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USE OF NATURAL WASTE CARRIER IN ENZYME IMMOBILIZATION: CATALASE IMMOBILIZATION ONTO EGGSHELL MEMBRANE

Ceyhun IŞIK*, Chemistry Department, Faculty of Science, Muğla Sıtkı Koçman University, Muğla, Türkiye,
cyhn87@yahoo.com
(https://orcid.org/0000-0001-6883-0180)

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*Corresponding author
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Abstract

The immobilization process improves the stability properties of enzymes and reduces production costs in industrial processes due to its reuse feature. In this study, catalase was immobilized onto eggshell membrane (ESM), which is a natural carrier macromolecule, via adsorption and cross-linking methods. Experimental parameters such as enzyme concentration, ESM amount, cross-linking agent concentration and adsorption time were determined. Then, optimization studies were performed for both free catalase and catalase immobilized ESM. It was observed that ESM improved the stability properties of catalase enzyme such as optimum temperature, thermal stability and optimum pH after the immobilization. Especially in alkaline conditions, catalase immobilized ESM showed higher activity than free enzyme. The catalase immobilized ESM was able to maintain more than 50% of its activity even after repeated 25 uses. In addition, characterization studies of ESM and catalase immobilized ESM were performed using Fourier transform infrared (FTIR) spectroscopy and scanning electron microscopy (SEM).

Keywords: Catalase, immobilization, adsorption, cross-linking, eggshell membrane

ENZİM İMMOBİLİZASYONUNDA DOĞAL ATIK TAŞIYICI KULLANIMI: YUMURTA KABUĞU MEMBRANI ÜZERİNE KATALAZ İMMOBİLİZASYONU

Özet

İmmobilizasyon işlemi, enzimlerin stabilite özelliklerini iyileştirir ve yeniden kullanım özelliğinden dolayı endüstriyel işlemlerde üretim maliyetlerini düşürür. Bu çalışmada, katalaz, doğal bir taşıyıcı makromolekül olan yumurta kabuğu membranı üzerine adsorpsiyon ve çapraz bağlama yöntemleri kullanılarak immobilize edilmiştir. Enzim derişimi, yumurta kabuğu membranı miktarı, adsorpsiyon süresi ve çapraz bağlayıcı ajan derişimi gibi deneysel parametreler belirlenmiştir. Daha sonra hem serbest katalaz hem de katalaz immobilize yumurta kabuğu membranı için optimizasyon çalışmaları yapılmıştır. İmmobilizasyon işleminden sonra yumurta kabuğu membranının, katalaz enziminin optimum sıcaklık, termal kararlılık ve optimum pH gibi stabilite özelliklerini iyileştirdiği görülmüştür. Özellikle alkali koşullarda katalaz immobilize yumurta kabuğu membranı, serbest enzime göre daha yüksek aktivite göstermiştir. Katalaz immobilize yumurta kabuğu membranı 25 tekrar kullanımdan sonra bile aktivitesinin %50'sinden fazlasını koruyabilmiştir. Ayrıca, yumurta kabuğu membranı ve katalaz immobilize yumurta kabuğu membranının karakterizasyon çalışmaları için taramalı elektron mikroskobu (SEM) ve fourier dönüşüm kızılötesi (FTIR) spektroskopi analizi de yapılmıştır.

Anahtar Kelimeler: Katalaz, immobilizasyon, adsorpsiyon, çapraz bağlama, yumurta kabuğu membranı

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1. Introduction

Enzymes are biomolecules that specifically and efficiently catalyze the chemical reactions [1]. Enzymes, which are natural biological catalysts, are widely used in many fields such as biosensors, polymerase chain reactions, pharmacy, bioremediation, medicine and food [2,3]. However, because they are soluble, sensitive to temperature and pH changes in the environment, and disposable, the use of enzymes in industrial applications is not economical, which limits the use of enzymes [4,5].

In order to overcome these limitations, enzyme immobilization, which is defined as the attachment of enzymes to a solid, water-insoluble and inert material by chemical or physical forces, has been a promising approach for the last two decades [6,7]. The immobilization process can provide the enzyme with a more suitable microenvironment and improve the specificity and activity of the enzyme, as well as making the enzyme more resistant to temperature and pH changes [8]. In the immobilization process, carriers that

are resistant to microbial factors, mechanically resistant and resistant to chemical attacks are preferred. For this reason, many natural and synthetic polymer support materials have been used in enzyme immobilization studies [9].

Eggshell membrane is one of the natural biopolymers widely used in immobilization studies owing to its unique properties like porous structure, structural strength, permeability, biodegradability, non-toxicity and large surface area [10]. The eggshell membrane consists of lysines, minerals, carbohydrates, collagens, proteoglycans, glucosamines, lipids and other proteins. With the emergence of the properties and content of ESM, this natural biopolymer, which is accepted as a waste material, has become the focus of researchers in many different fields, especially enzyme immobilization, thanks to its amide, carboxyl, hydroxyl, amino and thiol groups [11, 12].

Catalase, which has a protective feature against oxidative stress and a high turnover number, catalyzes the hydrolysis of hydrogen peroxide to molecular oxygen and water [13]. Catalase is a tetramer, and each tetramer consists of a polypeptide chain containing more than 500 amino acids. In addition, the enzyme has four porphyrin heme (iron) groups that allow catalase to hydrolyze hydrogen peroxide [14]. This enzyme is found in all organisms and is therefore an important enzyme used in many fields, especially in paper production, textile, food processing, medicine, pharmacy and wastewater treatment [15].

Immobilized catalase is a biological catalyst used to remove hydrogen peroxide wastes in biomedical and biosensor applications. In addition, it is also used in the dairy industry to decompose the hydrogen peroxide released during the cold pasteurization process of milk [16]. It has been reported that catalase enzyme has been immobilized on particles, cryogels, gels, polymeric nanostructures, nanoparticles, nanocomposites and nanofibers in the literature [17-21].

In this study, catalase was immobilized on ESM using adsorption and cross-linking methods. Household eggshell waste was used as an ESM source. It was observed that the enzymatic properties of catalase such as kinetic parameters, thermal stability, optimum temperature, pH stability and optimum pH were improved after immobilization. In addition, the results obtained from reusability experiments showed that catalase immobilized ESM is a new, economical, easily applicable, advantageous and reusable method for industrial applications.

2. Experimental

2.1. Materials

Coomassie Brillant Blue G-250). Catalase (bovine liver, 3390 U/mg), H_2O_2, hydrochloric acid, glutaraldehyde (GA), bovine serum albumin (BSA) and all other chemicals were obtained from Sigma-Aldrich. The eggs were supplied from the local market in Muğla.

2.2. Preparing the ESM for the immobilization

The egg was splinted and white parts of the egg and the yolk was removed from the shell. The shell of the egg was washed many times with water and it was kept in distilled water for one night. The ESM was peeled from the shell by hand. The ESM was held in 0.5 M HCl for 6 hours to remove remaining protein and shell residues. ESM was placed in 0.1 M NaOH solution and then washed with distilled water. Then it was completely dried at room temperature and made ready for the immobilization process [12].

2.3. Immobilization of catalase on ESM

Catalase was immobilized onto ESM surface via adsorption and cross-linking methods. Firstly, the known quantity of ESM (5, 7.5, 10, 12.5 and 15 mg) was added to 1 ml enzyme solution (0.5, 0.75, 1.0, 1.25, 1.5 and 2.0 mg/ml) and mixed at 25 °C during the fixed times (5, 10, 15, 20, 25 and 30 min). Then, different concentrations of glutaraldehyde (1, 2, 3, 4 and 5% v/v) were added to cross-link the enzyme molecules to the ESM and mixed for 15 minutes. ESM was removed from the solution and washed many times with distilled water.

2.4. Determination of protein amount

The Bradford method at 595 nm was used to determine the amount of protein present in the medium before and after immobilization [22].

2.5. Determination of catalase activity

In order to determine the activity of free catalase and catalase immobilized ESM, measurements were taken at 240 nm by spectrophotometric method [23]. 2.5 ml of 50 mM hydrogen peroxide (pH 7.0, 50 mM in phosphate buffer) was added onto 20 µl of enzyme solution and kept for 2 min at 25 °C. Then, 500 µl of 1 M HCl was added to the medium to stop the enzymatic reaction and the absorbance was measured spectrophotometrically at 240 nm. The degradation of 1 mmol of H_2O_2 in one minute at 25 °C and pH 7 was defined as one unit of catalase activity. The specific activity was calculated as U/mg protein by dividing the calculated activity by the amount of protein in the medium.

2.6. Characterization of enzymatic properties of catalase immobilized ESM

2.6.1. Determination of optimum temperature and optimum pH

In order to find the optimum temperature values of free catalase and immobilized catalase, activity determination was made at temperatures varying between 20-60 °C.

In order to find the optimum pH values of free catalase and immobilized catalase, activity determination was made using substrate solutions prepared with buffer solutions at different pH values (pH 3-10).

2.6.2. Thermal stability and pH stability

Free and immobilized catalase were incubated at temperatures ranging from 4-70 °C for 1 h to determine their thermal stability properties.

Free catalase and catalase immobilized ESM were incubated in buffer solutions at different pH values (pH 4-9) for 1 h and pH stability properties were determined by enzyme activity determination.

2.6.3. Kinetic parameters

In order to compare the kinetic properties of free catalase and catalase immobilized ESM, enzyme activity was determined using different concentrations of H_2O_2 (2.5-50 mM). Based on the Michaelis-Menten equation, kinetic parameters (V_{max} and K_m) were calculated using Lineweaver-Burk plots.

2.6.4. Reusability

Catalase activity determination was performed 26 times to find the number of reuses of the catalase immobilized ESM. At the end of each activity analysis, just before adding 1 M HCI to the reaction medium, the immobilized ESM was removed and washed with distilled water. In this way, the loss of activity that may occur in the immobilized enzyme was prevented by the addition of HCl. Then, the activity was determined again by adding fresh substrate to the medium.

2.7. Characterization of the surface morphology and chemical interaction of ESM before and after immobilization process

ATR-FTIR (Thermo Scientific Nicolet iS-5ATR/FTIR spectrometer) was used to determine the interactions between the groups on the surface of the ESM and the catalase molecules after immobilization. Scanning electron microscope (JOEL JSM 7600F) was used to determine the changes in the surface morphology of the ESM after immobilization.

3. Result and Discussion

3.1. Optimization of immobilization conditions of catalase

In order to immobilize catalase to ESM, which is a natural carrier membrane, first the adsorption method and then the cross-linking method were used. The amount of enzyme, amount of carrier, amount of crosslinker and adsorption time were investigated as optimization parameters of immobilization.

Enzyme solutions containing different amounts (0.5-1.5 mg/ml) of catalase were prepared and the optimum enzyme amount was found with the help of activity assay. The optimum amount of catalase for catalase immobilization was found to be 1 mg/ml. The reason for the decrease in the activity at enzyme amounts above 1 mg/ml may be the insufficient amount of carrier in the medium. Catalase was immobilized on p(HEMA-CTS)-Cu nanospheres and the optimum catalase concentration was determined as 3 mg/ml [24].

The amount of carrier suitable for enzyme immobilization was determined as 10 mg. In ESM amounts below 10 mg, there may not be enough carrier groups to immobilize the catalase enzyme molecules. In amounts above this value, excess ESM in the medium may have acted as a steric barrier during the substrate reaching the enzyme. In a previous study, the optimum amount of carrier was determined as 0.1 mg [24]. In the other study, lipase (from *Acinetobacter haemolyticus*) was immobilized on ESM and optimal amount of ESM was determined as 7.5 mg [12].

Immobilization was performed for 5-30 min and the optimum adsorption time was determined. After 20 min, it was observed that the carrier surface reached saturation with catalase molecules. Enzyme molecules may have been desorbed from the ESM surface at times above the optimum adsorption time. Catalase was immobilized on Fe_3O_4 particles and $Fe_2O_3NiO_2.H_2O$ particles, and it was reported that these carriers reached saturation after 10 and 15 min, respectively [25].

The amount of cross-linking agent, which is another optimization parameter, was determined by using the GA solution at concentrations ranging from 1% to 5%. The optimum amount of crosslinking agent was found to be 4%. The cross-linking agent in the medium in amounts below this value may be inadequate for catalase molecules to be immobilized on the carrier. At amounts above the optimum value, the GA enzyme molecules may be denatured.

The activity properties for free and immobilized catalase were given in Table 1. When the specific activity of free catalase was 14 U/mg protein and its protein content was 1.3 mg, these values were found to be 9.03 U/mg protein and 0.62 mg respectively for immobilized catalase. After the immobilization process, approximately 47.7% of the proteins in the medium were immobilized and the specific activity efficiency was calculated as 64.5%.

Table 1. Activity properties for free and immobilized catalase.

	Activity (U)	Protein (mg)	Specific activity (U/mg)	Bound protein (%)	Specific activity yield (%)
Free catalase	18.2	1.3	14	-	100
Immobilized catalase	5.6	0.62	9.03	47.7	64.5

3.2. Stability properties for catalase immobilized ESM

3.2.1. Temperature properties

In order to find and compare the optimum temperature of free and immobilized catalase, activity determination was made at different temperatures (20-70 °C). The optimum temperature for free catalase was found to be 30 °C, and this value was found to be 35 °C for catalase immobilized ESM (Figure 1). The optimum pH increase observed after immobilization may be due to amino acids rich in -OH groups in the ESM structure. It has been

reported in the literature that carriers rich in -OH groups protect enzymes at temperature increases [26].

Free and immobilized catalase were incubated at different temperatures (4, 20, 30, 35, 40, 45, 50, 60, 70 °C) for 1 h, and then their thermal stability properties were found by the activity assay (Figure 2). It was observed that catalase immobilized ESM was less affected by temperature changes and more stable than free catalase at all temperatures. After 1 h at 60 °C, free catalase retained only 30% activity, while catalase immobilized ESM was able to retain approximately 65% of its activity. The high thermal stability of the catalase immobilized ESM after the immobilization process can be explained by the fact that the groups on the ESM surface protect the enzyme molecules against the heat caused by the temperature increase. It has been reported that thermal stability properties generally improve after enzyme immobilization [27].

3.2.2. pH properties

Since enzymes are in protein structure, they are affected by pH changes of the environment. In addition, the effect of changing the pH of the environment on the activity varies depending on the groups on the carrier surface. In order to determine the effect of pH on the activity of free catalase and catalase immobilized ESM, activity determination was performed using buffer solutions at different pH values. The optimum pH value was found to be 7.0 for free catalase and 7.5 for immobilized catalase (Figure 3). In addition, catalase immobilized ESM showed higher enzymatic activity at all pH values. Therefore, it can be said that the multi-point coordinations formed between the catalase molecules and the groups on the carrier after immobilization increase the stability of the immobilized catalase. After enzyme immobilization, there are studies in which an increase in the optimum pH value was observed [27, 28]. While Kaushal et al. found the optimum pH 7.5 for free catalase, this value was found to be 8 for immobilized catalase [14].

pH stability results of free catalase and catalase immobilized ESM were given in Figure 4. It was noticed that pH stability properties increased after immobilization. Free catalase showed 30% activity, while catalase retained 55% of the immobilized ESM activity at pH 4. When the free enzyme maintained 60% of its activity at pH 9, the catalase immobilized ESM retained almost 90% of its activity under the same conditions. This can be explained by secondary interactions such as hydrogen bonds, polar and ionic interactions between the enzyme molecules and the carrier.

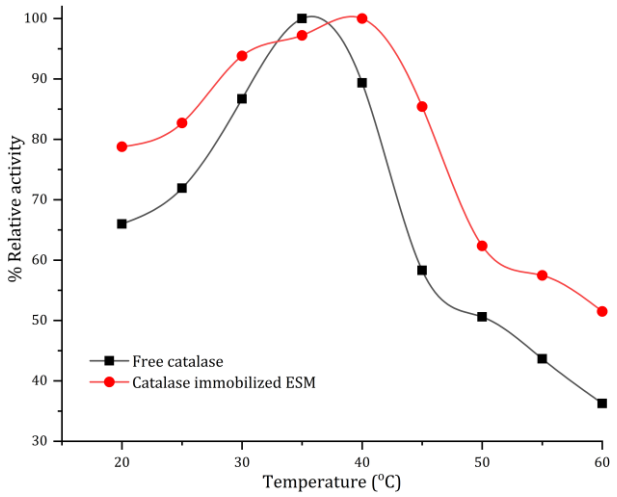

Figure 1. The optimum temperature of free catalase and catalase immobilized ESM.

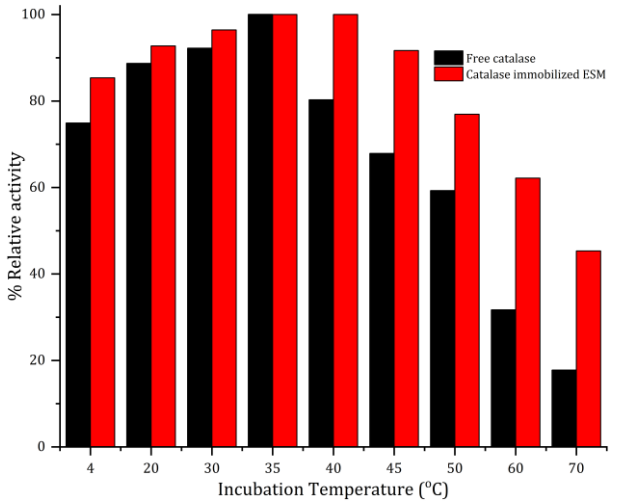

Figure 2. The thermal stabilities of free catalase and catalase immobilized ESM.

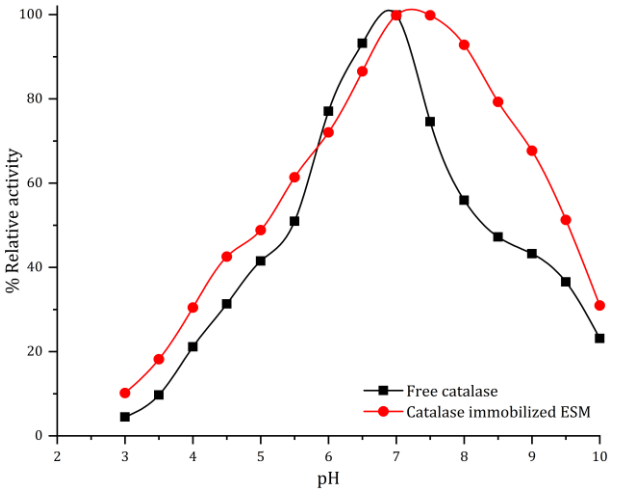

Figure 3. The optimum pH of free catalase and catalase immobilized ESM.

3.2.3. Kinetic parameters

Different concentrations of H_2O_2 were used as substrate to determine the kinetic parameters of free and immobilized catalase. The V_{max} and K_m values were found 2.09 x 10^{-4} µmole H_2O_2 x min^{-1} and 14.2 mM for free catalase and 1.25 x 10^{-4} µmole H_2O_2 x min^{-1} and 15.4 mM for catalase immobilized ESM, respectively. A slight increase in Km was observed after the immobilization procedure. This can be interpreted as the substrate molecules encountering some steric barriers in reaching the active site of the enzyme after immobilization. There are similar studies in the literature in which the K_m value increased and the V_{max} value decreased after immobilization [12, 29].

3.2.4. Reusability

One of the most important advantages of immobilization for industrial applications is its reuse. The reusability performance of catalase immobilized ESM was given in Figure 5. The catalase immobilized ESM maintained approximately 70% of its activity after 20 reuses while it lost nearly 50% of its activity after 25 reuses. Erol et al. reported that catalase immobilized poly(HEMA-GMA) cryogel lost 69% of their activity after 15 reuses [29]. In the other study, catalase p(HEMA-CTS)-Cu was immobilized on nanospheres and it was reported that 80% of the activity was preserved after 5 reuses and roughly 50% of the activity was lost after 12 reuses [24]. Lipase from *Acinetobacter haemolyticus* was immobilized on the ESM and it was observed that the activity decreased below 50% after 18 reuses [12].

3.3. Morphological analysis

SEM images of ESM at different magnifications were given in Figure 6. As seen in Figure 6, it was observed that the ESM had a porous structure rather than a fibrous structure and there were gaps between the pores. After the immobilization of catalase on the ESM by adsorption and cross-linking, it was observed that the spaces between the pores of the ESM were filled with enzyme molecules and the surface of the ESM was covered with enzyme molecules (Figure 7).

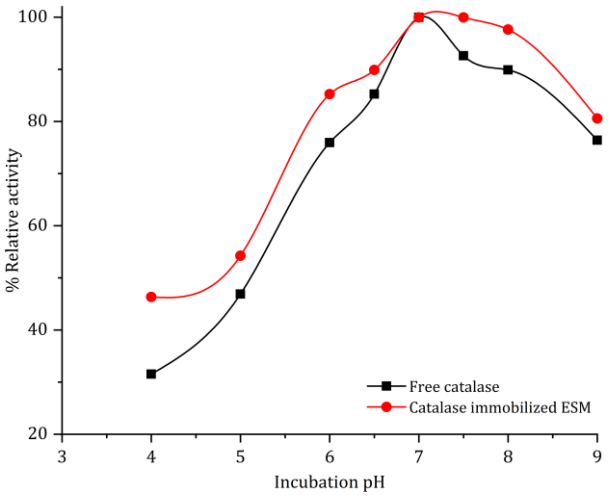

Figure 4. The stabilities of free catalase and catalase immobilized ESM.

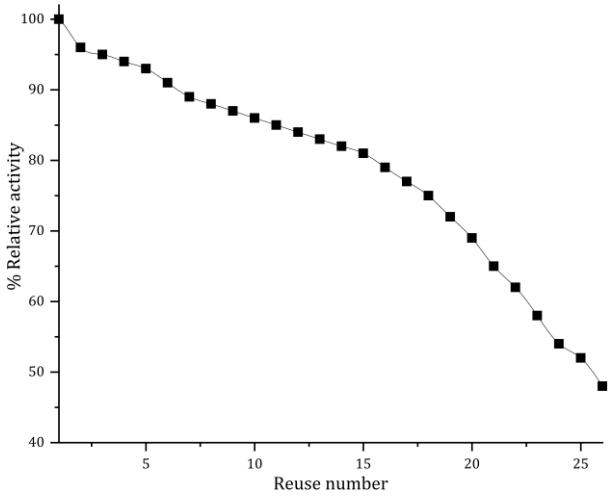

Figure 5. The reuse number of catalase immobilized ESM

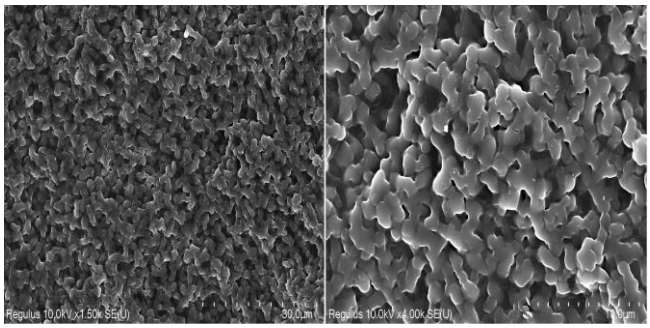

Figure 6. SEM images of ESM.

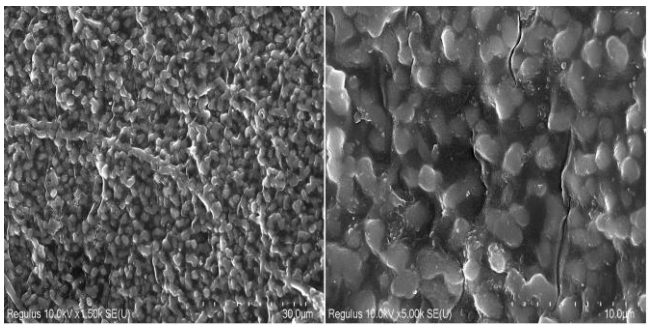

Figure 7. SEM images of catalase immobilized ESM.

3.4. ATR-FTIR

After immobilization, ATR-FTIR analysis of ESM and catalase immobilized ESM was performed to determine interactions between groups and enzyme molecules on the ESM surface. As seen in Figure 8, the stretching band belonging to -OH and -NH groups was seen in the spectrum of ESM at 3280 cm^{-1}, originating from the peptides found in the structure of the ESM [30, 31]. In the =CH and $=CH_2$ groups, bands at 3060, 2925 and 2860 cm^{-1} were seen due to the asymmetric stretching of the CH bonds [12]. Corresponding bands of amide I (C=O), amide II (N-H) and amide III (C-N) vibrations in the ESM structure were seen at 1638, 1527 and 1230 cm^{-1}, respectively [32]. Bands observed due to stretching of C=C, C–O and C-S bonds were seen at 1150, 1075 and 610 cm^{-1}, respectively [33]. After immobilization of catalase on the ESM by adsorption and crosslinking, no significant changes were observed when the ESM was compared

with the ATR-FTIR spectrum. Decreases in the intensity of the peaks of the ESM and shifts in the bands were observed. It can be said that after the immobilization process, the catalase molecules are attached to the ESM surface by non-covalent interactions and adsorption.

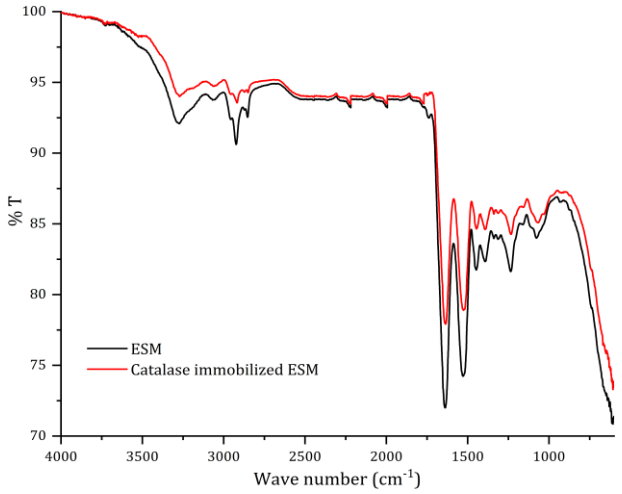

Figure 8. ATR-FIR spectrum of ESM and catalase immobilized ESM.

4. Conclusion

Catalase was successfully immobilized to ESM using adsorption and crosslinking methods. After immobilization, morphological and chemical interactions occurring on the ESM surface were determined using SEM and ATR-FTIR. ESM provided catalase molecules with large surface area and enhanced the stability properties of catalase such as thermal stability, pH stability, optimum pH, optimum temperature and reuse. After 1 h at 60°C, catalase immobilized ESM preserved 60% of its activity, free catalase lost 70% its activity at the same time. In addition, catalase immobilized ESM showed better enzyme activity than free catalase in alkaline conditions. The catalase immobilized ESM maintained more than 50% of its activity after 25 reuses. ESM, which is a natural carrier, offers a large surface area for catalase immobilization and improves the stability properties of the enzyme and for these reasons, it can be said that catalase immobilized ESM can be used for H_2O_2 removal in food, textile and biomedical applications.

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