

Biochemical Studies and Characterization of Immobilized Alkaline Phosphatases on Carboxyl-Functionalised Carbon Nanotubes

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ABSTRACT

Alkaline phosphatase has been isolated from the seeds of 'Arachis hypoghaea' by ammonium sulphate fractionation (30-80%) and ion exchange chromatography. The isolated enzyme was studied for its biochemical properties viz. optimum pH and temperature. The purified enzyme was immobilized using carboxyl-functionalised carbon nanotubes (CNTs) by cross linking with epichlorohydrin (ECH) as well as a matrix of ethyl cellulose. The resulting two matrices were characterized by FTIR and SEM to obtain structural and morphological properties. By both methods the immobilised enzyme showed good activity comparable with the native enzyme. Reusability of the immobilised enzyme was demonstrated up to 8-10 cycles. The immobilized enzyme showed higher temperature stability. The investigation of cutting-edge nanomaterial with the help of enzyme immobilisation using exceptional techniques makes nanobiocatalyst of promising thirst for knowledge for biosensor applications.

Keywords: Alkaline phosphatase, Immobilization, CNTs, Biochemical studies

I. INTRODUCTION

Immobilisation of enzymes have been well-defined as enzymes which are actually confined or localised, although without a losing their catalytic activity, and can be used repetitively and continuously [1]. Immobilisation of enzymes on a matrix offers significant cost benefits for industrial and clinical processes, since it enables enzyme recycling, enables enhancements in thermo-stability which ultimately reduces enzyme inactivation and permits for better control of enzyme activity [1, 2]. Likewise, Nanobiocatalysis is also a speedily increasing research ground which increases to the application of enzymes immobilized materials. A Nano material contributes some benefits over the bulk solid materials, specifically the high surface area which can lead to higher enzyme loading, the

nanoscale dispersion and the ease of surface functionalization [3]. Carbon nanotubes (CNTs) have fascinated significant benefits among nanostructured materials for their unique mechanical, thermal and electrical properties as well as their biocompatibility [4].

In this present work, Alkaline phosphatase has been isolated from the seeds of 'Arachis hypoghaea' and was then immobilised onto the carboxyl functionalised multi-walled carbon nanotubes (MWNTs) supports using various cross linkers viz. Epichlorohydrin (ECH) as well as a matrix of Ethyl cellulose. The biopolymer may also serve as a potential cheap and easily available biosorbent for environmentally harmful metal ions [5-9]. The enzymatic activity was then measured according to the typical process. Structural and morphological

changes for immobilized enzyme were analysed using various spectroscopy techniques. The catalytic efficiency of the immobilised alkaline phosphatase, in terms of thermal stability and reusability, was also studied.

II. METHODS AND MATERIAL

A. Isolation and purification of Alkaline phosphatase

'*Arachis Hypoghaea*' seed meal (100 g) was extracted with 500ml of physiological saline (0.145 M NaCl) for 4 hours at 4 °C. The extract was centrifuged at 16,000 rpm and subjected to fractional precipitation with ammonium sulphate. The proteins precipitating between 30% and 80% saturation of ammonium sulphate were collected by centrifugation, dissolved in minimum amount of distilled water, extensively dialysed against distilled water and finally against Tris buffer (pH 8.0, 0.1 M). The dialyzed protein solution, clarified by centrifugation (Fraction A) was used to isolate alkaline phosphatase by ion exchange chromatography on a UNOsphere-Q column an anion exchanger.

The optimum pH of the enzyme was determined as follows; a test system containing 200 µl Tris buffer (0.1 M pH 6-10) + 200 µl substrate (*p*-nitrophenyl phosphate) and 200 µl enzyme was incubated for 30 minutes. The reaction was arrested by adding 1 ml 0.5 M NaOH. The pH stability of the enzyme was determined by pre-incubating 200 µl of enzyme with different buffers (200 µl) for 8 hrs. along with corresponding control. The amount of *p*-nitrophenol liberated was estimated at 410 nm.

To study effect of temperature on the enzyme activity, the reaction was carried out at temperatures ranging from room temperature 20 °C to 90 °C all for 30 min. To determine the temperature stability of the enzyme, the pre-incubated enzyme (200 µl) in buffer (200 µl) was incubated at varying temperature (20-80 °C) each

for 30 min. Then 200 µl of substrate was added to it and the reaction mixture was incubated at room temperature for 8 hrs.

Effect of metal ions on enzyme activity was checked by preincubating the demetallised enzyme with 5 mM metal ions (Ca²⁺, Mg²⁺, Zn²⁺, K⁺, Fe³⁺, Mn²⁺, Ba²⁺, Mo⁵⁺, Ni²⁺, Al³⁺, Na⁺, Cu²⁺, Co²⁺, Be²⁺, V⁵⁺.) for 30 minutes at 60 °C. The enzyme activity was checked after arresting reaction. Corresponding controls without metal ions were run simultaneously.

B. Immobilization of Alkaline phosphatase on MWCNTs by various cross linkers.

Alkaline phosphatase was immobilized on commercially available carboxyl functionalized multi walled carbon nanotubes MWCNTs by cross-linking with various cross linkers viz. Epichlorohydrin (ECH), Citric acid, Glutaraldehyde and 1-Ethyl -3-(3-dimethyl amino propyl) carbodiimide (EDC) as coupling agents.

C. Immobilization of Alkaline phosphatase on MWCNTs by various cross linkers and Ethyl cellulose sponge matrix.

Also, the immobilized enzyme activity studies were also studied using ECH-Ethyl cellulose Matrix and MWCNTs. For this, Ethyl cellulose is dissolved in minimum required Ethanol and then continuously mixed with ECH with constant stirring followed by MWCNTs.

The optimum pH, pH stability, optimum temperature, temperature stability and effect of metal ions also studied for both types of immobilized enzyme. The effect of concentration of enzyme and composite matrix was also studied.

III. RESULTS AND DISCUSSION

A. Biochemical studies of native and immobilized enzyme

The effect of pH on alkaline phosphatase activity from '*Arachis Hypoghaea*' seeds is shown in Figure 1.

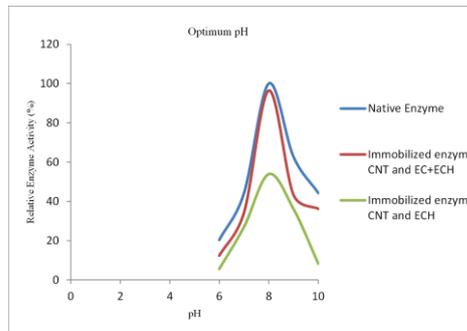


Figure 1. Optimum pH for Native and Immobilized enzyme

The optimum pH for alkaline phosphatase activity found to be 8.0. Epichlorohydrin (ECH) as cross linker is finalised based on the results of enzyme activity for 8-10 cycles when compared to other cross linking agents such as citric acid, glutaraldehyde and EDC with the 0.3M concentration. Figure 2 depicts the effect of temperature on activity of native and immobilized enzyme. The optimum temperature for alkaline found to be 60°C for native as well as immobilized enzyme. For all studies, the reusability of enzyme showed up to 8-10 cycles.

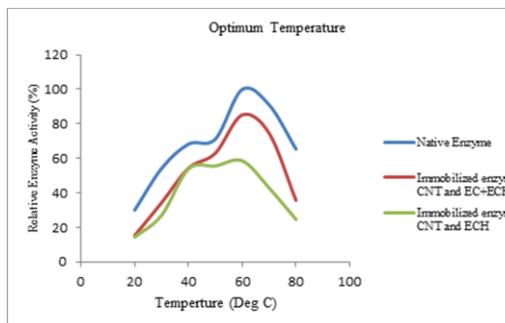


Figure 2: Effect of Temperature on Native and Immobilized Enzyme

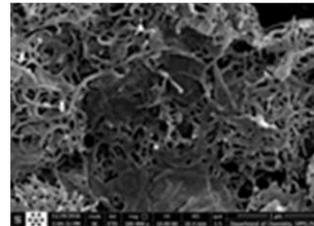


Figure 3a: MWCNT-ECH Matrix without enzyme

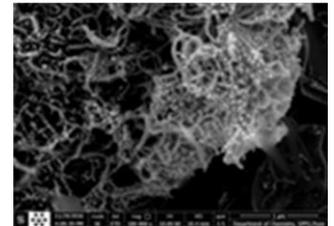


Figure 3b: MWCNT-ECH Matrix with enzyme

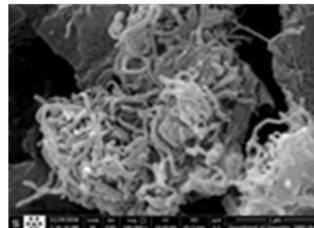


Figure 3c: MWCNT-ECH and Cellulose Matrix without enzyme

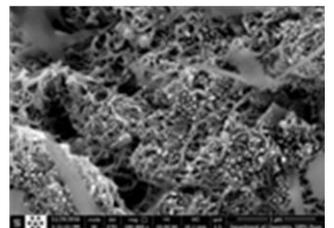


Figure 3d: MWCNT-ECH and cellulose Matrix with enzyme

FESEM images of MWCNT-ECH matrix and MWCNT-ECH-Cellulose sponge matrix with and without immobilized enzyme clearly shows as in Figure 3a, 3b, 3c, 3d that MWCNT-ECH-Cellulose sponge matrix exhibited randomly distributed micro-porous structures and highly 3D porous networks. As shown in Figure 3c and 3d this sponge matrix exhibits an interconnected porous structure with the pore sizes ranging from quite a few to one hundred microns which allows more free surface area for enzyme binding.

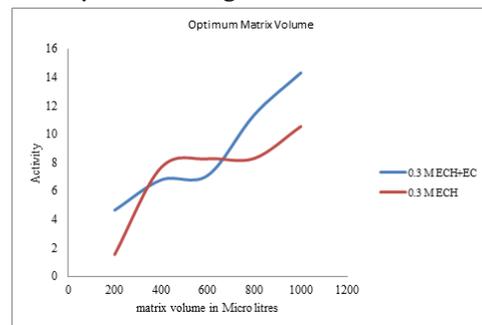


Figure 4: Effect of volume of matrix on Enzyme activity

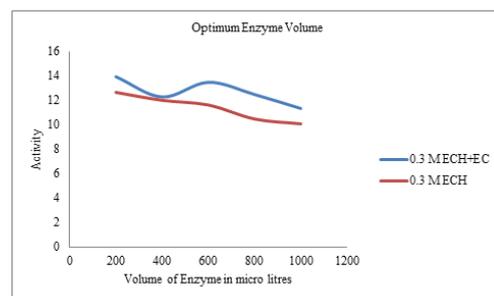


Figure 5: Effect of volume of enzyme on Enzyme activity

Superhydrophobic ethyl cellulose (SEC) sponges were prepared by cross-linking EC with epichlorohydrin (ECH) and complexing with Multiwall carbon nanotubes (MWCNTs) with ratio 1: 1. Due to the presence of residual hydroxyl groups in EC backbone, EC can be cross-linked by ECH in alkaline conditions. Chemical cross-linking can increase the mechanical properties of the Sponges [10]. Also cellulose favours the high light transparency [11].

Based the enzyme activity results shown in Figure 5 and Figure 6 we finalized optimum Matrix Volume 1000 micro litre and optimum Enzyme Volume 1000 micro litre. Mixing time of MWCNT, ECH and enzyme has optimised for 2 hrs.

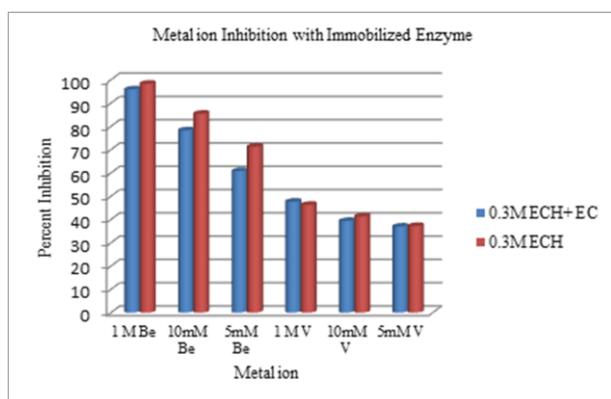


Figure 6b: Effect of Different metal ions on Immobilized enzyme activity
As seen in Figure 6a and 6b, remetallization of *Arachis hypogaea* alkaline phosphatase with 5 mM Be^{2+} resulted in restoration of more than 100% of the enzyme activity. Al^{3+} , K^+ , Ca^{2+} and Ni^{2+} had no effect on the enzyme activity. Alkaline phosphatase was found to be strongly inhibited by V^{5+} whereas moderate inhibition was observed with Mn^{2+} , Ba^{2+} , Mo^{5+} . Considering harmful effects of Beryllium and Vanadium, we carried out enzyme activity studies for these metal ions for immobilized enzyme which depicts similar results.

IV.CONCLUSION

In this report, we explored the immobilizations of Alkaline Phosphatase from *Arachis hypogaea* on MWNTs under several different conditions.

The characterization studies have revealed that the enzyme immobilization took place more efficiently at even 60 °C temperature ionic liquid as the medium for the better dispersion of carbon nanotubes, and the resultant immobilized enzyme displayed a good performance like Native enzyme. Further purification is in progress.

Arachis hypogaea alkaline phosphatase can be a used as sensor for vanadium detection since its immobilization increases its reusability without affecting enzyme activity.

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