RESEARCH ARTICLE

Starch
www.starch-journal.com

α-Amylase Immobilization on Ceramic Membranes for Starch Hydrolysis

*Elena A. Nazarova, Ekaterina D. Yushkova, Andrei I. Ivanets, Vladimir G. Prozorovich, Pavel V. Krivoshapkin, and Elena F. Krivoshapkina**

α-Amylase has many applications in various fields, especially in the food industry where it is used in starch hydrolysate production. However, the use of free enzymes is disadvantageous as the enzymes cannot be recovered from the reaction medium and are therefore used only once. The challenge of the biological pollution of wastewater can be tackled using immobilized enzymes as they possess higher stability as well as the possibility of repeated and continuous use. In this paper, adsorption and covalent methods of α-amylase immobilization on ceramic membranes are developed and their advantages and disadvantages are identified. For these purposes, silica ceramic membranes with a microfiltration layer (pore sizes 1–5 μm) are first used. The dependences of immobilized α-amylase activity on enzyme concentration and reaction time are determined and compared with the free enzyme. The influence of pH, temperature, and reusability on adsorbed enzyme activity is investigated. Thus, a novel combination of filtration and biocatalytic functions on one membrane is introduced. The results obtained are crucial for controlling certain characteristics of hydrolysis during industrial processes.

1. Introduction

α-Amylase catalyzes the hydrolysis of α-1,4-glucosidic bonds in starch into oligosaccharides. It plays a key biological function, for example, in the process of plant seed germination, converting starch, a necessary food reserve, into soluble maltodextrins, which are subsequently hydrolyzed to maltose and glucose.[1] The cleavage of the substrate is random and can be either single or multiple when several fragments are sequentially separated from the starch molecules.

E. A. Nazarova, E. D. Yushkova, P. V. Krivoshapkin, E. F. Krivoshapkina
SCAMT Institute
ITMO University
Lomonosova Street 9, St. Petersburg 191002, Russian Federation
E-mail: kef@scamt-itmo.ru
A. I. Ivanets, V. G. Prozorovich
Institute of General and Inorganic Chemistry of National Academy of Sciences of Belarus
Surganova Street, 9/1, Minsk 220072, Belarus

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/star.202100017

DOI: 10.1002/star.202100017

This enzyme is used in several industries, such as food, pharmaceuticals, textile, and paper among others. One of the main products of starch hydrolysis is maltooligosaccharide, which is applied as a coating agent, viscosity producer, flavor carrier, and low-calorie sweetener, as well as source of dietary fiber. Additionally, maltose is a major ingredient in maltose syrup production. The composition of oligomers is different for each application.[2–5] It is therefore necessary to control starch hydrolysis in order to produce oligosaccharides of the required polymerization degree. Usually, industrial starch hydrolysis is performed in batch reactions or continuous processing where α-amylase is mixed with a substrate solution. However, this method has some economic disadvantages because the enzymes in soluble form cannot be recovered from the reaction mixture and are therefore used only once. Applying immobilized enzymes can solve this problem.[6]

Immobilized enzymes refer to "enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities."[7] There are many advantages associated with using immobilized enzymes as opposed to free enzymes. A major benefit is their reusability, which makes the industrial process less expensive.[8] They possess longer half-lives and higher stability, and provide a simple means of initiating and quenching a reaction.[9] Enzyme immobilization also prevents substrate contamination with the enzymes themselves or other compounds, resulting in products with high purity.[10–12] Although enzyme activity is often hindered by immobilization due to conformational changes in the biomolecule structure and mass transfer limitations, their positives far outweigh the negatives, making them applicable in different biotechnology fields.[11,13]

In the past few decades, many immobilization methods have been investigated. The most common methods include physical adsorption, entrapment/encapsulation, cross-linking, and covalent bonding. Physical adsorption, which involves various noncovalent interactions such as van der Waals, hydrogen bonds, and others, is one of the simplest.[14] Despite showing high activity, enzymes physically adsorbed on a surface may detach and go into the substrate solution, resulting in undesirable contamination.[7,10]

The entrapment method involves trapping enzymes in the lattice of a material or in polymer membranes.[13,15,16] In the cross-linking method, enzymes themselves are carriers that are both expensive and ineffective. Furthermore, the enzymes show low activity. As a result, this method is often paired with other immobilization methods.[7,11]

Covalent bonding is one of the most widely used and researched methods for enzyme immobilization. It provides strong interactions between enzyme and support, results in high enzyme stability and reusability, and prevents their release into the reaction mixture in spite of decreasing their mobility and conformational changes.[6,13]

Many materials may be used as a support for enzyme immobilization. The choice of material depends on the method and enzyme application. Materials such as cellulose, synthesized polymers, metal oxides, polymeric gels, and ceramics are oftentimes used as carriers.[11,17] Supports should be non-toxic and biocompatible, feasible for regeneration, and have a high affinity for protein.[7,18] Inorganic supports have several advantages over their organic counterparts, including higher chemical, thermal, and mechanical resistance, and enhanced reusability. Furthermore, such materials confer resistance to microbial contamination.[13]

Today, among inorganic supports for enzyme immobilization, porous (mesoporous) materials or membranes are the preferred materials. These include aluminum, zirconium, silicon, and titanium oxides (Al_2O_3, ZrO_2, SiO_2, and TiO_2).[19] Silica (SiO_2) is the most studied porous material for enzyme immobilization and has found application in clinical diagnostics and biotechnology as well as in other areas.[20–22] Porous materials have a large surface area per unit volume.[23] At the same time, their production is easy and inexpensive due to the low cost of raw materials used. Continuous membrane reactors based on these materials are applied in the food industry as an alternative to batch reactors.[2,24] The main advantage of membrane reactors is the separation of key products from starting reagents and unreacted substrate from the reaction mixture through membrane pores.[25] Furthermore, continuous membrane reactors have high efficiency and low labor cost. There is a method in which ceramic membrane reactors are used in starch hydrolysate production.[26,27] In this method, however, the α-amylase needed to catalyze the starch hydrolysis process is mixed with the substrate solution, complicating enzyme recovery. Thus, to the best of our knowledge, there have been no studies on the immobilization of α-amylase on silica membranes for starch hydrolysis.

Therefore, the immobilization of enzymes on membranes, especially on ceramic ones, combines the advantages associated with both membranes and enzymes. Membranes with immobilized enzymes perform biocatalytic and separation functions, making the processes both energy efficient and environmentally friendly. Moreover, enzyme stability and reusability significantly increase.[13]

This study compares the adsorption and covalent methods of α-amylase immobilization on silica ceramic membrane microfiltration layers. These enzyme-ceramic systems are developed for the first time, and their improved properties are discussed. Enhanced product formation by such an enzymatic membrane promises to reduce the cost of technological processes in the food industry.

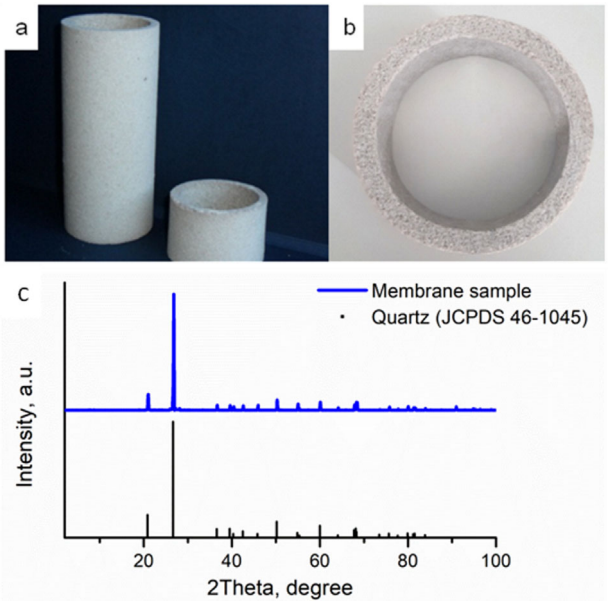

Figure 1. Tubular ceramic membrane samples: A) general view, B) cross-section, C) XRD pattern of membrane material (a.u., arbitrary units).

2. Experimental Section

2.1. Materials

α-Amylase from *Bacillus subtilis* (powder, 50 units mg^{-1}, Product Number: 10070) was obtained from Sigma-Aldrich, Germany. Potato starch (soluble), used as a substrate for hydrolysis, was obtained from Vekton Russian Company. Carbonate-bicarbonate and phosphate buffers were purchased from Sigma-Aldrich, Germany. Quartz sand from the Republic of Belarus was used as the raw material for obtaining ceramic membranes. Bradford reagent for 0.1–1.4 mg mL^{-1} protein (Product Number: B6916) was obtained from Sigma-Aldrich, Germany. Glutaraldehyde (GA) solution (50% (w/w) in H_2O, Product Number: 340855), gelatin from porcine skin (gel strength 300, Type A, Product Number: G2500), and bovine serum albumin (lyophilized powder, ≥96%, Product Number: A2153) were purchased from Sigma-Aldrich, Germany. Deionized (DI) water was purified using a Milli-Q system from Millipore.

2.2. Membranes

Ceramic microfiltration membranes were made according to the two-step method previously described.[28,29] First, tubular porous ceramic substrates (diameter 65 mm, length 500 mm) were prepared, followed by the application of the microfiltration membrane layer at the second stage (**Figure 1**A,B). The substrates were fabricated by isostatic pressing. Quartz sand was used as the main component of the ceramic mixture. An aluminosilicate binder (aqueous solution of sodium aluminosilicate), an organic burning additive (flour), a plasticizer (clay mineral raw material), and a nonionic surfactant (1% (w/w) solution of OS-20—a mixture of polyethyleneglycol esters of higher fatty alcohols) were used. In order to optimize the

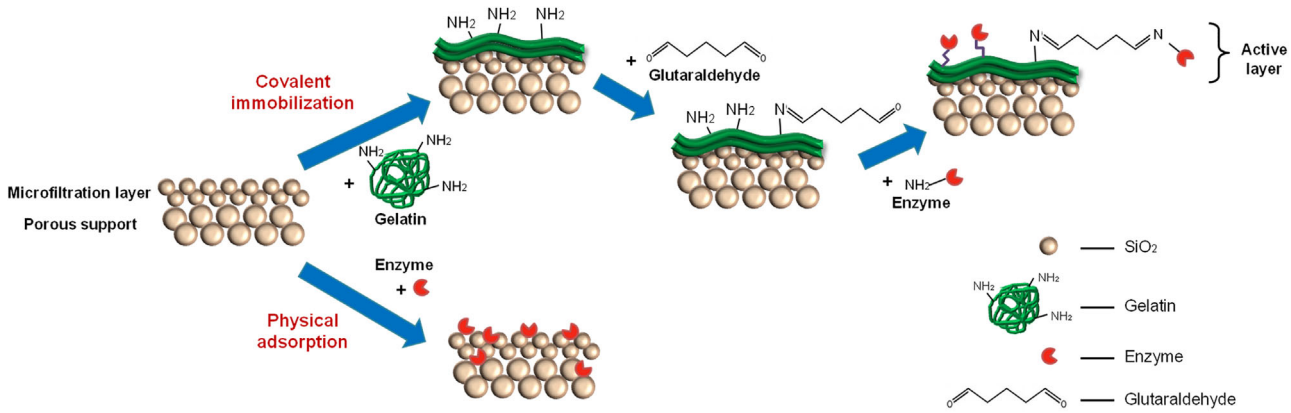

Figure 2. The scheme of enzyme immobilization on silica-based membrane for covalent and adsorption immobilization methods.

composition and thermal treatment conditions, pressed sample tablets (19 mm in diameter and 10–12 mm in height) were prepared and tested. Sintering of the final samples was conducted in an SNOL 7.2/1100 laboratory oven, Umega Group in air at 850 °C. Microfiltration membrane layers were applied using 30–50% (w/w) aqueous suspension of fine-dispersed crystal silicon dioxide (particle size of 40–70 and <40 μm) with the aluminosilicate binder (10% (w/w)). Membrane layer fixation was performed by drying at room temperature for 24 h with subsequent thermal treatment at 600 °C for 5 h using a heating rate of 5 °C min^{-1}.[30]

2.3. X-Ray Diffraction

To characterize the membrane material, an X-ray diffractometer (SmartLab 3, Rigaku Corporation) was used. Measurement conditions include Cu Kα radiation (1.54 Å), step size 0.01°, angle range 5–90° at a speed of 5° min^{-1}.

2.4. Enzyme Immobilization

Covalent immobilization on ceramic membranes consisted of four main steps: surface hydration, functionalization with a biopolymer (gelatin), activation with a cross-linking agent (GA), and enzyme attachment.[13,31]

First, the ceramic membranes were hydrated by submerging them in pure water. Next, they were immersed several times in a 4% (w/w) aqueous gelatin solution prepared in 0.2 M carbonate buffer (pH 9.2) via the dip coating method to form a layer of gelatin on the outer microfiltration surface. After this, the membranes were immersed in a 4% (v/v) aqueous GA solution prepared in 0.2 M carbonate buffer (pH 9.2), using the dip-coating method and left to react for 15 min. The next step was enzyme grafting, where the membrane samples were covered with 50 μL of the aqueous enzyme solution. Finally, the active membranes with immobilized α-amylase were dried and stored at room temperature before use. After each stage, the ceramic membrane samples were washed with DI water to remove excess compounds. A schematic of the enzyme immobilization process is shown in **Figure 2**.

The adsorption method was realized by immersing ceramic membrane samples in 5% (w/w) aqueous enzyme solution for 30 min followed by washing any unattached enzymes from them.[12]

The amount of immobilized enzymes was calculated by subtracting the unbound enzymes from the total added. Protein concentration was determined using Bradford's method.

2.5. Scanning Electron Microscopy with Energy Dispersive X-Ray Analysis

To visualize changes in surface morphology, the scanning electron microscopy (SEM) and energy dispersive X-ray (EDX) images of the ceramic membranes before and after enzyme immobilization were obtained using SEM (Tescan Vega 3). Ceramic membrane samples with and without immobilized enzymes were sprayed with gold-palladium alloy in an argon atmosphere using an SC7620 sputter coater prior to imaging.

2.6. Bradford Assay

Before each experiment on enzyme activity, enzyme-membrane systems were analyzed for α-amylase release from the support by washing the system three times with DI water. For this purpose, in both adsorption and covalent immobilization methods, a Bradford protein assay was used.[32] Bovine serum albumin was used as a standard for measuring protein concentration in three technical replicates. It was observed that three washings were enough to remove unattached enzymes from the carrier.

2.7. Fourier Transform Infrared Spectroscopy

Functional composition was analyzed using Fourier transform infrared spectroscopy (FTIR Spectrometer IR-Prestige 21, Shimadzu). The spectra were recorded within the range 4000–400 cm^{-1}. GA was measured directly as a thin film between the potassium bromide (KBr) windows. Dry samples were mixed with crystalline KBr (2 mg of sample per 700 mg of KBr) and then pressed into a disk. The spectra were collected at a resolution of 0.5 cm^{-1} with 20 cumulated scans, Happ-Genzel apodization, and a signal-to-noise ratio of 40 000:1.

2.8. Starch Hydrolysis Using Immobilized and Free α-Amylase

Both physically and chemically immobilized enzyme activities were determined by starch hydrolysis reaction. Various α-amylase concentrations (1%, 5%, and 10% (w/w)) were used for immobilization and study of the hydrolysis reaction over time. For experiments with a free enzyme, the amount of α-amylase corresponded to the amount bound to the membrane after immobilization in 1%, 5%, 10% solutions.

Each sample of ceramic membrane with immobilized enzymes was immersed in 10 mL of the substrate solution (1% (w/v) soluble starch with 0.067 M phosphate buffer (pH 6.0)) and another 10 mL of starch solution with an enzyme-free membrane was used as a control. Therefore, the results reflect only the catalytic activity of the enzyme immobilized on the membrane. The solutions were incubated for timed intervals at 30 °C, followed by sampling on successive occasions during a time-dependent experiment. Then, 0.25 mL aliquot from each sample was added to 25 mL iodine solution[33] and a color change was observed.

The starch concentration was determined by spectrophotometry, using a Cary 60 UV-Vis Spectrophotometer (Agilent) in accordance with the Beer–Lambert law. The thickness of the absorbance layer was 1 cm. The absorbance was read against distilled water at wavelength of 670 nm.

The degree of starch hydrolysis (C) was determined by the relative difference of absorbance between the control and sample solutions. The following formula (1) was used for its calculation:

$$C = (A_{st} - A_i)/A_{st} \tag{1}$$

where A_{st}—optical density of control starch, A_i—optical density of i-sample.

The same procedure of starch hydrolysis analysis was used for soluble enzyme form.

2.9. The Optimal pH and Temperature Conditions

The optimal pH of free and immobilized α-amylase was determined by adding the soluble enzyme and 5% biocatalytic membrane samples into 10 mL of substrate solution (1% w/v) with different pH (5.0–8.0, 0.067 M) for 10 min, and the temperature was maintained 30 °C. Buffers were prepared by mixing solutions of 0.067 M sodium phosphate dibasic and 0.067 M potassium phosphate monobasic in volume ratios corresponding to a specific pH (1:99 for pH 5.0, 12:88 for pH 6.0, 61:39 for pH 7.0, and 97:3 for pH 8.0, respectively). In addition, in order to investigate the effect of temperature on free and immobilized enzyme activity, the samples were incubated with starch solution (1% w/v) prepared with 0.067 M phosphate buffer (pH 6.0) at different temperatures (30, 37, 45, 52, and 60 °C) for 10 min. For experiments with free enzyme, the amount of added α-amylase was calculated as the amount bound to the membrane after adsorption in 5% enzyme solution. Then, the normalized to unity value of starch hydrolysis degree by free and immobilized enzyme samples was determined with the highest value of each set being assigned the value of 100% hydrolysis.

Table 1. Microfiltration ceramic membrane technical characteristics.

Ceramic tubular substrate	Pore size	50–100 μm
	Open porosity	30%
	Outer diameter	65 mm
	Length	500 mm
	Wall thickness	4 mm
Microfiltration ceramic membrane	Average pore sizes	1–5 μm
	Microfiltration layer thickness	150–200 μm
	Water permeability	25.0–45.0 m^3 per m^2 h bar

2.10. Determination of Kinetic Parameters

To determine the kinetic parameters, Michaelis–Menten constant (K_m) and maximum reaction velocity (V_{max}), the activity of free and immobilized enzyme was measured at different starch concentrations (2.5, 5, 7.5, 10, and 20 mg mL^{-1}) prepared with 0.067 M phosphate buffer (pH 6.0) and incubated at 30 °C for 10 min. Lineweaver–Burk plots were depicted and kinetic parameters (K_m and V_{max}) were calculated by the following equation:

$$\frac{1}{V} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}} \tag{2}$$

2.11. Reusability

The reusability (number of cycles) of the enzyme-membrane systems obtained via the adsorption and covalent immobilization method was investigated, and for adsorption at various temperatures. For this purpose, 1% (w/v) starch solution was used and enzyme activity in starch hydrolysis reaction was determined after 10 min for adsorption and after 24 h for covalent immobilization. The temperature range was 30–60 °C.

3. Results and Discussion

3.1. Membrane Characteristics

Porous ceramic tubular substrates for microfiltration membranes were obtained by isostatic pressing. Microfiltration ceramic membrane key parameters were presented in **Table 1**.

Ceramic membranes were analyzed using X-ray diffraction (XRD) and their material was determined to be quartz since all the typical peaks belonging to it were registered. The images and XRD pattern of tubular ceramic membranes are shown in Figure 1C.

In this study, a method to obtain enzyme-ceramic membranes via covalent immobilization was developed and compared with the physical adsorption approach. A schematic of enzyme immobilization by both techniques is shown in Figure 2. The macroporous layer of the enzyme membrane plays a supporting role. The microfiltration layer that coats the porous surface performs the filtering function. The active layer is the immobilized enzymes on the microfiltration layer using gelatin and GA as functionalization and activation agents, respectively. It provides catalytic function in the starch hydrolysis reaction.

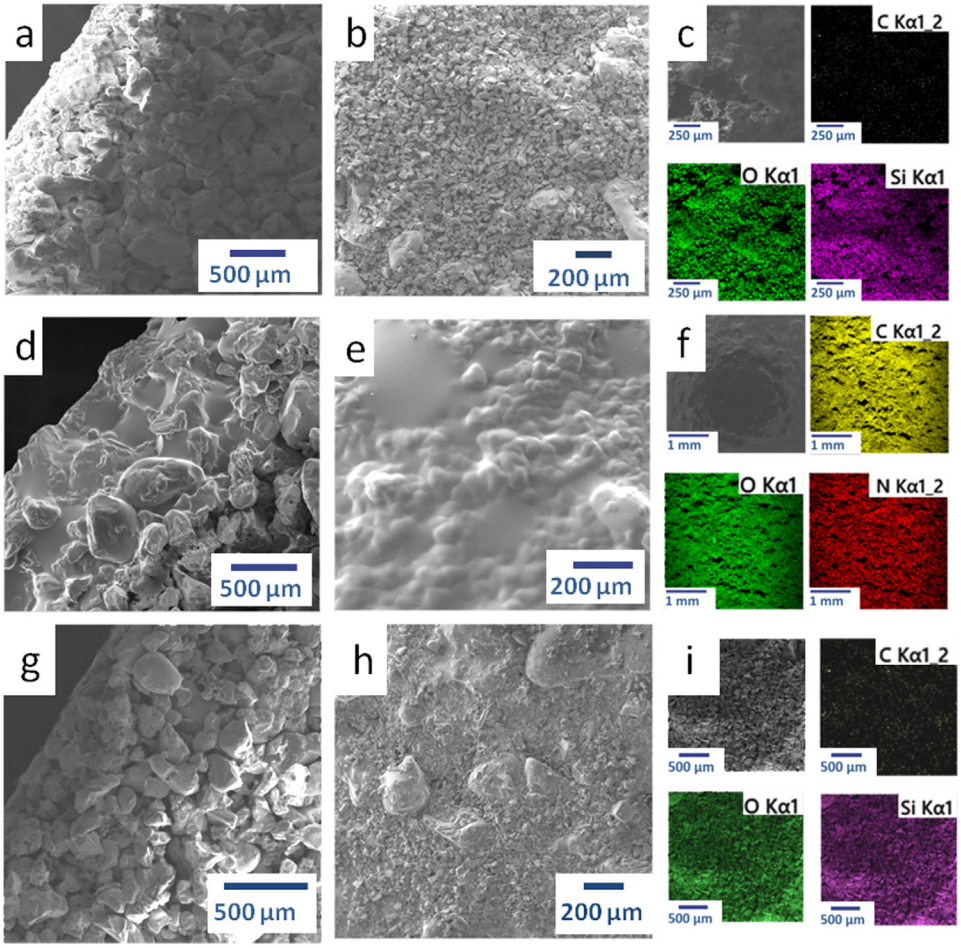

Figure 3. SEM and EDX images of membrane samples: A–C) cross-section, microfiltration surface, and elemental mapping for initial membrane, D–F) for membrane with active layer obtained by covalent bonding, and G–I) membrane with physically adsorbed enzyme. C,F,I) EDX analysis presents the mapping distribution of carbon (C, yellow), oxygen (O, green), silicon (Si, purple), and nitrogen (N, red) atoms on the membrane surface.

In order to accommodate the covalent attachment of the enzymes, the membrane surface needs to be modified. First, it is covered by gelatin that contains free NH_2 groups for the ensuing bond formation. Second, GA, which possesses two carbonyl groups, is used as cross-linker. It acts as a "bridge" between the amino groups of gelatin and the enzymes. The bonds formed during the reaction between the C = O and NH_2 groups of GA and proteins, respectively, are called Schiff bases. As a result, such interactions provide strong attachment of the biocatalyst to the membrane surface. Contrarily, the enzyme immobilization process by physical adsorption is very simple. The biocatalysts are fixed in the pores of the ceramic membrane, but due to the weak bonds, enzyme leakage from the support structure is observed.

3.2. SEM

The images of the initial silica ceramic membranes (A–C) and the carriers after enzyme covalent immobilization (D–F) and physical adsorption (G–I) are shown in **Figure 3**. On the cross-section of the membrane before immobilization (Figure 3A), the porous support and microfiltration layer with hierarchical porosity are observed. After covalent immobilization (Figure 3D–F), the uniform active layer consisting of gelatin, GA, and enzymes is formed on the microfiltration surface. This is also confirmed by EDX mapping.

Elemental analysis showed that the enzyme-free support mainly consists of oxygen (O) and silicon (Si) while, after covalent immobilization, carbon (C) and nitrogen (N) were also present, confirming enzyme immobilization effectiveness.

The influence of adsorbed enzymes on the sample surface was also studied. It was observed that there are no significant changes in the ceramic surface after enzyme immobilization by the physical adsorption method (Figure 3G–I). It may also be noted that the SEM technique does not allow direct observation of the enzymes.

3.3. FTIR Spectroscopy

Using the IR-spectroscopy method, a study of covalent bonding immobilization mechanism through the Schiff base formation during the reaction between carboxyl (C = O) group of GA and

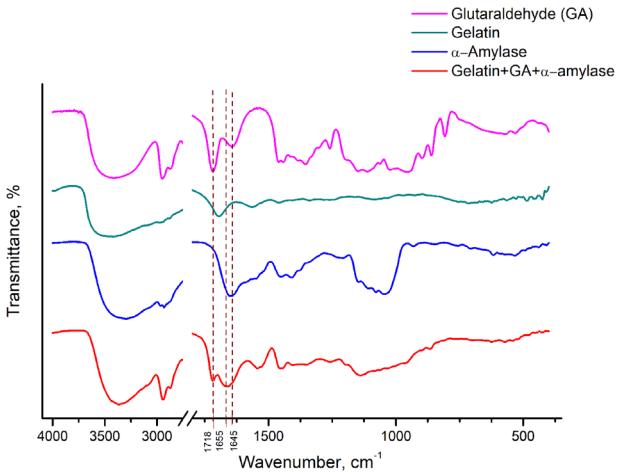

Figure 4. IR-spectra of glutaraldehyde (GA), gelatin, α-amylase, and their mixture.

amino (NH_2) group of proteins was conducted. A schematic of this reaction (3) is presented below:

$$\begin{matrix} R \\ \diagdown \\ H \end{matrix}\!\!\diagup C = O + H_2N - R \xrightarrow[-H_2O]{} \begin{matrix} R \\ \diagdown \\ H \end{matrix}\!\!\diagup C = N - R \qquad (3)$$

The FTIR spectra of GA, gelatin, α-amylase, and their mixture are shown in **Figure 4**. Two characteristic IR peaks for α-amylase were observed in the amide I band (1650 cm^{-1}) and the amide II band (1540 cm^{-1}) due to stretching C = O bonds (amide I) and planar deformation vibrations of the N–H bond (amide II), respectively. The variations in the peaks between 3200 and 3500 cm^{-1} appeared due to the changes in the stretches of the hydroxyl (O-H) and amines (N-H) groups of gelatin and the enzymes. Two sharp peaks of medium intensity near 2941 and 2870 cm^{-1} on the violet and red curves are C-H stretching vibrations of organic compounds. The peak at 1718 cm^{-1}, related to bending vibrations of C = O of carbonyl groups, was present for GA. The peak broadening at 1655 cm^{-1} on the "Gelatin+GA+amylase" curve corresponds to –C = N– bond formation between 1630 and 1690 cm^{-1}.

3.4. Starch Hydrolysis by Immobilized and Free Enzymes

The influence of time and initial α-amylase solution concentration on immobilized and free enzyme activity in starch hydrolysis was investigated. Three experiments for covalent and adsorption method were conducted with 1%, 5%, and 10% (w/w) α-amylase solution. It was found that the degree of starch hydrolysis increases with reaction time, followed by saturation. Each curve was obtained by averaging the three parallel experiments. Here and elsewhere, the error bars represent the standard deviation from the mean. The dependences obtained are shown in **Figure 5**.

The optimal concentration was defined. It was observed that the 1% α-amylase solution, used for enzyme immobilization, showed low activity in starch hydrolysis. Showing a much greater activity for covalent method, the 10% solution had high viscosity, solution inhomogeneity, and tended to sediment, which are the notable disadvantages in terms of work with this concentration.

When α-amylase concentration reached 5%, the adsorbed α-amylase activity almost achieved its highest value (Figure 5B), which indicated that all surface sites were occupied by enzymes through adsorption, and α-amylase activity is unlikely to improve significantly with concentration increase. The 5% α-amylase solution had optimal properties for the both methods; therefore, it was chosen for the following study. For experiments with a free enzyme, where the amount of free α-amylase was calculated as the amount bound to the membrane, the lower activity of soluble α-amylase form at small concentrations could be associated with lower biocatalyst stability.[16] This effect disappeared for a 10% enzyme solution.

3.5. The Optimal pH and Temperature Conditions

As shown in **Figure 6**A, both free and adsorbed α-amylase had the same optimal pH at 6.0, which corresponded with other enzyme

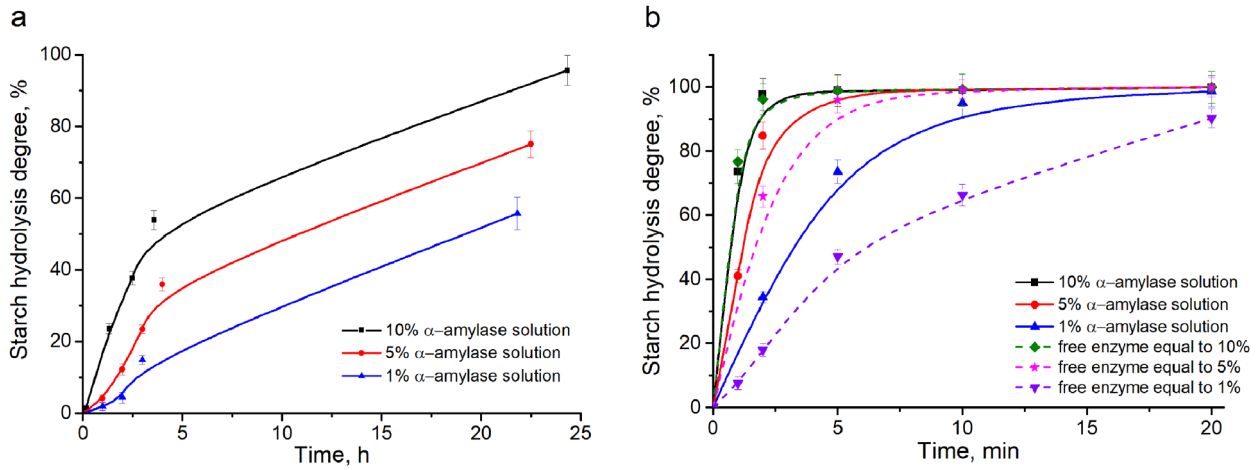

Figure 5. The dependence of starch hydrolysis degree on time and α-amylase solution concentration for: A) covalent immobilization and B) adsorption immobilization and free enzyme. Each point represents the mean of three experiments ± SD.

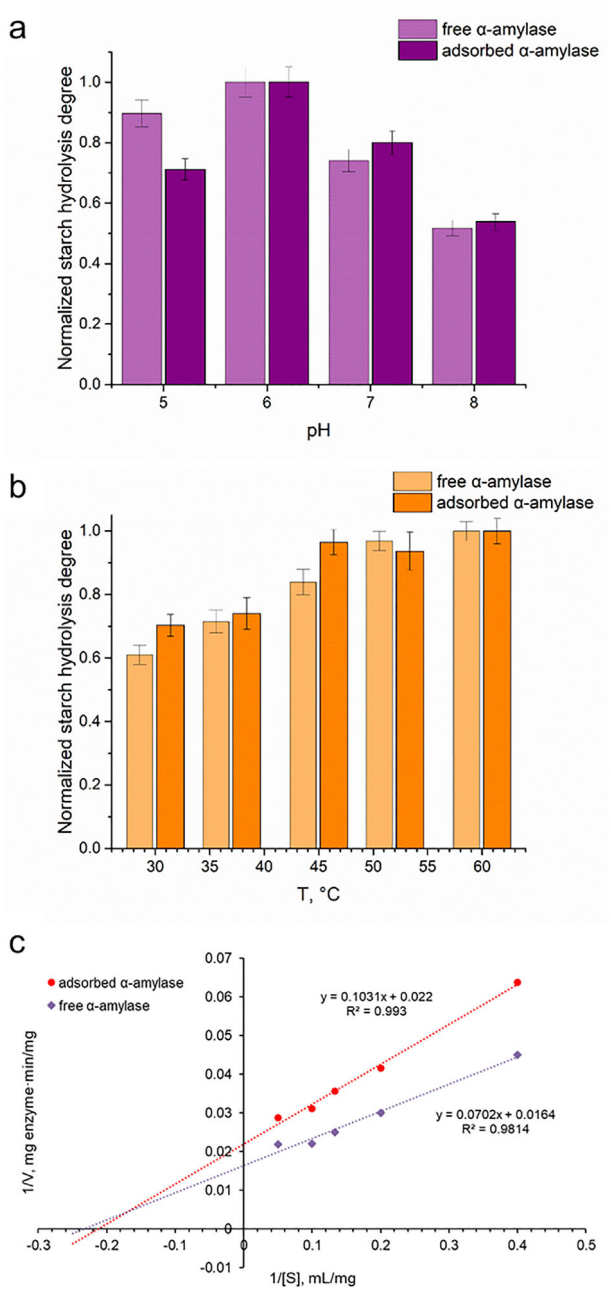

Figure 6. The effect of the A) pH and B) temperature on starch hydrolysis degree for free and physically adsorbed α-amylase. Each point represents the mean of three experiments ± SD. C) Lineweaver–Burk plots of free and physically adsorbed α-amylase.

immobilization research.[2] Although the optimal pH value did not change after immobilization, adsorbed α-amylase exhibited a higher relative starch hydrolysis degree above pH 6.0. This indicated that immobilized enzyme could maintain upper activity in an alkaline pH range compared with free α-amylase. A similar improvement in the enzyme activity was reported with another bacterial α-amylase and might be attributed to the altered configuration under immobilized environment.[4] Extremes in pH can accelerate gelatin degradation in solution, so pH 6.0 is the most appropriate for long-time hydrolysis by covalent systems.[34]

In the temperature range of 30–60 °C, the activity of free and adsorbed enzymes increased with saturation after 45 °C (Figure 6B) due to the thermostable properties of α-amylase. The temperature profile of the immobilized enzyme was slightly broader than that of the free one up to 45 °C. This higher thermal stability of the immobilized enzyme compared to its soluble form may be associated with lower denaturation rates corresponding to adsorption and reduced enzyme flexibility.[35] The optimal reaction temperature for immobilized α-amylase in terms of repeated uses will be discussed below.

3.6. Kinetic Parameters

Lineweaver–Burk plots of free and adsorbed α-amylase are shown in Figure 6C. Affinity of the enzyme for the substrate was indicated through its K_m value. The values of K_m for free and immobilized α-amylase were found to be 4.3 and 4.7 mg mL^{-1}, respectively. This decrease in the affinity is caused by structural changes in the enzyme introduced by the immobilization procedure and lower accessibility of the substrate to the active site of the adsorbed enzyme.[15] The V_{max} value for α-amylase adsorbed on silica membrane were lower than that of the free enzyme (45 and 61 mg_{starch} $(mg_{enzyme}$ $min)^{-1}$, respectively) indicating a decrease in enzyme activity because of immobilization. The decline in V_{max} after immobilization corresponds to the mass transfer limitation of the diffuse layer around the biocatalyst particle.[35] Other researchers have also obtained noticeably larger K_m and lower V_{max} for immobilized α-amylase compared to the free form.[2,15]

However, kinetic parameters of the free and immobilized α-amylase for starch are of the same order of magnitude. This indicates that the catalytic function of α-amylase was not highly suppressed by this immobilization method.

3.7. Reusability of Immobilized Enzyme

An experiment on the reusability of ceramic membranes with the biocatalyst immobilized by covalent bonding was conducted for three replicate samples (**Figure 7**A). Enzyme activity was determined as a mean value after 24 h of use at 30 °C. In each case, the enzymes continued to work, but the activity loss was observed due to conformational changes in enzyme structure during immobilization. A similar study also reported a drop in the residual activity after the second cycle.[4] An increase in temperature leads to inactivation of biocatalytic systems due to thermal instability of the gelatin layer.

To determine the optimum temperature, at which the physically adsorbed enzymes work most effectively with the repeated use, 30, 45, and 60 °C were used. The dependences of the degree of starch hydrolysis on the number of cycles at various temperatures are presented in Figure 7B. It was shown that the optimum temperature is 45 °C,[2,35] at which the enzymes retain their activity for a longer time and have a smoother rate of activity decrease. For all temperatures, enzyme-membrane system is more efficient than single-use free enzymes.

Between the first and second cycles, a significant decrease in starch hydrolysis is observed, since the availability of the enzyme

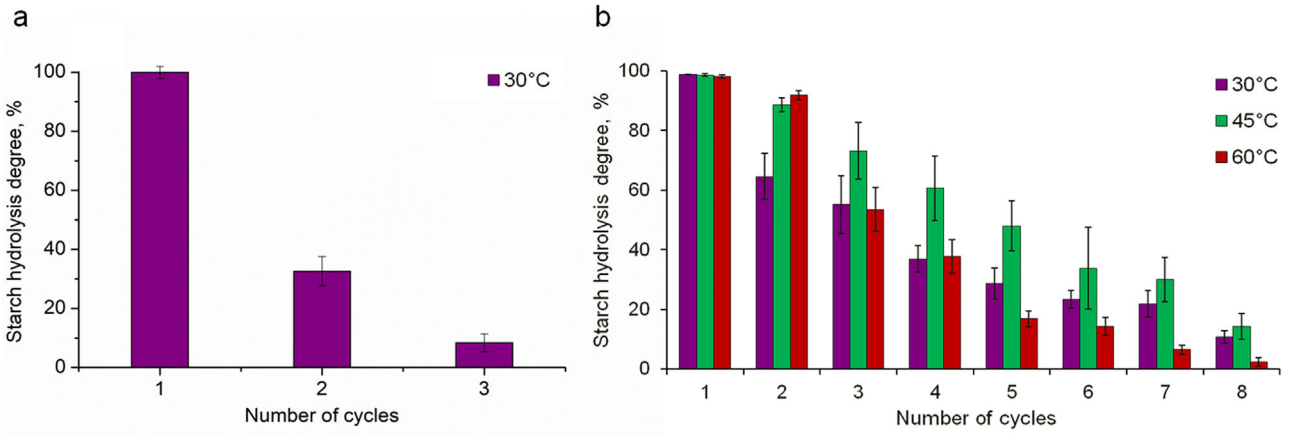

Figure 7. A) The dependence of covalently immobilized α-amylase activity on the number of cycles and B) the dependences of adsorbed α-amylase activity on temperature and number of cycles. Each point represents the mean of three experiments ± SD.

to starch is maximum in the first cycle. Then, due to the filling of the membrane pores with the substrate, the phenomenon of mass transfer limitation begin to appear.[13] In subsequent cycles, the decrease is very negligible, since the effect of the phenomenon becomes permanent. It is also worth noting that in fifth, sixth, and seventh cycles there are quite significant deviations in measurements.

In this work, the regeneration of ceramic membrane samples was conducted at high temperatures. The weight and appearance of the membrane samples before and after immobilization did not change. Therefore, they may be used as carriers multiple times.

4. Conclusion

In this study, silica ceramic membranes were prepared and used as supports for α-amylase immobilization. They can enhance enzyme reusability and reduce wastewater biological pollution making the technological process of starch conversion more effective. The main advantages of the proposed enzymatic ceramic membranes are easy immobilization protocol, the possibility of key product separation from other reagents, and the enzyme recovery from the reaction mixture for the followed repeated use. Furthermore, such supports possess high thermal and mechanical resistance and can be easily regenerated.

On the one hand, the covalent method of immobilization with gelatin as the functionalization agent and GA as the cross-linker generates strong bonds between the ceramic support and the enzymes. On the other hand, the adsorption immobilization method provides minimal enzyme conformational changes and leads to high immobilized molecule activity retention. The correlation of enzyme activity and number of operation cycles were found. The covalent immobilization method allows enzymes to retain their catalytic activity for two cycles with its complete loss by the third one. The loss occurs due to conformational changes caused by the immobilization process and subsequent decrease in biocatalyst mobility. Contrarily, the adsorption method provides α-amylase operational stability for more than eight cycles because of the minimum influence on enzyme molecule structure during attachment to the membrane. This study is promising for the food industry. The use of such heterogeneous catalysts allows to perform enzymatic process continuously and to control the rate of the catalyzed reaction as well as the yield of the product by changing the flow rate. In the future, it is planned to conduct similar experiments on these immobilization methods for various other enzymes and thereby expand the field of application of biocatalytic membrane technologies.

Acknowledgements

E.A.N. is grateful to the Council on grants of the President of the Russian Federation (Project No. MK-1632.2020.11). E.F.K. is grateful to the Ministry of Science and Higher Education of the Russian Federation (Project No. 075-15-2019-1896). The XRD studies were performed using the equipment of the Engineering Center of the St. Petersburg State Institute of Technology (Technical University).

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

adsorption, ceramic membranes, covalent immobilization, enzymes, immobilized α-amylase

Received: January 22, 2021
Revised: July 12, 2021
Published online: September 12, 2021

[1] Y. Zhao, W. Ma, *JSM Biotechnol. Bioeng.* **2018**, *5*, 1086.
[2] T. Mardani, M. S. Khiabani, R. R. Mokarram, H. Hamishehkar, *Int. J. Biol. Macromol.* **2018**, *120*, 354.

[3] B. L. Tee, G. Kaletunç, *Biotechnol. Prog.* **2009**, *25*, 436.
[4] R. J. Shukla, S. P. Singh, *Int. J. Biol. Macromol.* **2016**, *85*, 208.
[5] Y.-K. Liu, P.-M. Lien, *J. Taiwan Inst. Chem. Eng.* **2016**, *62*, 162.
[6] Z. Bayramoglu, U. Akbulut, S. Sungur, *Biomaterials* **1992**, *13*, 704.
[7] N. R. Mohamad, N. H. C. Marzuki, N. A. Buang, F. Huyop, R. A. Wahab, *Biotechnol. Biotechnol. Equip.* **2015**, *29*, 205.
[8] D. Gangadharan, K. Madhavan Nampoothiri, S. Sivaramakrishnan, A. Pandey, *Food Res. Int.* **2009**, *42*, 436.
[9] E. T. Hwang, M. B. Gu, *Eng. Life Sci.* **2013**, *13*, 49.
[10] C. Spahn, S. Minteer, *Recent Pat. Eng.* **2008**, *2*, 195.
[11] N. S. Hettiarachchy, D. J. Feliz, J. S. Edwards, R. Horax, in , *Proteins in Food Processing*, Elsevier, Amsterdam, **2018**, pp. 569–597.
[12] F. Gashtasbi, G. Ahmadian, K. A. Noghabi, *Enzyme Microb. Technol.* **2014**, *64–65*, 17.
[13] S. B. Sigurdardóttir, J. Lehmann, S. Ovtar, J. Grivel, M. D. Negra, A. Kaiser, M. Pinelo, *Adv. Synth. Catal.* **2018**, *360*, 2578.
[14] M. P. Cabrera, T. F. da Fonseca, R. V. B. de Souza, C. R. D. de Assis, J. Quispe Marcatoma, J. da C. Maciel, D. F. M. Neri, F. Soria, L. B. de Carvalho, *Appl. Surf. Sci.* **2018**, *457*, 21.
[15] G. Bayramoğlu, M. Yilmaz, M. Y. Arica, *Food Chem.* **2004**, *84*, 591.
[16] S. B. Jadhav, R. S. Singhal, *Carbohydr. Polym.* **2014**, *105*, 49.
[17] Z. Wu, W. Qi, M. Wang, Y. Wang, R. Su, Z. He, *Biochem. Eng. J.* **2013**, *77*, 190.
[18] S. A. Ansari, R. Satar, S. K. Zaidi, M. I. Naseer, S. Karim, M. H. Alqahtani, M. Rasool, *Food Bioprod. Process* **2015**, *95*, 298.
[19] L. Treccani, T. Y. Klein, F. Meder, K. Pardun, K. Rezwan, *Acta Biomater.* **2013**, *9*, 7115.
[20] S. Mura, G. Greppi, A. M. Roggio, L. Malfatti, P. Innocenzi, *Microporous Mesoporous Mater.* **2011**, *142*, 1.
[21] S. Tanvir, J. Pantigny, P. Boulnois, S. Pulvin, *J. Memb. Sci.* **2009**, *329*, 85.
[22] W. Cheng, Y. Li, X. Li, W. Bai, Y. Liang, *J. Taiwan Inst. Chem. Eng.* **2019**, *104*, 351.
[23] N. Carlsson, H. Gustafsson, C. Thörn, L. Olsson, K. Holmberg, B. Åkerman, *Adv. Colloid Interface Sci.* **2014**, *205*, 339.
[24] L. Słomińska, J. Niedbacha, *Desalination* **2009**, *241*, 296.
[25] R. Abejón, C. L. Gijiu, M. P. Belleville, D. Paolucci-Jeanjean, J. Sanchez-Marcano, *J. Memb. Sci.* **2014**, *473*, 189.
[26] A. Grzeækowiak-Przywecka, L. Slomińska, *Desalination* **2005**, *184*, 105.
[27] A. Grześkowiak-Przywecka, L. Słomińska, *J. Food Eng.* **2007**, *79*, 539.
[28] A. I. Ivanets, A. I. Rat'ko, T. A. Azarova, S. M. Azarov, S. H. Al-Khowaiter, O. Al-Harbi, S. V. Shemchonok, V. A. Dobysh, V. A. Tarasevich, V. E. Agabekov, A. A. Rat'ko, *Ceram. Int.* **2014**, *40*, 12343.
[29] A. I. Ivanets, T. A. Azarova, V. E. Agabekov, S. M. Azarov, C. Batsukh, D. Batsuren, V. G. Prozorovich, A. A. Rat'ko, *Ceram. Int.* **2016**, *42*, 16571.
[30] A. I. Ivanets, V. E. Agabekov, *Pet. Chem.* **2017**, *57*, 117.
[31] S. Ben Ameur, C. Luminiţa Gîjiu, M. P. Belleville, J. Sanchez, D. Paolucci-Jeanjean, *J. Memb. Sci.* **2014**, *455*, 330.
[32] M. M. Bradford, *Anal. Biochem.* **1976**, *72*, 248.
[33] State Standard of the Russian Federation GOST R 54330—2011 Enzyme preparations for food industry. Methods for determination of amylase activity (in Russian), Standartinform, Moscow **2012**, 14 p. Approved: Federal Agency for Technical Regulation and Metrology, 6/15/2011.
[34] K. C. G. Silva, A. I. Bourbon, L. Pastrana, A. C. K. Sato, *Food Funct.* **2020**, *11*, 8331.
[35] S. A. Ahmed, F. A. Mostafa, M. A. Ouis, *Int. J. Biol. Macromol.* **2018**, *112*, 371.