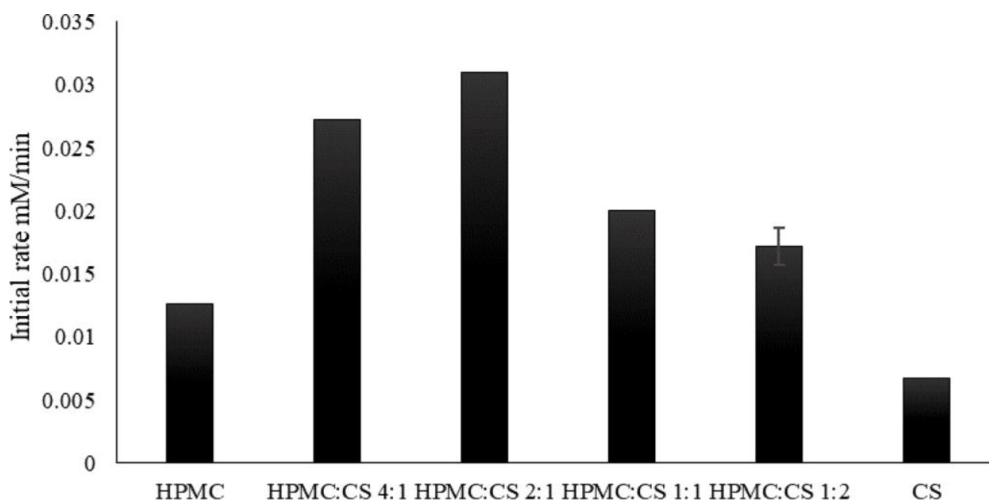


**Fig. 1.** Effect of polymer ratio (HPMC:CS) on the activity of *M. miehei* lipase as expressed by the initial rate of esterification reaction of lauric acid with 1-propanol. Reaction conditions as described in the Materials and Methods section.

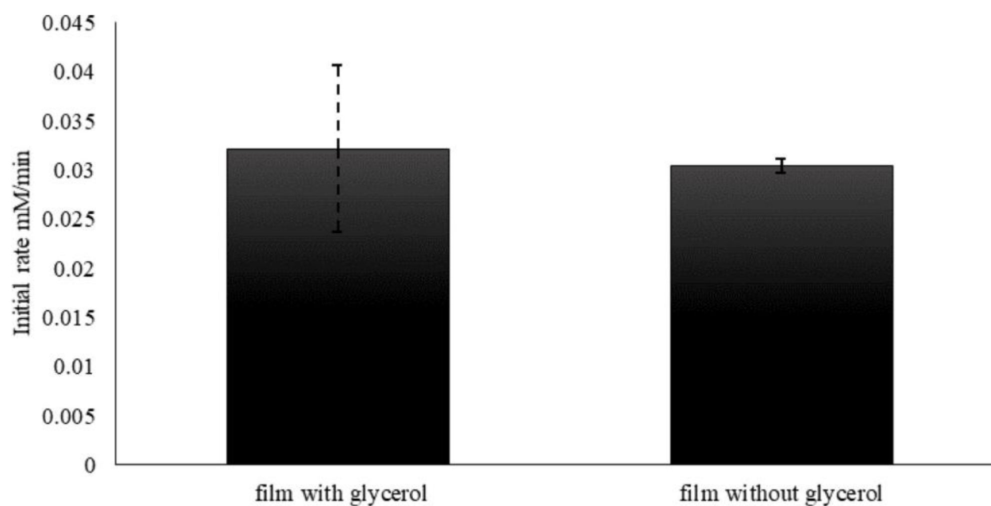


**Fig. 2.** Synthetic activity of *M. miehei* lipase immobilized on film in the presence and absence of glycerol, as expressed for the esterification of lauric acid with 1-propanol (100 mM each). HPMC:CS = 2:1; 1 mg lipase from *M. miehei* / g of film; Glycerol: 0.5 g / g of film.

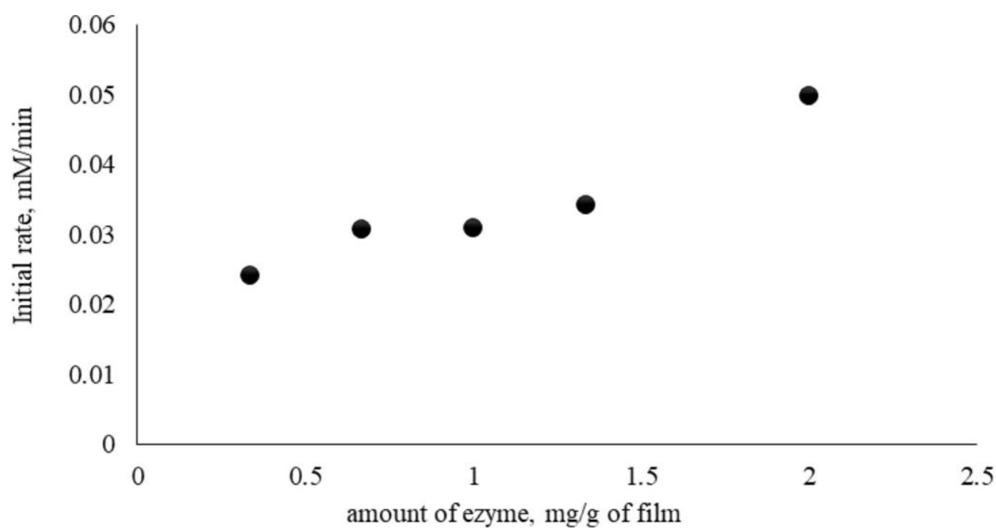


**Fig. 3.** Effect of the amount of *M. miehei* lipase immobilized on HPMC:CS=2:1 film, calculated as mg of lipase per g of film, on the esterification rate of 1-propanol (100 mM) with lauric acid (100 mM).

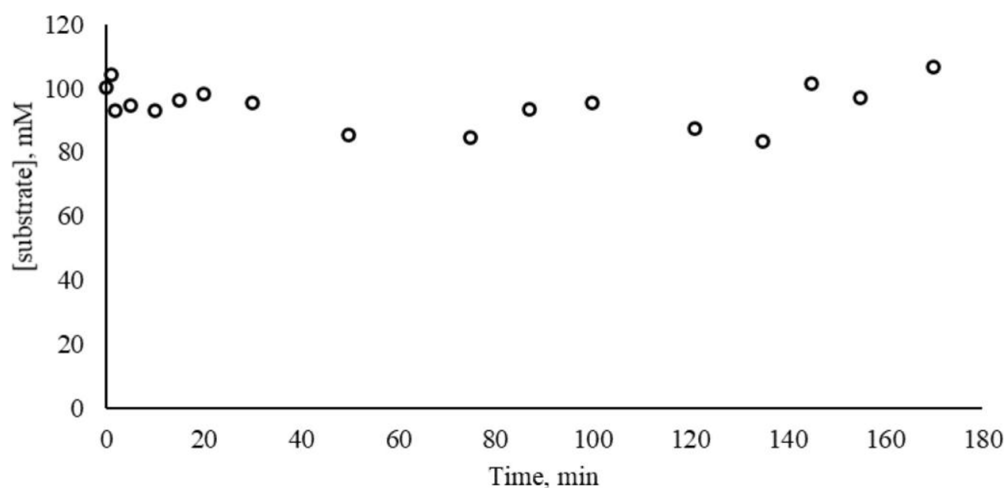


**Fig. 4.** Concentration of butyl laurate in the external solvent (isooctane), at 25 °C. Initial concentration 100 mM; solvent volume 10 mL. Film with ratio HPMC:CS = 2:1.

this increase is not linear. This lack of linearity can be attributed to mass transfer limitations at lower enzyme amounts, as linearity is consistent with kinetically controlled procedures [38].

### 3.4. Diffusion of products

During heterogeneous catalysis diffusion of substrates and products into the catalyst plays an important role, as it may be the reaction' s limiting step. Since in our case changing the amount of immobilized enzyme does not linearly affect the esterification rate of propyl laurate synthesis (Fig. 3) indicating mass transfer limitations, an effort to clarify this was made by studying the diffusion of the products into the matrix. Therefore, films were prepared in the absence of enzyme and placed in vials with isooctane as an external solvent containing butyl laurate as a reaction product. In defined time intervals, samples were withdrawn and analyzed by GC to study the changes in product concentration in the external solvent.

As shown in Fig. 4, the concentration of butyl ester is constantly changing, causing a dynamic equilibrium between the ester in the film and the ester in the external solution, in which approximately 90% of the total amount of ester is present. Therefore, although mass diffusion limitations are present (Fig. 3), they do not restrict the use of the film as a suitable lipase carrier for esterification reactions.

### 3.5. Effect of energy input

The use of mechanical agitation can enhance the reaction rate not only by offering energy to the system but also by assisting substrate diffusion, overcoming, thus, diffusing limitations. However, in some cases agitation fails to increase mass transfer [39]. To take a step further, the use of other energy sources such as ultrasound to increase the initial rate of reaction and enhance mass transfer can be considered as an alternative energy-efficient technique [40]. In general, the use of ultrasound reduces energy consumption almost by 25 – 50% as compared to mechanical agitation [41].

To study the effect of these parameters on the esterification rate, energy was supplied to the reaction via different methods. In the first case reaction took place in controlled temperature, at 45 °C. In the second case mechanical agitation was used by using an orbital shaker at 200 rpm and in the third case energy input took place by using sonication. As a control, a reaction took place at room temperature, without stirring or any energy input. In each case, biocatalysts were formed with HPMC:CS ratio of 2:1, containing lipase from *M. miehei* and were used for propyl laurate synthesis. The results are shown in Fig. 5.

It is clear from Fig. 5 that the use of ultrasound significantly accelerates the catalysis, with the initial rate of the reaction being almost double in the first four hours of observation. These results are in accordance to the findings of relevant studies [42 – 45], where the use of ultrasound dramatically increases the catalytic activity when compared to mechanical agitation. In particular, Deshmane, et al. [44] reported that the use of ultrasound increases the yield of isopropyl esters from palm fatty acid distillate by 75% in 6 h, whereas with mechanical agitation the equilibrium conversion was at 65% in 7 h. Xiao, et al. [45] reported 98% conversion of glucose to glucose ester with sonication, compared to 48% without sonication, within two hours.

Temperature increase, as shown in Fig. 4, causes a small increase to the reaction rate, up to 7%. In previous studies of our group involving chitosan [5] and HPMC [46] gels, it has been stated that temperature increase leads to increase of the reaction rate, however, higher temperature than 50 °C could cause the loss of lipase activity due to enzyme denaturation [5,46].

Mechanical agitation increased the initial rate of the reaction as expected. The increase was higher than that induced by temperature increase; however, the result was not as pronounced as in the case of sonication. This is not the first time that similar results have been observed. Xiao et al. [45] reported that the reaction yield under ultrasonication in the first 2 h was double that under shaking. Moreover, Brenelli and Fernandes reported that rates of acyl transfer increased up to 10 times using ultrasonication as compared to magnetic stirring [47].

### 3.6. Effect of incubation temperature

Temperature is a crucial parameter when it comes to biocatalytic reactions. It affects enzyme activity in two different ways. On one hand, the energy provided accelerates the mobility of the substrates. On the other hand, the catalytic rate is confined due to possible enzyme denaturation at high temperatures, which redound to enzyme activity loss. For this purpose, the activity of the biocatalyst was studied by incubation at a range of temperature between 30 and 60 °C.

As can be seen from Fig. 6, the higher initial rate was observed at 40 °C. As stated in Section 3.5, studies have shown [5,46] that even though temperature increase leads to increase of the reaction rate due to the energy input, higher temperature than 50°C could cause the loss of lipase activity. This could be attributed to either enzyme denaturation or destruction of the immobilization matrix. It should be mentioned here that the films studied at all temperatures did not show any

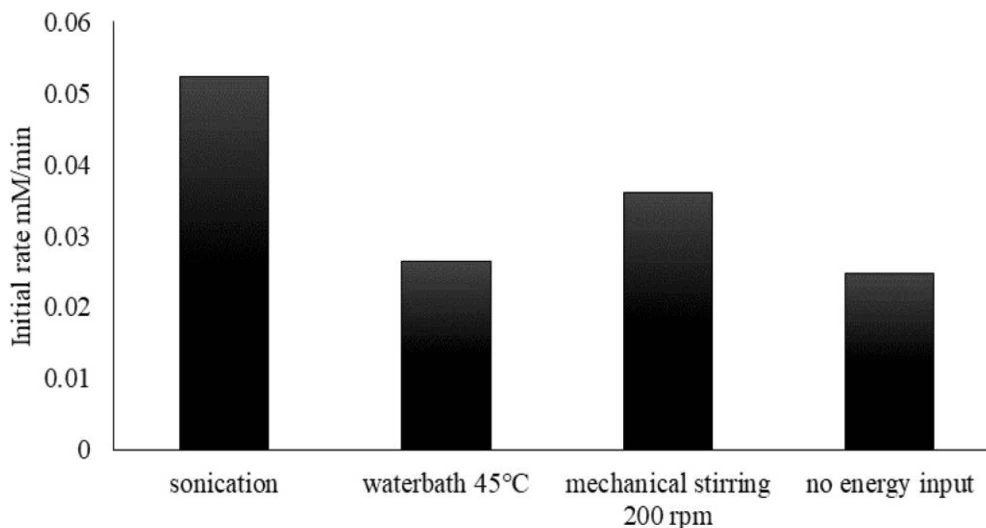


**Fig. 5.** Influence of energy input on the synthetic activity of M. miehei lipase (1 mg / g of film) immobilized on CS:HPMC =1:2 film, as expressed for the esterification of lauric acid with 1-propanol (100 mM, each).

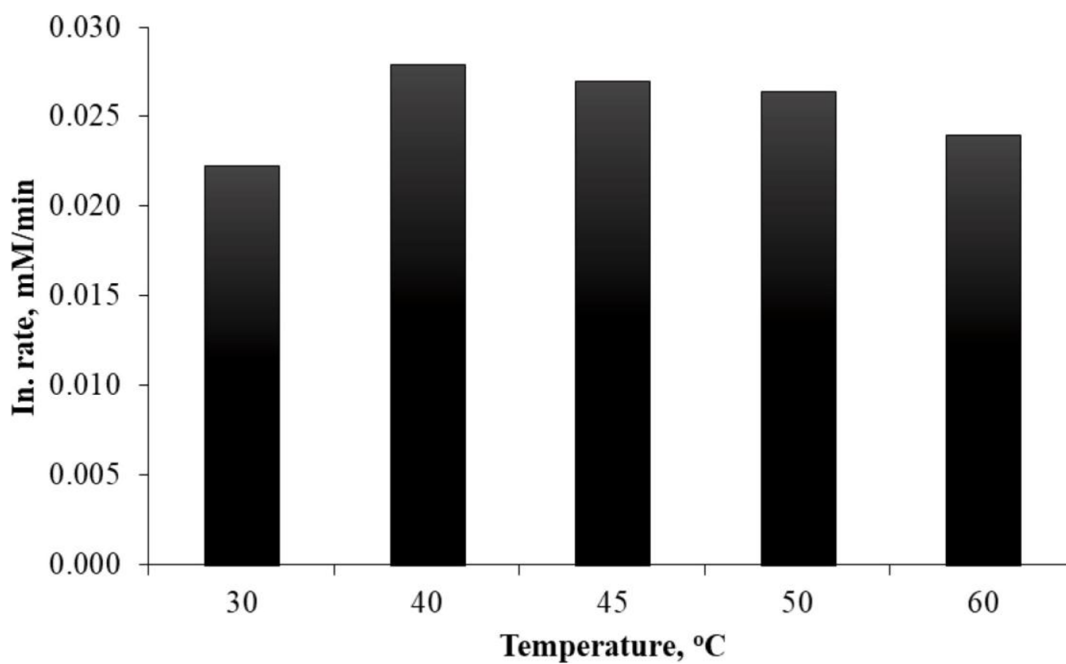


**Fig. 6.** Influence of temperature on the synthetic activity of *M. miehei* lipase (1 mg / g of film) immobilized on CS:HPMC =1:2 film, as expressed for the esterification of lauric acid with 1-propanol (100 mM, each).

macroscopically noticeable change in their structure, however, in microscale the increased temperature may have caused changes in film structure.

Similar results were found for the same lipase immobilized on chitosan hydrogels where the optimum temperature was found to be 30 °C [5], while for the same lipase immobilized on HPMC organogels this was not the case as increased temperature led to increased reaction rate up to 70 °C [46]. Another study [48] reported a similar temperature profile for *C. rugose* lipase immobilized on chitosan beads towards the hydrolysis of p-nitrophenyl palmitate.

The fact that the reaction rate only slightly changes upon temperature increase (the highest increment is almost 14% for temperature increase from 30 to 40 °C) suggests that the reaction rate is limited by mass transport phenomena and not by the reaction steps. This is in agreement with the results presented in paragraph 3.3 where the addition of enzyme did not affect linearly the reaction rate, leading to the same conclusion.

Moreover, the activation energy was calculated for the range of temperatures shown in Fig. 6. The Arrhenius plot for the synthesis of propyl laurate using the films is shown in Supporting Material (Fig. S1). The apparent activation energy was calculated and found to be 6 kJ/mol. The order of magnitude of the activation energy calculated here is similar to that calculated for the same lipase immobilized on organogels based on either HPMC or agar [46] and to the activation energy measured by Hedström et al. for immobilized *C. antarctica* lipase [49].

### 3.7. Effect of reaction medium

To study the influence of the reaction solvent on the enzymatic activity, several solvents were tested for the esterification reaction of lauric acid with 1-propanol. The solvents tested were alcohols, hydrocarbons and esters, either branched or non-branched. Fig. 7 presents the initial rate of propyl laurate synthesis in different solvents.

Although the film retained integrity in all solvents used, as can be

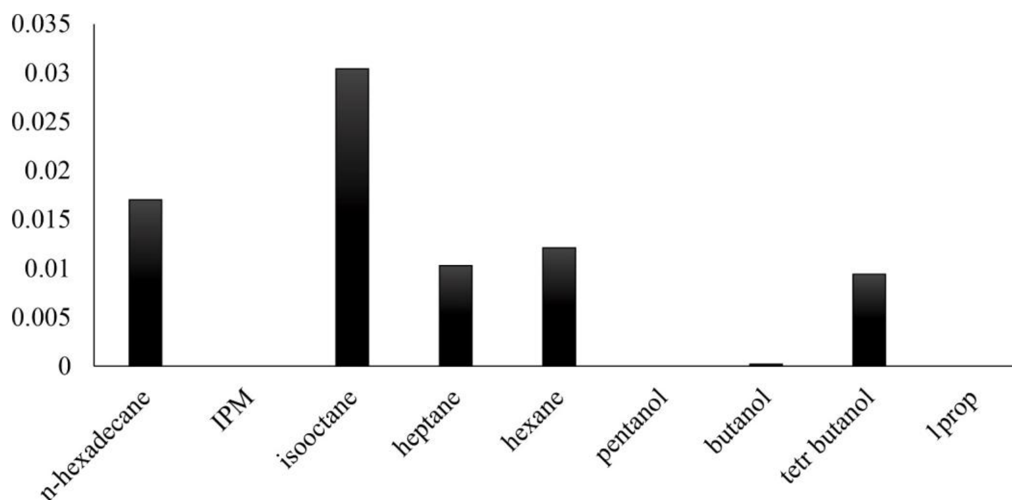


**Fig. 7.** Effect of the reaction solvent on the esterification rate of propyl laurate synthesis. HPMC:CS= 2:1, lipase from *M. miehei* 0.1 mg / g of gel. (IPM: Isopropyl myristate).

seen from Fig. 7, the nature and the polarity of the organic solvent influence the activity of the immobilized lipase. Thus, as it can be observed, polar solvents such as alcohols seem to create a non-favorable environment for the enzyme. This could occur due to the tendency of polar solvents to replace or remove essential water from the enzyme surface, leading to its inactivation. The highest lipase activity was observed in the less polar solvents, i.e. in hydrocarbons.

The highest reaction rate can be observer in the presence of isooctane and that could be explanted by the branched structure of its molecules that keeps them from packing tightly, offering thus higher diffusion of the substrates [5,46]. Branched hydrocarbons, especially isooctane, have been reported as the optima solvents by many authors in studies of enzymes immobilized on organogels [5,38,50,51].

Under the optimum conditions as determined thus far (room temperature, HPMC:CS = 2:1, isooctane as solvent, and enzyme concentration 1 mg/g of film) the final yield of propyl laurate synthesis in 24 h is 96%. Under these conditions, a kinetic study was conducted for the enzyme upon propyl laurate synthesis. The kinetic study revealed an Ordered Bi Bi mechanism with inhibition by both substrates at concentrations higher than 75 mM. However, the calculation of the kinetic constants was not feasible as significant mass transfer limitations effect the reaction and are more pronounced for low substrate concentrations. The effect of lauric acid concentration on the initial reaction velocity of the esterification for constant 1-propanol concentration can be seen at the Supporting Material (Fig. S2).

### 3.8. Reusability

For any industrial application of immobilized enzymes, the feasibility of reuse of the biocatalyst is important for the economic viability of a biosynthetic process. Therefore, the ability of the biocatalyst to be reused was tested for repeated cycles of propyl laurate synthesis.

Fig. 8. shows the ability of the biocatalyst to be reused, for the esterification reaction of propyl laurate synthesis. The film appears to have an excellent performance since the enzyme maintained its full activity for 35 uses. After the 35th use, the initial rate of the reaction shows a decrease down to 79% at 36th and rapidly to 60% at 39th use. These results are remarkable, as in other studies where films based on natural polymers have been used as carriers for lipases [52,53], the repeated use of films under optimized reaction conditions, showed the ability to be reused for five times, while the conversion decreases up to 69% at the fourth or fifth reuse. Similarly to these studies, in our case by performing the same test for the glycerol-containing films, we found that the reaction rate decreased to 64% at the 13th use (data not shown).

The decrease of the activity after several uses could be due to leaching of lipase from the support during each use by contact with the solvent, after each use by the contact with the washing solution, or gradual inactivation (i.e. from the stress upon each use or storage in refrigerator after each use) [3,54]. Since the loss of activity for 35 uses is insignificant, we can assume that it is caused by the gradual inactivation of the enzyme and not by leaching or washing it out.

### 3.9. Morphological analysis of the surface

Atomic Force Microscopy (AFM) is used to obtain images with atomic resolution of up to one tenth of nanometers. This type of microscopy can be effectively applied in the field of polymers to study the surface characteristics of polymer film samples [55]. Furthermore, AFM can offer information on the interaction between the matrix and the immobilized enzyme. Based on these information, catalyst optimization is possible by tuning the surface properties [56]. For the surface examination of the biocatalyst, four samples were analyzed, namely, (A) a HPMC:CS = 2:1 film, (B) a HPMC:CS = 2:1 film with immobilized *M. miehei* lipase, (C) a HPMC:CS = 2:1 glycerol-containing film and (D) a HPMC:CS = 2:1 glycerol-containing film with immobilized *M. miehei* lipase. The AFM height images are reported in Fig. 9 for $10 \times 10$ μm$^2$ and $1 \times 1$ μm$^2$. The other scan sizes are not shown.

For sample A corresponding to the biopolymer mixed film, the existence of domains with different heights can be observed. These domains show that the polymer film is nanostructured. The Fourier Tranform of a $60 \times 60$ μm$^2$ image does not allow defining a characteristic size of these domains. They are polydispersed in size and shape. At a smaller scan size (Fig. S3, Supporting Material), it appears that the light domains exhibit themselves a height variation at a smaller scale and some fibrils seems to be present. The roughness of the film (size 10 μm x10 μm), Rq, is equal to 42.8 nm. When the enzyme is added to the film (sample B), the domains are smaller and the roughness decreases by almost a factor 8 as Rq = 5.55, showing that the film is much smoother. For glycerol containing films (sample C), the domains have disappeared and small nodules are visible (scale of the order of 20 nm). The film roughness is equal to 12.8 nm. The picture of the film containing both glycerol and the enzyme (sample D), is very similar to the previous one and nodules are also visible. The film roughness, Rq, is equal to 8.3 nm.

To summarize, pure biopolymer films exhibit a nanostructuration, with a large size and shape distribution and a roughness of the order of 43 nm. The enzyme levels the film. So does also the glycerol in a smaller extent. Adding the enzyme to the film containing glycerol has only a small additional smoothing effect and does not alter the presence of

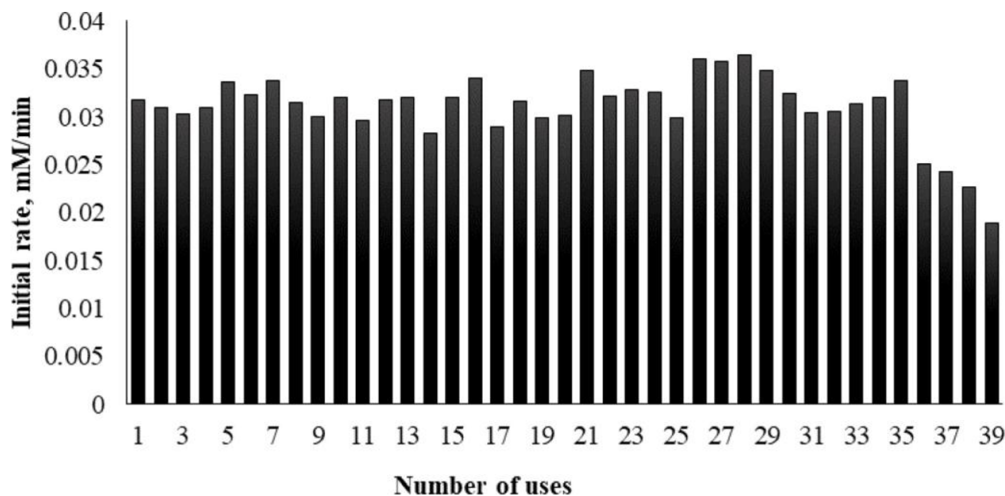


**Fig. 8.** Reaction rate as a function of number of uses for M. miehei lipase immobilized on the HPMC:CS=2:1 film, in successive batch syntheses of propyl laurate. Solvent: isooctane; lipase from M. miehei 1 mg / g of film; ambient conditions.

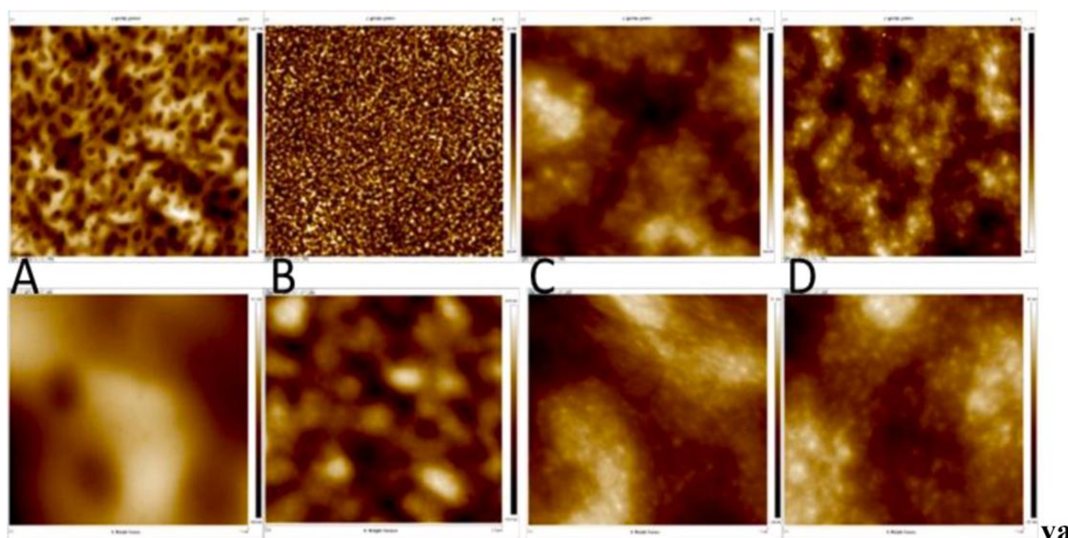


**Fig. 9.** Height imaging of films with scan size 10 μm; (A) HPMC:CS= 1:2 film; (B) HPMC:CS = 2:1 film with immobilized lipase, (C) a HPMC:CS = 2:1 glycerol-containing film, (D) a HPMC:CS = 2:1 glycerol-containing film with immobilized lipase. Lipase: *M. miehei* 1 mg / g of film; Glycerol: 0.5 g / g of film. First row scan size of $10 \times 10$ μm² and second row scan size of $1 \times 1$ μm². The height scale is not the same for all images.

nodules due to glycerol.

## 4. Conclusion

Chitosan and cellulose derivative HPMC were combined for the preparation of a film that was used as an enzyme carrier. Lipase from *Mucor miehei* was immobilized on the films and its activity and stability in organic media were studied. The novel catalyst permits easy diffusion of the substrates and products, although mass transfer limitations are present for the lower enzyme concentrations. Several ratios of two polymers were examined to optimize the biocatalyst over the esterification of propyl laurate synthesis, with the ratio of HPMC:CS=2:1 presenting the highest reaction yield. The immobilized lipase maintains its activity in non-polar solvents, showing higher activity in isooctane. Moreover, energy input via ultra-sonication increases enzyme activity more than thermal energy or mechanical agitation. The most remarkable result of the study is the excellent biocatalytic performance that occurred when testing the reusability of the biocatalyst, with the activity of the enzyme remaining up to 60% for 39 uses. Lastly, the films were investigated in terms of surface texture and morphology via Atomic Force Microscopy (AFM). AFM showed a nanostructuration of the polymer blend films. The addition of the enzyme led to a smoother film. Glycerol was also responsible for a smoother film. Moreover, nodules of about 20 nm are visible with glycerol. As it would be interesting to further characterize these nanostructurations using X-ray scattering techniques, the present AFM study can serve as preliminary work to our next steps.

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## CRediT authorship contribution statement

**Evdokia Vassiliadi:** Data curation, Formal analysis, Conceptualization, Writing – original draft, Visualization. **Anastasios Aridas:** Data curation, Formal analysis. **Véronique Schmitt:** Data curation, Formal analysis, Writing – review & editing. **Aristotelis Xenakis:** Conceptualization, Writing – review & editing. **Maria Zoumpanioti:** Conceptualization, Data curation, Writing – original draft, Writing – review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Supplementary materials

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