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Immobilization of catalase onto chitosan and chitosan–bentonite complex: A comparative study

Jyoti Kaushal, Seema, Gursharan Singh, Shailendra Kumar Arya*

University Institute of Engineering and Technology, Panjab University, Chandigarh, India

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ABSTRACT

The immobilization of catalase onto chitosan and chitosan–bentonite was investigated and immobilization yield of 95.91 and 95.26 was obtained respectively. The optimum pH and temperature were found as 7.5 and 8.0 at 40 °C for free and immobilized enzyme. The value of V_{max} decreased by 33,000–26,300, 24,500 μmol (min mg protein)$^{-1}$ and K_m increased by 12.5–25 and 20 mM for free and immobilized on chitosan and chitosan–bentonite respectively. The thermal stability, half life, FTIR analyses of the beads was also performed in order to characterise the structural differences. The remaining immobilized catalase onto chitosan and chitosan–bentonite activity was 50% and 70% after 20 cycles respectively. The storage stability were found as 22%, 60%, and 70% from its original activity in case of free enzyme and immobilization of chitosan, chitosan–bentonite beads respectively after 60 days.

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1. Introduction

Catalase (EC 1.11.1.6) enzyme is an oxidoreductase enzyme as it plays a crucial role in quenching the reactive oxygen species (ROS), i.e. hydrogen peroxide, often produced as a by-product of aerobic respiration [1], by converting it into oxygen and water. Hydrogen peroxide metabolism is mainly regulated by this enzyme. Catalase is a common enzyme found in nearly all living organisms aerobic as well as anaerobic. It has one of the highest turnover of all enzymes as it has the capacity to decompose more than one million molecules of hydrogen peroxide per molecule of enzyme [2–5]. Catalase is a tetramer of four polypeptide chains, each over 500 amino acids long. It contains four porphyrin haem (iron) groups that allow the enzyme to react with the hydrogen peroxide. Catalase has a fairly broad range of working from optimum pH between 4–11 [6–8]. Catalase is usually located in a cellular, bipolar environment organelle called the peroxisome. As this enzyme is found in mainly all organisms (aerobic and anaerobic), it has been exploited in many applications including food processing, textile, paper, pharmaceutical industry, medical areas, and also in the field of bioremediation as one of the upcoming areas of its application [9–13].

Catalase has been immobilized on various supports for use in the numerous industrial prospects. The immobilization of catalase onto chitosan beads is one of the mainly used techniques because of its being simple and cheap [14]. Chitosan proves to be attractive for the purpose of enzyme immobilization because of its inert nature; moreover it is an inexpensive and hydrophilic support material and is also biocompatible, biodegradable and non toxic, making it one of the main immobilization methods [15–17]. Amino groups that are present on chitosan facilitate the binding of the enzyme covalently to the support [18,19]. The literature suggests that the immobilization can be either by the entrapment of the enzyme in the chitosan beads, or by the covalent binding to the chitosan transparent films [20–23]. The use of glutaraldehyde as a cross linked for chitosan beads are more applicable in biochemical engineering due to their good biocompatibility and mechanical strength. However their minor operational defect like density (close to water) causing, it floats easily and surface is also soft. To overcome this problem chitosan was mixed with some inactive materials like clays [24].

Bentonite is one of the clay minerals that have been proven to be of great importance for the immobilization of catalase. Bentonite is a sedimentary rock with a three layered structure (smectites) like nontronite, montmorillonite, etc. and the presence of quartz, zeolites, feldspars are also frequently found in it. Bentonite is an inexpensive matrix and in addition to its low cost, it has several other advantages that include its high chemical reactivity, low toxicity, and availability of functional groups that allow the easy fixation of the enzyme [25,26].

The main aim and objective of this research paper was to form a cost effective immobilization using natural clay ingredient i.e.

* Corresponding author.
E-mail address: skarya@pu.ac.in (S.K. Arya).

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bentonite for the formation of the chitosan beads instead of glutaraldehyde. The chitosan-bentonite beads were formed for the purpose of catalase immobilization and the stability and working of the catalase immobilized chitosan-bentonite complex beads was compared to the glutaraldehyde reinforced chitosan beads, so that these can replace the commonly used catalase beads formulations requiring glutaraldehyde as a cross-linker. Therefore, the activity, pH, temperature dependence, kinetics and other physical parameters of both free and immobilized enzyme were studied.

2. Materials and methods

2.1. Chemicals used

Hydrogen peroxide was used as a substrate and obtained from Fisher Scientific, Bovine liver catalase (15,200 U/mg), Glutaraldehyde, Acetic acid, Chitosan, Bentonite, NaOH were obtained from HIMEDIA.

2.2. Immobilization of catalase on chitosan beads

The chitosan (2.5 g) was dissolved in 1% acetic acid (100 ml) solution prepared by mixing one hour at room temperature. After the completion of the mixing, the beads were formed by pouring the solution drop wise into 2% of NaOH. Chitosan beads were formed in the precipitating solution [16,17]. To prepare the crosslinking solution, 200 μl of glutaraldehyde was added to 100 ml of distilled water and mixed thoroughly. The beads prepared were added into this crosslinking solution and kept at room temperature for 3 h. The reinforced beads were then collected in a sieve and stored at room temperature before immobilization of the enzymes onto the beads. The immobilization of the enzyme onto chitosan beads were immersed in 10 ml of catalase solution (2.0 mg ml^{-1}) in 0.5 mM Phosphate buffer (pH 7.5). The beads were kept in this solution for 5 h at 25 °C in a rotating incubator. The immobilized beads were washed with cold phosphate buffer (pH 7.5) thoroughly and stored for further study [4,16,20].

2.3. Immobilization of catalase on chitosan–bentonite complex beads

The chitosan (2.5 g) solution was prepared by dissolving 1% acetic acid (100 ml). In this 50 mg bentonite was separately activated by mixing it with 10 ml catalase (2.0 mg ml^{-1}) in 0.5 mM phosphate buffer (pH 7.5). These two solutions prepared separately were then mixed together in a beaker and subjected to magnetic stirring for 4 h at 4 °C to form a uniform mixture. After the completion of the mixing, the beads were formed by pouring the solution drop wise into 2 M NaOH precipitating solution. Thereafter the beads were washed with cold phosphate buffer (pH 7.5) [2,28].

2.4. Enzyme activity of free and immobilized catalase

The catalase activity was determined by spectrophotometrically by measuring the decrease in absorbance of H_2O_2 at 240 nm in a reaction mixture containing 3.0 ml of substrate (10 mM H_2O_2 in pH 7.5 phosphate buffer) and 1.0 ml of the Catalase (2 mg ml^{-1}) solution. The reaction mixture was kept at 40 °C temperature for 15 min and stop by adding 0.5 ml of 1 M HCL. For activity of immobilized enzyme (approximate 25 mg) were mixed substrate as above prepared at 40 °C temperature for 15 min and stop by adding 0.5 ml of 1 M HCL after removal of the beads. The activity of free and immobilized catalase were expressed as μmol min^{-1} and μmol (min mg protein)$^{-1}$ respectively [16,17]. One unit of enzyme activity was defined as the amount of enzyme which converts 1 μmol H_2O_2 in to product per minute at pH 7.5 and 40 °C.

2.5. Determination of total protein and immobilization yield

Total protein concentration was determined according to Lowry's method using Bovine serum albumin (BSA) as a standard [29]. The amount of bound protein was determined by subtracting the amount of protein in supernatant after immobilization from the total amount of protein used for immobilization. The protein loading and immobilization efficiency of immobilization onto chitosan and chitosan bentonite was calculated using formula [30]

$$\text{Protein loading}\ (\%) = \frac{\text{Amount of immobilized protein}}{\text{Amount of initial protein}} \times 100$$

$$\text{Immobilzation efficiency}\ (\%) = \frac{\text{Specific activity of immobilized enzyme}}{\text{Specific activity of free enzyme}} \times 100$$

2.6. Effect of pH and temperature on enzyme activity

The effect of pH on free and immobilized catalase activity was carried out at different pH values ranging from 3 to 9 using 0.5 M citric acid (pH 3.0–4.0), 0.5 M sodium acetate (pH 4.0–6.0), 0.5 M sodium phosphate (pH 6.0–8.0), 0.5 M Tris–HCl (pH 8.0–9.0), 0.5 M glycine–NaOH (pH 9.0). The highest value of enzyme activity in each set was assigned as 100% activity [31,32]. The effect of temperature on free and immobilized catalase was tested by performing the activity in the various temperatures ranging from 5 to 60 °C with the interval of 10 °C.

2.7. Thermal stability and half life of enzyme

The thermal stability and half life of the free enzyme and the enzyme immobilized on the two beads were determined by monitoring the activity of catalase within temperature range of 20–60 °C at time intervals of 0, 30, 45 and 60 min respectively against H_2O_2 as a substrate [20]. The results were obtained by plotting a graph of $-\ln E/E_0$ (E_0: Initial enzyme activity and E: enzyme activity at time t) on Y axis against time (t) on the X axis. The slope of the graph gives K_d of the enzyme and $t_{1/2}$ is calculated as:

$$t_{1/2} = \frac{0.6932}{K_d}$$

2.8. Determination of kinetic parameters

The kinetic parameters of the immobilized catalase and free catalase were determined by measuring the rates of the reaction at various substrate concentrations ranging from 2 to 10 mM at optimum temperature and pH. The kinetic parameters K_m and V_{max} were calculated from the lineweaver–burk plot [20].

2.9. FT-IR spectroscopy

The characteristic of a molecule can be well determined by the vibrational spectrum of that molecule. It is considered as one of the most fundamental tool for characterisation of a molecule [17]. It serves as an impression for the identification by comparing the spectrum of our unknown sample with previously recorded reference spectra. In the case of chitosan beads prepared for immobilization as well as chitosan-bentonite complex beads, Fourier Transform Infrared Spectroscopy (FTIR), is a popular tool

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