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CHARACTERIZATION OF FREE AND IMMOBILIZED CATALASE PURIFIED FROM *CONVOLVULUS ARVENSIS* L.

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Abstract

A novel catalase named CaCAT was extracted from non-commercially plant, *Convolvulus arvensis*. The enzyme was purified to homogeneity and biochemical properties of free enzyme were improved by immobilization. Free and immobilized catalases may be having a potential role in biotechnological processes. Fold and yield of catalase obtained by final step of purification were 14.6 and 23.41 % respectively. The immobilized CaCAT displayed 88 % from activity of free enzyme. The molecular weight determined by Sephacryl S-200 was ~ 230 kDa. Free and immobilized CaCAT showed 100% of activity at pH 7 and 25 °C with a greater activity for immobilized enzyme at other values. Respectively, the free and immobilized CaCAT were exposed ~ 0-42 and ~ 33-72 % from their original activity when treated with inhibitors. Fe^{+2} ion was enhancing the activity of both enzymes unlike other ions with a higher resistance of immobilized catalase. Storage at 4 °C the free CaCAT has no activity for 30 days meanwhile the immobilized was retained about 76% of its activity. At 25 °C of the storage period for 15 days free CaCAT has 0 % of activity while immobilized lost only ~ 29 %. Immobilized CaCAT recovers more than 75 % of the activity at eight times of reusing. K_m and V_{max} free and immobilized were 15.3, 250 and 21.73 mM, 212 mM min^{-1} respectively. Thus, *Convolvulus arvensis* could be used as an inexpensive and safe source of catalase for immobilization by entrapment or other alternative methods to produce enzyme can works at wide pH and temperature in addition be able to resists inhibitors and heavy metal ions.

Key words: Catalase, CaCAT, *Convolvulus arvensis*, Purification, DEAE cellulose, Sephacryl S-200, Immobilization, Characterization.

Introduction

The great progress in the use of biocatalysts in medical and industrial fields opens doors to find cheap and safe enzymatic resources from non-commercially plants, one of these enzymes is catalase. Catalase (hydrogen-peroxide:hydrogen-peroxide oxidoredectase, EC 1.11.1.6, CAT) is the hydrogen peroxide decomposer to water or to water and oxygen when acts as peroxidase and hyperoxidase respectively (Luhová *et al.*, 2003). Generally, it is tetrameric manganese or iron containing, monofunctional or bifunctional enzyme present in the plants, animals and all aerobic microorganisms (Klotz *et al*., 1997; Wu *et al.*, 2004). In the plants main functions of catalase involve reduce excessive concentration of toxic hydrogen peroxide to protect the cells against deleterious influence of oxidative stress, metabolism, pathogenesis-related responses and signal perception (Mhamdi *et al.*, 2010). Localization of catalase in the different cytoplasmic organelles of plant parts were reviewed by Anjum *et al.*, (2016) included the peroxisomes, glyoxysomes, unspecialized peroxisomes and mitochondria. Liberate of catalases extracellularly from cytoplasm of the cells of leaves, fruits, seeds and roots require optimum conditions for extraction and powerful purification techniques such as precipitation by ammonium sulfate or acetone, ion exchange and gel filtration chromatography. Separation of catalases from others contaminated proteins are routinely used for evaluate degree of purity and the yield. Plants are adopted to extract and purify of their catalases by these techniques reviewed by Arabaci and Usluoglu (2013) involved greening pumpkin cotyledons, tobacco leaves, sunflower cotyledons, cotton seeds, loblolly pine seeds, van apple, parsley leaves, dill and black gram seeds. However, wide applications of catalases in the brewing, dairy, textile and wastewater treatment (Aehle, 2007) required immobilization of the free purified enzymes for improve their properties like stability to wide range of the pH and temperature, inhibitors and metal ions resistance. In

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addition, the immobilization contributes to lowering of cost, storing for long period, allows for continuous processes and reusing (Kennedy *et al*., 1990). Thereby, our work focused on enhancement of the features of the free purified catalase from *Convolvulus arvensis* by alginate immobilization. New free and immobilized enzymes perhaps useful in medical or industrial applications.

Materials and Methods

Chemicals

Sodium phosphate monobasic, Sodium phosphate dibasic Citric acid, Sodium citrate, Tris (hydroxymethyl) aminomethane, Hydrochloric acid, Sodium hydroxide, PVPP, Bovine serum albumin, Ammonium sulfate, EDTA, Hydrogen peroxide, Sodium alginate, Calcium chloride, Ferritin, Aldolase, Lysozyme, Potassium cyanide and Ferric, Copper, Nickle, Mercuric, Zinc chlorides were from BDH. Diethylaminomethyl cellulose. Sephacryl S-200, Blue dextran 2000, Sodium azide were from Pharmacia.

Plant Samples

Fresh and healthy leaves, bended stems and capsules (fruits with its seeds) of wild *Convolvulus arvensis* (bindweed) were obtained from cultivated area in Al Talee'a town, Babylon province, Iraq. The plant parts were sampled from July to September 2017. All samples were washed three times in tap water then rinsed with distilled water. At room temperature the collected parts were dried then stored in plastic dark container at -20°C for next experimental steps.

Methods

Extraction

In blender frozen selected samples (20g) were homogenized with 100 ml of 0.05 mM sodium phosphate buffer pH 7 containing 0.3 g PVPP for 5 min at 4°C. The homogenate was filtrated through three layers of cheesecloth. The crude CaCAT (supernatant) was isolated after the cool centrifugation of the filtrate at 10000 rpm for 30 min.

Purification

Each supernatant solution was fractionated separately with different weights of solid $(NH_4)_2SO_4$ at 4°C to obtain 20, 30, 40, 50, 60, 70 and 80% saturation. Saturated mixture was centrifugated at 10000 rpm for 30 min then resulting sediment was dissolved in 5 ml of 0.05 mM sodium phosphate buffer pH 7 and dialyzed overnight at 4°C against three changes of the same buffer. The active dialyzed solution of 30% sediment loaded directly onto 2×20 cm DEAE cellulose ion exchanger column was previously equilibrated in same buffer of dialysis. Rate of flow of the washing buffer was adjusted approximately at 20 ml h^{-1}. Fractions of unbounded proteins (2 ml) were collected and tested for H_2O_2 degradation until absorbance at 280 was less than 0.02. CaCAT was eluted as in washing manner except buffer contained liner gradient (0-1 M) of NaCl. Active fractions of CaCAT were pooled and applied onto 1.5×60 cm Sephacryl S-200 gel column was pre-equilibrated in 0.2 M sodium phosphate buffer pH 7 for remove trace charge of gel. Rate of flow and collect of fractions for H_2O_2 degradation were regulated as in ion exchanger column.

Table 1: Purification of CaCAT from *Convolvulus arvensis*.

Step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Degree of purity	Yield (%)
Crude leaves extract	18.7	4327.2	231.4	1	100
30 % $(NH_4)_2SO_4$	12	3180.3	265.02	1.14	73.49
DEAE cellulose	0.84	2349.76	2797.33	12.08	54.3
Sephacryl S-200	0.3	1013.1	3377	14.6	23.41

Immobilization

Two ml of free purified CaCAT was mixed with autoclaved of 0.18 M sodium alginate solution. Syringe with 3 mm needle was used to transfer the mixture drop by drop to Petri dish contained 0.09 M of $CaCl_2$. Resulting $CaCl_2$ beads were washed many times with $CaCl_2$ for remove un-immobilized CaCAT then stored at 4°C.

Catalase and Protein Assay

Activity of free enzyme was estimated spectrophotometrically at 240 nm and 25°C. CaCAT solution (0.02 ml) was added to 0.98 ml of 0.01 M H_2O_2 in 0.05 M sodium phosphate buffer pH 7. The decrease in absorbance of H_2O_2 in 1 ml quartz cuvettes was recorded during 2-5 min. One unit is defined as the amount of catalase inducing the degradation of 1 ìmol of H_2O_2 per minute calculated from the [(43.6 M^{-1} $cm^{-1)}$ for H_2O_2 at 240 nm (Aebi, 1984). Same conditions of free enzyme were prepared for immobilized CaCAT activity with some modifications involved add 50 mg of calcium alginate beads containing enzyme to 10 ml of H_2O_2 in buffer and elimination of beads from reaction mixture. The protein concentration in CaCAT solution was accomplished according to traditional Bradford method (Bradford 1976). BSA solution was used for built up of standard curve.

Characterization of free and immobilized CaCAT

Molecular Weight

Molecular weight of free CaCAT was calculated by using of gel column of Sephacryl S-200 was prepared

as above. The void volume (V_o) of the column was determined with blue dextran. The elution volume (V_e) of enzyme and marker proteins (Ferritin, Aldolase, BSA and Lysozyme) were also estimated. Log of molecular weight of marker proteins plotted against V_e/V_o to exhibit molecular weight of the free purified CaCAT.

Optimal pH and temperature

Different buffer systems were prepared to obtain pH range (3-9) for detect effect of pH on activity of free and immobilized CaCAT at 25°C. At optimal pH value, influence of temperature ranging from 10-80°C was carried out on both enzymes to investigate optimal temperature of activity.

Inhibitors and Metal Ions Resistance

Free and immobilized CaCAT were incubated independently with buffer containing 5 mM of EDTA, NaN_3, KCN, metal ions of Fe^{2+}, Cu^{2+}, Ni^{2+}, Hg^{2+} or Zn^{2+} for 30 min at 25°C then remaining activity was measured.

Stability of Storage and Recycling

Remaining activity of free and immobilized CaCAT stored in 0.05 M sodium phosphate buffer pH 7 at 4 and 25°C for 0-50 days was estimated to appearing the storage stability. Number of reusing for immobilized enzyme was assayed at optimal conditions by determine of relative activity.

Calculation of K_m and V_{max}

According to relationship between V^{-1} versus $[S]^{-1}$ of Lineweaver-Burk plot (Lineweaver and Burk 1934), K_m and V_{max} of the free and immobilized CaCAT were calculated at different concentrations (5, 10, 15, 20, 25, 30, 35 and 40 mM) of H_2O_2.

Results and Discussion

Extraction and purification of CaCAT

The crudes of CaCAT leaves have specific activity 232.63 U mg^{-1} were purified to homogeneity while other crudes of capsules and stems that have less activity were ignored (Data not shown). At the final procedure of purification was reported in Table 1. The specific activity for H_2O_2 degradation, degree of purity and yield of free purified CaCAT were 3377 U mg^{-1}, 14.6 and 23.41 % receptively.

Immobilization

The calcium alginate bounded CaCAT was retained

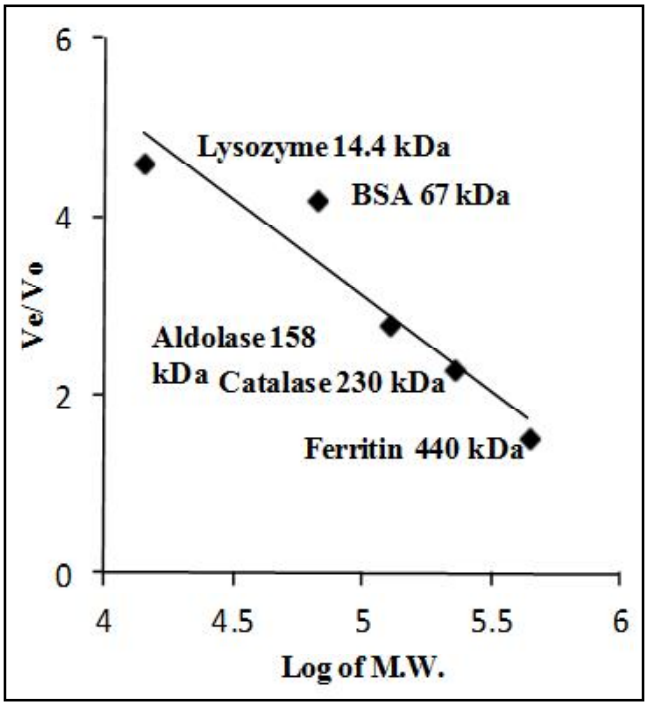

Fig. 1: Molecular weight of the free CaCAT (4 230 kDa) obtained by Sephacryl S-200 column. The void volume (V_o) of the column and elution volume (V_e) were determined with commercially blue dextran and standard proteins as described in chemicals: Ferritin (440 kDa), Aldolase (158 kDa), (BSA 67 kDa) and Lysozyme (14.4 kDa). Rate of flow was approximately at 20 ml h^{-1}.

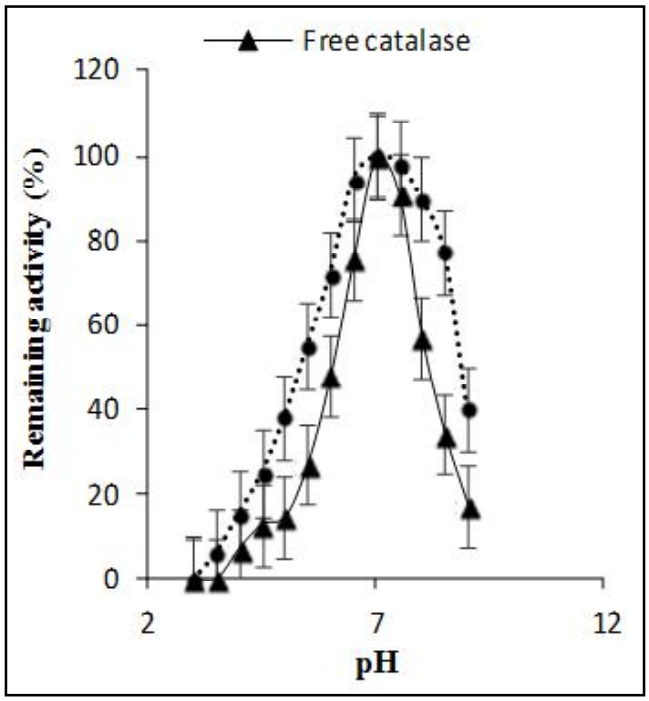

Fig. 2: Optima of pH for CaCATs activity. Optimal activity of the free and immobilized CaCAT were showed at pH values in the range of 3 - 9 using hydrogen peroxide as a substrate for 2-5 min at 25 °C. The following buffer systems were used; 0.05 M sodium citrate (pH 3 to 6), sodium phosphate (pH 6.5 to 8.0), Tris–HCl (pH 8.5 to 9.0). The optimal activity at pH 7 of both enzymes were 101.2 and 89.05 U ml^{-1} respectively. Remaining activity enzymes were detected by defining optimal activity as 100 %. The data are average of three independent experiments.

Table 2: Inhibitors and metal ions resistance of the free and immobilized CaCAT.

Inhibitors or Metals	Conc.	*Remaining activity (%)	
		Free	Immobilized
None	-	100	100
EDTA	5 mM	42.02 ± 0.01	72 ± 0.57
NaN_3	5 mM	0	33.12 ± 1.26
KCN	5 mM	0	40.46 ± 0.32
Fe^{2+}	5 mM	103.02 ± 0.6	103.7 ± 0.85
Cu^{2+}	5 mM	55.42 ± 0.87	72.42 ± 1
Ni^{2+}	5 mM	42.04 ± 1.15	63.57 ± 0.55
Hg^{2+}	5 mM	21.03 ± 0.96	32.36 ± 1.2
Zn^{2+}	5 mM	66.15 ± 0.8	75.25 ± 1.67

*Remaining activity of the free and immobilized CaCAT were measured by defining original activity as 100 % (101.2 and 89.05 U ml^{-1}). The data are average of three independent experiments.

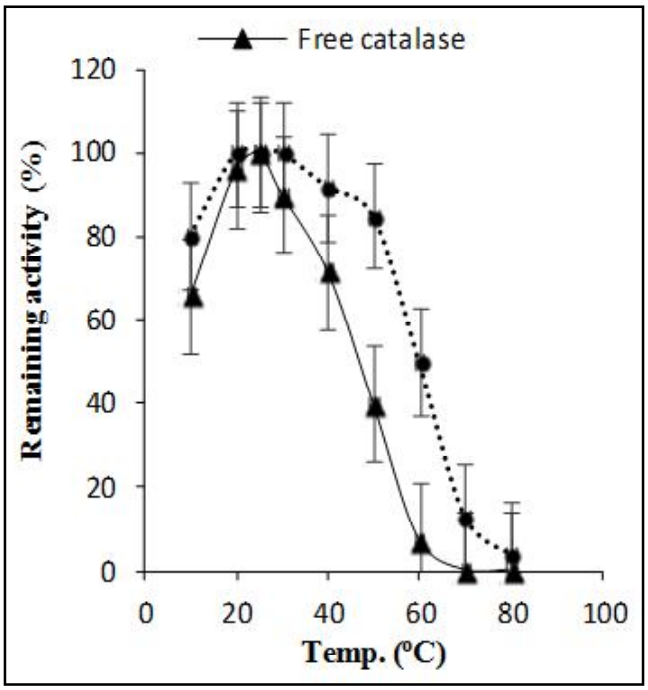

Fig. 3: Optima of temperature for CaCATs activity. Optimal activity of the free and immobilized CaCAT were showed at temperatures ranging from 10 to 80°C using hydrogen peroxide as a substrate for 2-5 min at pH 7. The optimal temperatures for catalases activity at 25°C were 101.2 89.05 U ml^{-1} respectively. Remaining activity of the enzymes were recorded by defining optimal activity as 100%. The data are average of three independent experiments.

about 88% of its pre-entrapped activity. Relative high activity of the CaCAT was observed post-entrapped due to the advantages of entrapping method for enzyme immobilization. Probably CaCAT structure was not subjected to hazard alterations, and immobilization protects the catalase against the influence of proteases

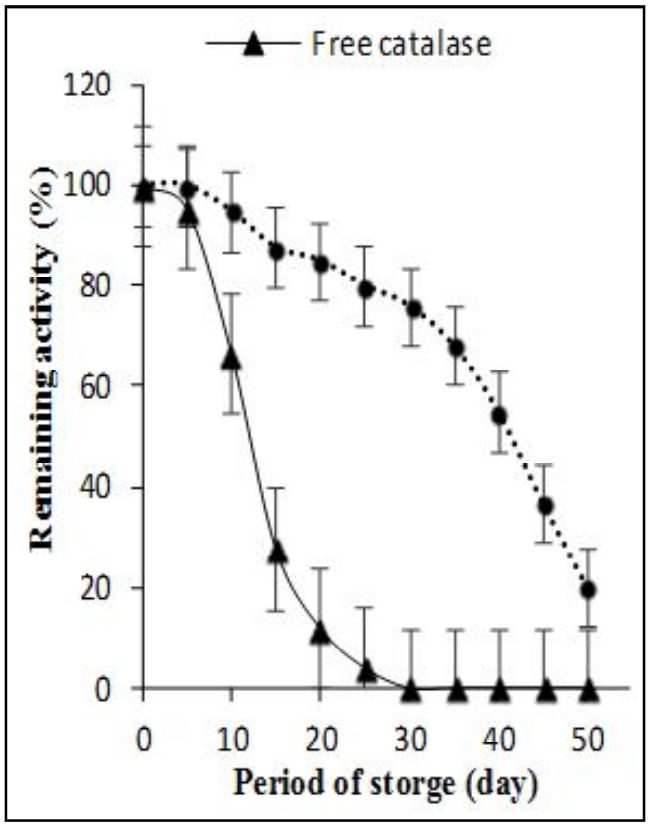

Fig. 4: Stability of storage of the free and immobilized CaCAT at 4°C. Enzymes were stored in 0.05 M sodium phosphate buffer pH 7 for 0-50 days. The original activity of two CaCATs were 101.2 and 89.05 U ml^{-1} respectively. Remaining activity of both catalases were recorded by defining original activity as 100%. The data are average of three independent experiments.

and biocatalyst inhibitors of high molecular weight. In addition, powerful electrostatic attachments between negatively charged groups of sodium alginate gel and positively charged amino acids of the CaCAT (Aehle, 2007; Guzik *et al.*, 2014).

Characterization of Free and Immobilized CaCAT

Molecular Weight

The molecular weight of free CaCAT was calculated by Sephacryl S-200 gel to be approximately 230 kDa (Fig. 1). Therefore, its near to molecular weight of whole tetrameric catalase purified from *Beta vulgavis var. cicla* (Dinçler and Aydemir, 2001), and unlike 160 kDa active dimeric or three subunits of catalase with molecular weights of 79, 74 and 62 kDa extracted from *Mesembryanthemum crystallinum* leaves (Niewiadomska and Miszalski, 2008). However, the molecular weights of the holoenzymes from different organisms fall between 220-270 kDa having large (> 75 kDa) or small (< 60 kDa) subunits.

Optimal pH and Temperature

Maximal activity of both CaCATs were indicated at

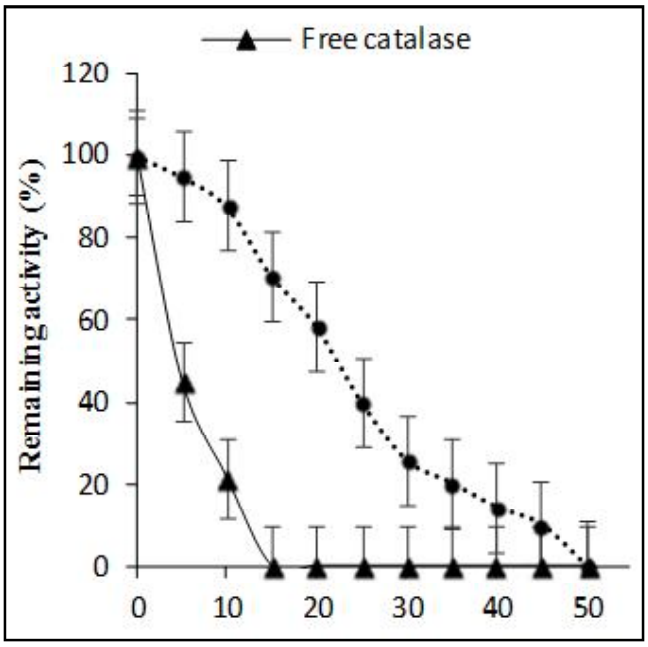

Fig. 5: Stability of storage of the free and immobilized CaCAT 25°C. Enzymes were stored in 0.05 M sodium phosphate buffer pH 7 for 0-50 days. The original activity of two CaCATs were 101.2 and 89.05 U ml^{-1} respectively. Remaining activity of both catalases were recorded by defining original activity as 100 %. The data are average of three independent experiments.

pH 7 but the immobilized catalase has residual activity more than the free enzyme in other values (Fig. 2). Over 94, 98 and 90 % of the maximal activity of the immobilized CaCAT were maintained at pH 6.5, 7.5 and 8 respectively and that reflect resistance of immobilized form to pH changing. Optimal pH 7 for both catalases indicate that the surface of beads was neutral and has no influence on the displacement of immobilized activity at this value (Anwar *et al.*, 2009). On the other hand, cationic or anionic beads able to generate shifting in optimal pH of immobilized enzyme (Norouzian, 2003).

At 25°C the free and immobilized CaCAT showed 100 % of their activity (Fig. 3). In addition, the total activity of the immobilized form was not altered at 20-30 °C and loss of its activity less than the free CaCAT at non-optimal temperatures. Retained of immobilized activity at higher temperatures compared with free CaCAT pointed out protection of 3-dimensional structure of enzyme by alginate beads (Anwar *et al.*, 2009).

Inhibitors and Metal Ions Resistance

Generally, as in Table 2 the free and immobilized CaCAT were exposed about 0-42 and 33-72% from their original activity when treated with 5 mM of inhibitors. Incubation of free and immobilized enzyme with 5 mM Fe^{2+} raised the activity ~ 3% whereas other ions (5 mM) dropped it ~ 34-79% and ~ 25-68% respectively. Loss about 58% of catalytic degradation of H_2O_2 by EDTA

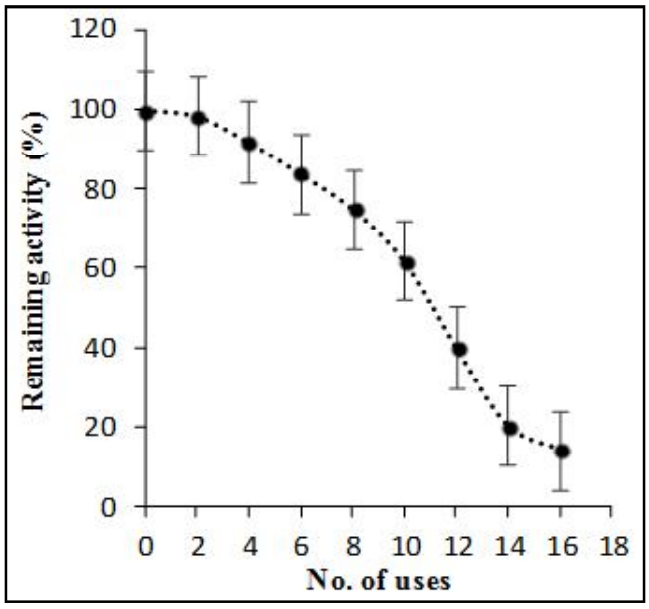

Fig. 6: Recycling of the immobilized CaCAT. Reusing was at optimal conditions, pH 7 and 25°C for determination of activity. The data are average of three independent experiments.

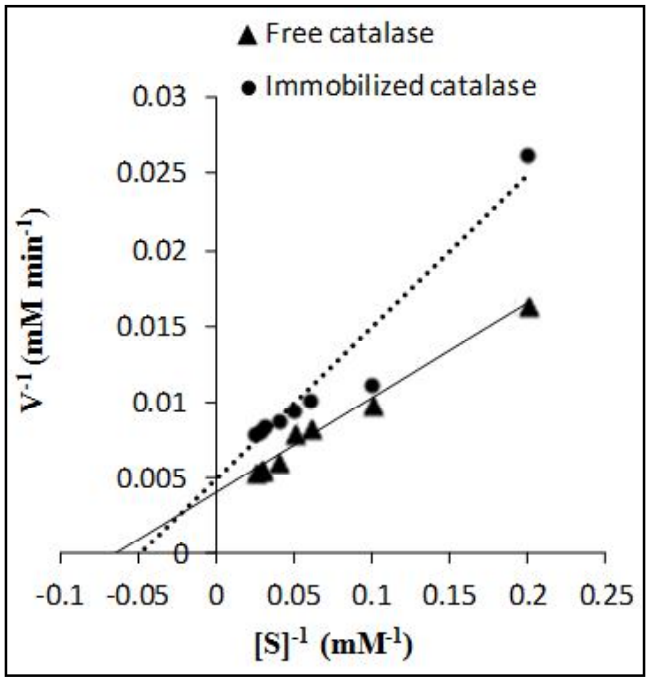

Fig. 7: Lineweaver-Burk plot for determination of K_m and V_{max} of the free and immobilized CaCAT using different concentrations of substrate.

and complete inhibition of the free CaCAT by NaN_3 and KCN refer that it belongs to metalloenzyme contain heme in active site. In contrast the Mn-catalase is insensitive to the heme inhibitors such as azide and cyanide. The Fe-catalase is very sensitive to lower concentrations of noncompetitive NaN_3 and KCN inhibitors (Ma *et al.*, 2017) while competitive EDTA inhibitor could exhibit partial inhibition activity (Lee *et al.*, 2010). Also, variations

of inhibitory effect on CaCAT depends on the metal, concentration, the tissue, and species (Atli *et al*., 2006). Different resistances of immobilized CaCAT to all inhibitors and metal may be useful in industrial sectors.

Stability of Storage and Recycling

In a period 30 days of storage at 4°C the free CaCAT has no activity meanwhile the immobilized CaCAT was maintained about 76% of its activity (Fig. 4). As well immobilized CaCAT showed significant activity (80-100%) during early period of storage. At higher temperature (25°C) of the storage period for 15 days free CaCAT has 0% of activity while immobilized lost only ~ 29%. Total collapse of immobilized CaCAT activity was reported within 50 days. (Fig. 5). Regardless of the values of activity, the immobilized enzyme was revealed more stability during days of storage at 4 and 25°C compared with free enzyme. As shown in Fig. 6 the immobilized CaCAT recovers about 75% of the activity when it reused eight times. In industrial applications bounded enzymes have advantages over free enzymes as a result of its stability and reusability (Liese and Hilterhaus, 2013).

Calculation of K_m and V_{max}

For more understanding of CaCAT *Km* and V_{max} parameters of the free catalase were estimated to be 15.3 mM and 250 mM min^{-1}. On other hand same parameters of the immobilized enzyme were 21.73 mM and 212 mM min^{-1} respectively (Fig. 7). The immobilization process may be altered structure of whole enzyme or the active site of CaCAT and then reduced entrance of H_2O_2 to the active site leading to decline in the affinity and maximal velocity (Arabaci and Usluoglu, 2013).

Acknowledgments

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