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Immobilization of Catalase onto Polylysine Modified Chitosan Polymer

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Abstract

In this study, chitosan polymer was modified in two different ways and used for the immobilization of bovine liver catalase (CAT). First, it was activated with glutaraldehyde (GAL), and then covalently immobilized covalently onto the support via lysine amino acid residue in CAT. In the second modification, GAL-activated chitosan was interacted with polylysine (pLYS), then reactivated with GAL and used in CAT immobilization. Samples of bare chitosan (Chi), GAL-activated (Chi_{GAL}), polylysine-modified (Chi_{GAL}-pLYS), reactivated with GAL (Chi_{GAL}-$pLYS_{GAL}$) and CAT-immobilized chitosan (Chi_{GAL} -CAT and Chi_{GAL}-$pLYS_{GAL}$-CAT) were all characterized by FTIR. The enzymatic activities of Free CAT, Chi_{GAL} -CAT and Chi_{GAL}-$pLYS_{GAL}$-CAT samples were investigated at different pH and temperatures and the values with the highest activity were determined. In addition, the effect of substrate concentration on activity under optimal conditions was investigated. Optimum pH values of Free CAT, Chi_{GAL}-CAT and Chi_{GAL}-$pLYS_{GAL}$-CAT samples were 7.5, 7.0 and 7.0, respectively; temperature values were determined as 25, 30 and 35° C, respectively. After immobilization, the Vmax values of the enzymes decreased, the Km values increased, and the efficiency of catalase immobilized to the polylysine modified support was found to be higher. It was observed that after 20 repeatedly use in the column reactor, Chi_{GAL}-CAT and Chi_{GAL}-$pLYS_{GAL}$-CAT enzymes retained 88 % and 76 % of their initial activities, respectively.

Keywords:
Catalase, chitosan, polylysine, immobilization
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Introduction

Catalase, (H_2O_2: H_2O_2 oxidoreductase E.C.1.11.1.6), is an enzyme that is very common in nature and breaks down hydrogen peroxide into water and molecular oxygen (Figure 1). CAT, which is found in almost all aerobic microorganisms, plant and animal cells, is rarely encountered in anaerobes. The enzymes involved in oxidation and reduction reactions are called oxidoreductases,

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and CAT is in the class of hydroperoxidases, a group of oxidoreductases. Hydroperoxidases use hydrogen peroxide or other organic peroxides as substrates, but also protect the living thing against harmful peroxides. The accumulation of peroxides causes the emergence of free radicals and disruption of the membrane structure, atherosclerosis and possibly cancer formation (Ozyilmaz et al., 2007; Yıldırım, 2010).

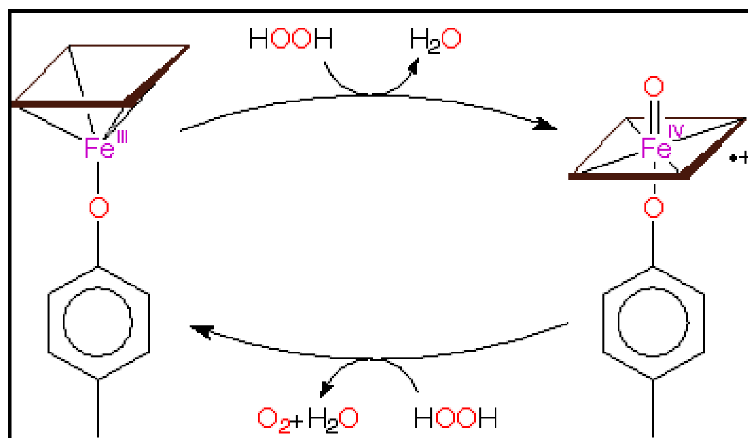

Figure 1. CAT activity.

Since catalase is an enzyme that breaks down H_2O_2, it can be used in all processes where H_2O_2 is used and the excess must be removed from the environment (Basak, 2011).

In the dairy industry, H_2O_2 is used to preserve via lactoperoxidase activity, which is naturally found in milk and needs H_2O_2 to show activity. However, excess H_2O_2 in milk must be removed before processing. This happens with the use of free or immobilized CAT. Thus, while excess H_2O_2 is broken down, milk enzymes and beneficial bacteria are preserved (Seriner, 2010). When it is desired to remove the H_2O_2 released in the reaction systems in which oxidases are involved, CAT enzyme is also used in addition to these enzymes. For example, while using glucose oxidase, which is used in canning and packaging of foods, desaccharification of some foods such as eggs and wine, and gluconic acid production, CAT should also be present in the environment. H_2O_2 can be used for oxidation and foaming in resin and plastic production or for sterilization in the food industry. Excess H_2O_2 can be broken down by treatment with CAT (Seriner, 2010).

Enzymes are extremely expensive biocatalysts because they are produced by living things. In addition, they cannot be removed from the reaction medium as they show activity in aqueous medium; for this reason, there are also disadvantages such as not being able to control the reaction rate and creating pollution in the environment. The immobilization of enzymes can offer several advantages, including reuse, ease of application of both batch and continuous systems, better control of reactions, ease of removal from the reaction medium, and improved stability (Eberhardt et al., 2004; Ozyilmaz et al., 2005).

There are a several carriers used for the immobilization of enzymes such as florisil, alumina, silica gel, bentonite, polymeric materials, hydrogel, chitosan etc. Chitosan is a poly-N-

acetylglucosamine-based cellulosic carbohydrate polymer obtained by deacetylation of chitin and its monomer is D-glucosamine (Figure 2) (Shentu et al., 2005). Chitin is found in the shells of arthropods such as crabs, shrimps, and lobsters, and in the cell walls of some bacteria and fungi (Çetinus et al., 2007; Collins et al., 2011). Due to its non-toxicity and bioactive properties, chitosan is also frequently used in the pharmaceutical industry and medical applications.

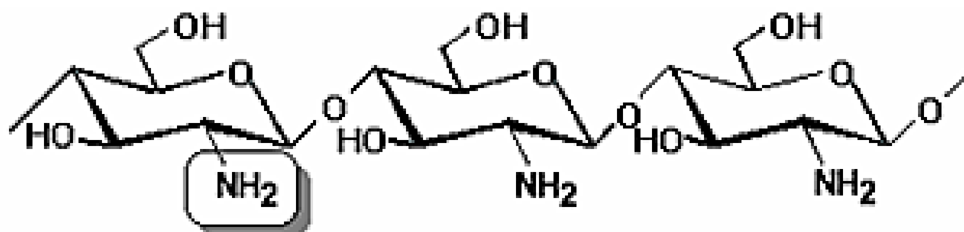

Figure 2. Chemical structure of Chitosan.

In this study, it was aimed to perform covalent immobilization and characterization of CAT enzyme onto chitosan and also onto polylysine modified chitosan support. The optimal pH and temperature values of the immobilized enzyme were compared with the free enzyme by determining its kinetic parameters; The reuse stability of immobilized enzymes was investigated. The fact that there are many industrial applications of the CAT enzyme, especially the immobilized form, has been an important point in the realization of this study.

Materials and Method

Bovine liver catalase, chitosan, glutaraldehyde, polyliysine were obtained from Sigma. All other chemicals used were at analytical grade.

Immobilization of CAT

Enzyme immobilization was carried out covalently by means of a Schiff base, which is easily formed between the free aldehyde groups to be formed on the support and the amine groups of the lysine amino acid residue within the enzyme protein (Tukel & Alptekin, 2004; Ozyilmaz et al., 2007). Chitosan support was used in the immobilization of the CAT enzyme by being modified in two different ways. In the first method, the chitosan support was interacted with glutaraldehyde (GAL), a dialdehyde, and the support was activated with the formation of a schiff base (Chi_{GAL}). Afterwards, CAT was covalently immobilized to the Chi_{GAL} surface by forming a schiff base (Figure 3). In the second method, the Chi_{GAL} support surface was modified through the formation of schiff base by interacting with polylysine (Chi_{GAL}-pLYS), and the obtained support was activated again by retreatment with GAL (Chi_{GAL}-$pLYS_{GAL}$). Thus, the enzyme was covalently immobilized onto the Chi_{GAL}-$pLYS_{GAL}$ support to obtain Chi_{GAL}-$pLYS_{GAL}$-CAT (Figure 3).

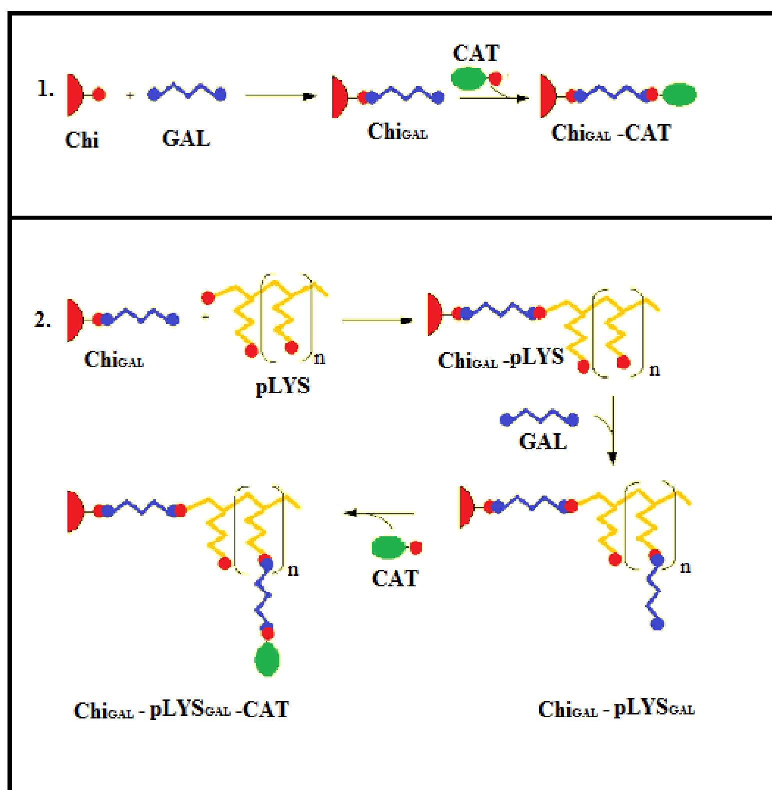

Figure 3. Schematic representation of CAT immobilization steps.

To immobilize CAT, one gram of activated carrier (Chi_{GAL} or Chi_{GAL}-$pLYS_{GAL}$) was mixed with 10 ml of CAT solution prepared at 2mg/ml concentration and at pH 7.0. Immobilization mixture was shaken gently during 2 hour of immobilization time and then washed with immobilization buffer solution until no protein was detected in the washing solution. The volume of the combined buffer and wash was measured and the amount of recovered protein determined. The amount of protein bound to the carrier was estimated from the difference between the amount of added and recovered protein by the method of Lowry. The amount of the bound protein was given as mg protein. g $carrier^{-1}$.

Activity of free and Immobilized CAT

Free and immobilized CAT activities were determined according to previous study spectrophotometrically by measuring the decrease in absorbance of H_2O_2 in the reaction medium (Ozyilmaz et al., 2007). Free or immobilized CAT enzymes were reacted with 10 mM 2.5 ml H_2O_2 solution for 2 minutes and the reaction was stopped by adding 0.5 ml 1 M HCl. The absorbances of the enzyme containing and enzyme-free solutions were measured at 240 nm, and the enzyme activity was calculated as U/mg protein based on the amount of decomposed H_2O_2.

Characterization of Free and Immobilized CAT

FTIR analysis of pure Chi, Chi from different stages, and CAT-bound supports was performed using Perkin Elmer spectrum 65 model with universal ATR. In order to determine the optimum pH

values of free and immobilized CAT enzymes, activities were measured at different pH values between pH 5.0 and 8.0 at 25°C without changing other operating parameters. The temperature-dependent activity profiles of free and immobilized enzymes were determined by changing the temperature at their optimum pH values. Kinetic parameters of enzymes were calculated by Lineweaver-Burk plot using activity values depending on substrate concentration. Finally, operational stabilities of immobilized CAT samples were investigated by 20 repeated uses of the same immobilized enzymes in the column reactor. To this, 100 mg of immobilized CAT sample (Chi_{GAL}-CAT or Chi_{GAL}-$pLYS_{GAL}$-CAT) was loaded into column reactor and 5 ml of H2O2 solution was added. After 2 minutes, the reaction mixture was removed from the column reactor and substrate was added, allowing the enzyme to react again. In this way, the H_2O_2 degradation rate of the enzyme, which was used 20 times, was determined and the remaining activity after each use was calculated as % of initial activity.

Results and Discussion

CAT was immobilized onto modified chitosan support and the bound enzyme was determined as 17.8 mg/g support and 9.2 mg/g support for Chi_{GAL}-CAT or Chi_{GAL}-$pLYS_{GAL}$-CAT. FTIR spectra of bare Chi**,** Chi_{GAL}, Chi_{GAL}-$pLYS_{GAL}$**,** Chi_{GAL}-CAT and Chi_{GAL}-$pLYS_{GAL}$-CAT were given in Figure 4. Since there are groups such as -OH, -NH_2, -C-C-, -C-O-C- in both the support and the enzyme, the spectra are similar to each other. The strong band seen in the 1085-1050 cm^{-1} band in all samples in the spectrum indicates the stretching of the primary alcohol group of chitosan. In addition, the broad peak seen in the 3300-3400 cm^{-1} band is caused by the primary amine and intramolecular alcohol group in the structure of the support. According to a study by Silvana et al. (2012), it was suggested that the band at 1410 cm^{-1}, which was not observed in the spectrum obtained for bare Chi, was caused by the Schiff base bond between chitosan and glutaraldehyde. The band at 1410 cm^{-1} is present in all samples except Chi.

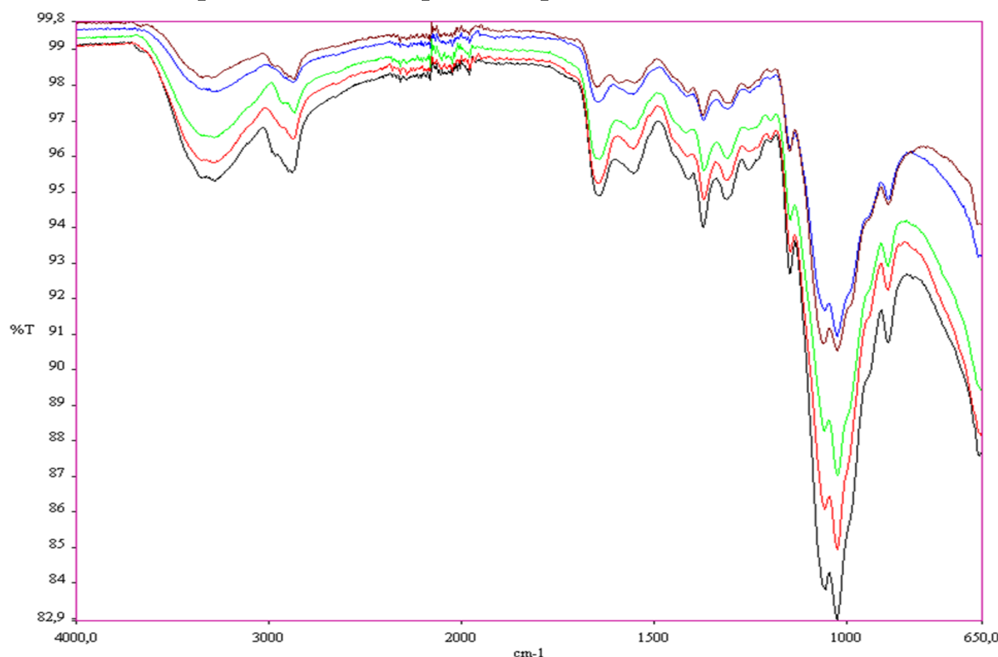

Figure 4. FTIR spectra of Chi (−), Chi_{GAL} (−), Chi_{GAL}-$pLYS_{GAL}$ (−), Chi_{GAL}-CAT (−), Chi_{GAL}-$pLYS_{GAL}$-CAT (−).

Figure 5 shows the effect of the reaction pH on the activities of free CAT, Chi_{GAL}-CAT and Chi_{GAL}-$pLYS_{GAL}$-CAT. As seen, the highest activity of free CAT was determined as pH 7.5, while immobilized CAT enzymes showed maximal activities at pH 7.0. It was revelaed that, the optimal pH of both immobilized CAT shifted to slightly acidic region than that of free enzyme. Chitosan support used to immobilize CAT and it was found that both free enzyme and immobilized enzyme showed the highest activity at pH 7.0 (Çetinus et al., 2007; Arabacı & Usluoğlu, 2012).

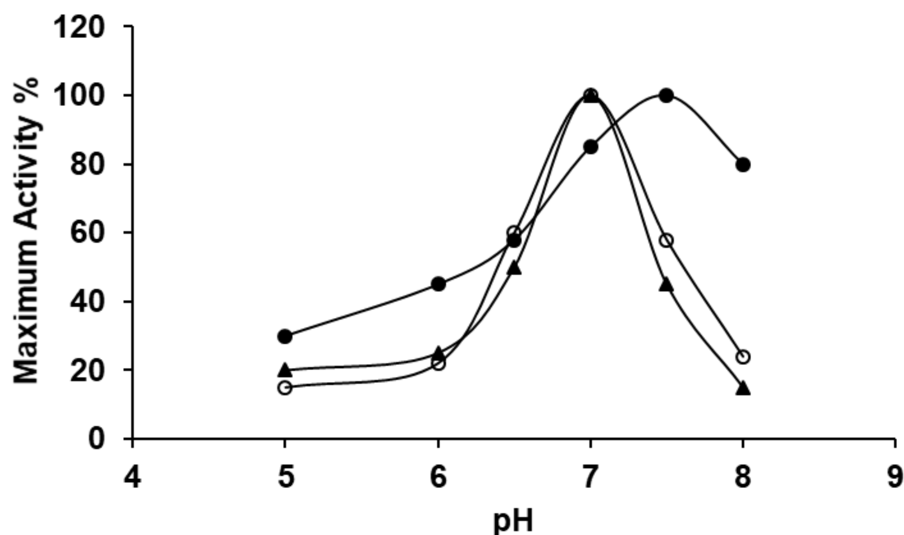

Figure 5. The effect of pH on the catalase activity, free CAT (●), Chi_{GAL}-CAT (○) and Chi_{GAL}-$pLYS_{GAL}$-CAT (▲).

The shift of the optimum pH value of immobilized enzymes to a more alkaline region compared to the free enzyme can be explained by the basic character of the microenvironment of the enzyme due to the amine groups in the chitosan carrier and polylysine.

The activity-temperature profiles of CAT samples are given in the Figure 6. It was observed from Figure 6, free CAT, Chi_{GAL}-CAT and Chi_{GAL}-$pLYS_{GAL}$-CAT showed the highest activity at 25, 30 and 35 °C, respectively. As can be seen, the immobilized enzymes showed the highest activities at higher temperature than the free enzyme. In the previous study, both free and immobilized CAT onto florisil showed the highest activity at 30°C (Ozyilmaz et al., 2007). Similarly, Arabacı & Usluoğlu (2012) covalently immobilized the CAT enzyme to Chi and found the optimum temperature values as 30 and 35 °C for free and immobilized CAT, respectively. Alptekin et al. (2010) covalently immobilized CAT to Eupegit C and reported the optimum temperature for free and immobilized CAT as 40 and 25 °C, respectively.

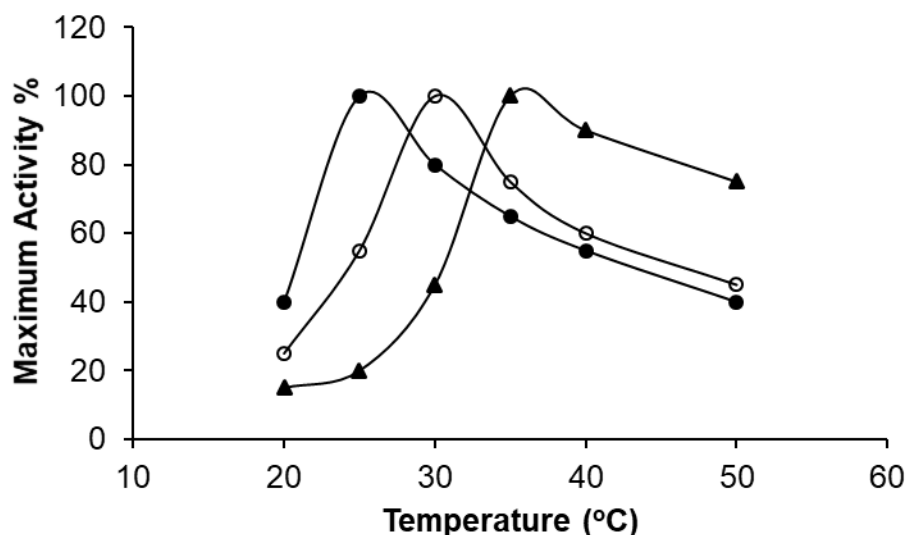

Figure 6. The effect of temperature on the catalase activity, free CAT (●), Chi_{GAL}-CAT (○) and Chi_{GAL}-pLYS$_{GAL}$-CAT (▲).

By determining the activity values depending on the substrate concentration, the kinetic parameters of the free and immobilized cat samples were calculated from the lineweaver-burk curve (Table 1). In general, immobilization is a process that reduces the catalytic activities of enzymes. The Km values of both immobilized enzymes were higher than the value of the free enzyme while the Vmax values were lower. There are many studies in the literature reporting that CAT activity is greatly reduced after immobilization (Ozyilmaz et al., 2007; Çorman et al., 2010; Alptekin et al., 2010; Bayramoglu et al., 2016; Barreca et al., 2018). In this study, Vmax/Km values, which are an indicator of the catalytic efficiency after immobilization, were determined as 28 % and 39.2 % of the free enzyme for Chi_{GAL}-CAT and Chi_{GAL}-pLYS$_{GAL}$-CAT, respectively. It is a generally observed situation that the optimum temperature values of immobilized enzymes shift to higher temperatures than the free enzyme (Bayramoglu et al., 2011; Arabaci & Usluoglu 2012; Bayramoglu et al., 2016).

Table 1. Kinetic parameters of CAT samples

CAT samples	Vmax (U/mg CAT)	Km (mM)	Vmax/Km (Efficiency)	%
Free CAT	170000	33	5105	-
Chi_{GAL}-CAT	50000	35	1429	28.0
Chi_{GAL}-pLYS$_{GAL}$-CAT	100000	50	2000	39.2

Reusability is one of the most important advantages of immobilized enzymes. Therefore, in this study, the operational stabilities of the immobilized forms of CAT, which is an important enzyme for industrial use, were investigated using a column reactor and results were given in

Figure 7. It was determined that Chi_{GAL}-CAT and Chi_{GAL}-pLYS$_{GAL}$-CAT enzymes continued to preserve 88% and 76% of their initial activities, respectively, after 20 consecutive uses.

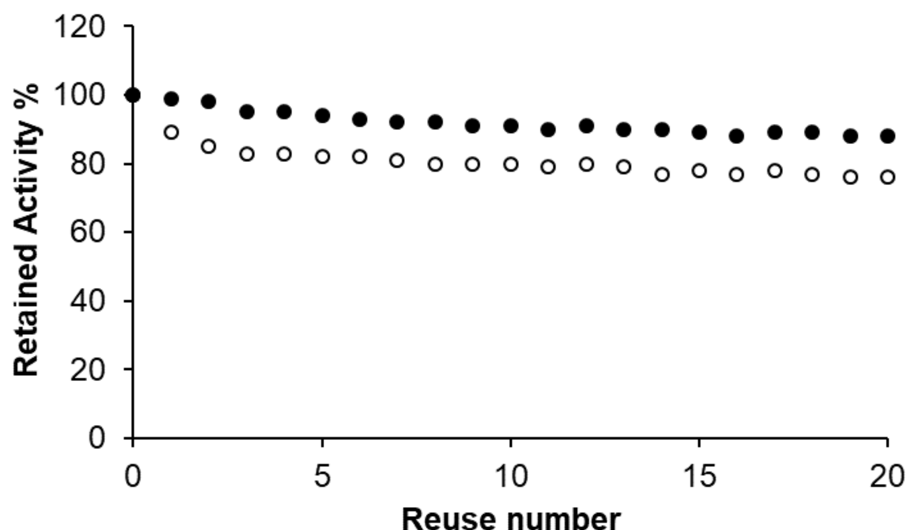

Figure 7. The operational stabilities of, Chi_{GAL}-CAT (●) and Chi_{GAL}-pLYS$_{GAL}$-CAT(○).

Reuse stability is a frequently investigated property of immobilized enzymes. Sel et al. (2021) immobilized the CAT enzyme to P (MMA-co-PEG500MA) gel and reported that it retained 21% of the initial activity after 20 uses. Bayramoglu et al. (2016) found that after 10 uses of catalase immobilized on silica-based support, there was a 23% decrease in activity compared to first activity value. Arabacı & Usluoğlu (2012) covalently immobilized catalase onto chitosan carrier via glutaraldehyde and determined that it preserved 70% of its initial activity after 10 uses. In our study, ChiGAL-CAT and ChiGAL-pLYSGAL-CAT enzymes showed 91% and 80% of their initial activities, respectively, after 10 uses. When compared with the literature studies, it was revealed that the reuse stability of ChiGAL-CAT and ChiGAL-pLYSGAL-CAT enzymes were higher.

The CAT enzyme was successfully immobilized to the Chi carrier by glutaraldehyde directly (Chi_{GAL}-CAT) and via polylysine spacer arm (Chi_{GAL}-pLYS$_{GAL}$-CAT). Catalytic efficiency of Chi_{GAL}-pLYS$_{GAL}$-CAT enzyme was higher, while Chi_{GAL}-CAT showed higher reuse stability. In this study, an effective method to get immobilized CAT with high industrial applicability has been demonstrated. The immobilized CAT forms prepared in this study can be used effectively in situations where the excess of H_2O_2 used needs to be broken down, such as in the dairy and textile industries.

Author Contributions

All work of the article, data analysis and preparation of the article was carried out by Gul Ozyilmaz.

Conflict of Interest

The author declares that she has no competing interests.

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