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Study of cross linked enzyme aggregate of glucose isomerase of *Streptomyces thermonitrificans* immoblised on magnetic particle

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Abstract

Magnetic cross linked enzyme aggregate of glucose isomerase were prepared by chemical cross-linking of enzyme aggregates with amino functionalized magnetic particle. The result of X-Ray Diffraction (XRD) has shown that the particles are in hematite phase (Fe_2O_3) and 47.84nm in size. Fourier Transform Infrared Spectroscopy (FTIR) confirmed the existence of the surface coating. Scanning probe microscopy (SPM) was used to confirm the presence of protein on the particle surface. The magnetic CLEAs displayed a shift in optimal pH towards the acidic side, whereas optimal temperature of magnetic CLEAs was shifted to higher temperature, making conditions favorable for commercial use. Moreover, the magnetic CLEAs retained 80% initial activity even after 6 cycles of reuse.

Keywords: Magnetic Particle, Cross linked Enzyme Aggregates, Glucose Isomerase, Immobilization, *Streptomyces thermonitrificans*

Introduction

Glucose isomerase (EC 5.3.1.5), is the third highest value enzyme after amylase and protease. It catalyzes the reversible isomerization of D-glucose to D-fructose. Conversion of glucose to fructose is of commercial importance in the production of High-Fructose Corn Syrup (HFCS). Glucose has 70-75% the sweetening strength of beet sugar (sucrose), but fructose is twice as sweet as sucrose, so high fructose syrup can be used to replace sucrose as a sweetener.

Although many organisms produce glucose isomerase, most of them are mesophilic and only a few are thermophilic. Since thermophilic organisms grow at high temperatures, their use not only helps to prevent microbial contamination but also permits optimum enzyme production in a shorter time than with mesophilic organisms. A thermophilic strain of *Streptomyces thermonitrificans* has been reported to produce high level of intracellular glucose isomerase [Deshmukh, Dutta and Shankar et al. 1993] and therefore, selected for the production of glucose isomerase.

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Traditional method can conveniently divided into three types: binding to a support (carrier), entrapment (encapsulation) and cross-linking [Sheldon et al. 2003].

Cross-linking of enzymes using glutaraldehyde was originally developed more than 40 years ago. However the cross-linked enzymes are difficult to handle, exhibiting low activity because of gelatinous nature, low stability and shelf life [Sheldon et al. 2011]. Recently, carrier-free enzyme immobilization methods are gaining attention due to advantages such as: high enzyme activity in the catalyst, high stability and less production cost because of elimination of an expensive solid carrier [Talekar et al. 2012].

Among these methods cross-linked enzyme aggregates (CLEAs) have appeared as a groundbreaking and multifaceted carrier-free immobilization technique. This methodology combines purification and immobilization into a single step, so crude enzyme extracts can be used. In this technique, first proteins are precipitated as physical aggregates by addition of salts, water miscible organic solvent or non-ionic polymers and then the formed aggregates are chemically attached to each other by a bifunctional reagent, usually glutaraldehyde. Immobilization in this way does not suffer from drawbacks such as diluted enzyme activity, mass transfer limitation and lower catalyst productivity. In addition, CLEAs exhibits enhanced storage and operational stability; facile separation and have low production cost as no expensive carrier is needed [Sheldon et al. 2011].

Recently, magnetic CLEA of α-amylase [Talekar et al. 2012] and xylanase [Bhattacharya et al. 2014] have been prepared, which can be easily separated using magnetic fields thereby eliminating the need for filters and centrifugal techniques.

In the present work, we developed magnetic CLEA of glucose isomerase obtained from *Streptomyces thermonitrificans*. The prepared Magnetic CLEA were characterized in terms of optimum temperature and pH, and compared with free enzyme. Lastly, the reusability of magnetic CLEA of glucose isomerase was also measured.

Materials and Methods

Material

[3-(2-aminoethylamino)propyl]trimethoxysilane (APTS) were purchased from Sigma Aldrich. Glutaraldehyde (25% v/v), Casamino acid, cysteine Hydrochloride, carbazole and xylose were obtained from Himedia. All other reagents were used of analytical grade.

Microorganism and Growth

The thermophilic strain of ***Streptomyces therrnonitrificans*** (NCIM 2007) was routinely maintained on MGYP slants (malt extract, 0.3%; yeast extract, 0.3%; peptone, 0.5%; glucose, 1%; and agar, 2%; adjusted to pH 7.0 with NaOH) at 50°C. The vegetative growth was colorless to pale yellow turning slightly darker in 2-3 days. Greyish white aerial mycelium developed after 36-48 hr and turned dark grey in 72 hr.

Figure 1. Growth of *Streptomyces thermonitrificans* on MGYP slant.

Production of Glucose isomerase.

The inoculum was prepared in 50ml Callens' Medium[Callens et al. 1985](sorbitol, 2%; Casamino acid, 1%; Yeast extract, 0.5%; KH_2PO_4, 0.27%; K_2HPO_4, .52%; $MgSO_4.7H_2O$, 0.2%; NH_4NO_3, 0.3%; Xylose, 1%) by inoculating with a well sporulated slant of Streptomyces culture, followed by incubating at 50^0C with shaking (200rpm) for 24hr.
Enzyme production was carried out by transferring inoculums (10%) to 50 ml of the same medium, in each of several 250-ml Erlenmeyer flasks, and then incubated at 50°C for 16 h with shaking (200rpm).

Preparation of Crude Enzyme Extract

The cells, from 50ml culture, were separated by centrifugation at 10000rpm for 20 min, washed twice with distilled water and suspended in 30 mL of 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM Co^{2+} and 5 mM Mg^{2+}. The cells were sonicated using a hielscher sonicator for 5 min (Cycle .5% and Ampltitude-70%) and the cell debris was removed by centrifugation at 10000rpm for 20 min. The supernatant was brought to 30% saturation of ammonium sulphate. The precipitate obtained by centrifugation at 12000rpm for 20min was discarded and supernatant was used as crude enzyme. Salting out was done to partially purify the enzyme.

Protein Estimation

Protein was estimated according to Biuret test using BSA as standard.

Preparation of Magnetic particle

Magnetic particles were prepared by method described by Reza et al. (2010), Talekar *et al*. (2012) & brett *et al*. (2014) with certain modifications. For the synthesis of magnetite particles, 1.8 gm of Ferric chloride and 1.2gm of ferrous sulphate were mixed with 30 mL deionized water, followed by dropwise addition of ammonium hydroxide until the visible appearance of the precipitates. The solution was allowed to stand for 1 h at room temperature and then solution was decanted keeping the magnetic particle at the base using magnetic field. In order to remove the residual ions, the obtaining precipitate was washed several times with deionized water until a pH value of 7 was obtained; the powder was dried at 100^0C for 2 h.

Aminosilane Coating

The surface of these particles was coated with [3-(2-aminoethylamino)propyl] trimethoxysilane (APTS) by a silanization reaction as described by Reza et al[10]. The process consisted of suspending magnetic particles (200mg) in [3-(2-aminoethylamino)propyl]trimethoxysilane (1ml), deionized water (250µL) and methanol (25mL). The mixture was sonicated for 30 min. After that, glycerol (15 mL) was added, and the solution was heated at 90^0C for 6 hr with continuous mechanical agitation. The obtained precipitate was washed with water and methanol for four times in each case and dried, yielding a fine powder.

Protein immobilization

Magnetic CLEAs of Glucose isomerase were prepared as described by Talekar et al. (2012). The amino functionalized magnetic particles (50mg) were mixed with 15mL of crude enzyme extract (50 mM potassium phosphate buffer, pH 7.0, containing 1 mM Co^{2+} and 5 mM Mg^{2+}) and shaken for 15 min at room temperature. Then ammonium sulphate (70%) was added with stirring at 4^0C for 30 min. After precipitation, 15ml glutaraldyhyde (1%v/v) was added into the suspension and stirred for 3 h at room temperature. After cross-linking, magnetic CLEAs were separated using magnet, washed for three times by sodium phosphate buffer and stored in 0.1 M phosphate buffer (pH 7.0) at 4^0C.

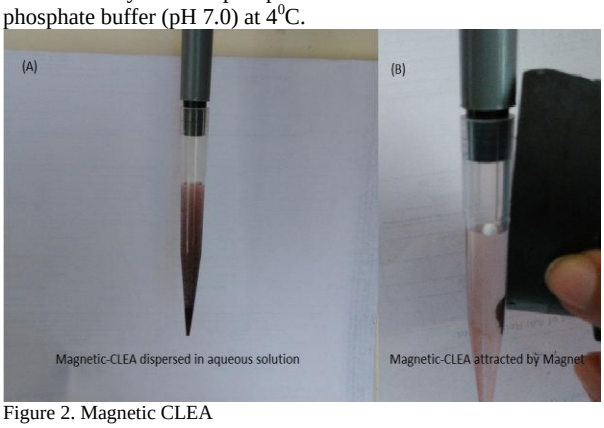


Figure 2. Magnetic CLEA

Determination of enzyme activity

Glucose isomerase activity was determined according to the method of Chen *et al.* (1979). The fructose formed was estimated spectrophotometrically, according to Dische and Borenfreund *et al.* (1951) and modified by Marshall and Kooi *et al.* (1957). One unit of the enzyme was defined as the amount of enzyme required to liberate 1 µmol of fructose/min under the assay conditions.

Characterization

X-Ray Diffraction (XRD) was used to identify the phase of Iron oxide (Fe-O). Fourier Transform Infrared Spectroscopy (FTIR) was used to confirm the existence of the surface coating, the products obtained at each step. Atomic Force Microscopy (AFM)(NT-MDT) was used to confirm the presence of protein on the particle surface.

Effect of pH and temperature on Glucose isomerase activity

The pH optimum of free glucose amylase and Mag-CLEA of Glucose isomerase was studied in the pH range of 4.5-8.5 using 200mM sodium phosphate buffer. The effect of temperature on enzyme activity of free enzyme and Mag-CLEA were determined in the temperature range of 40-90°C.

Reusability of Magnetic-CLEA of glucose isomerase

To determine the reusability of Magnetic CLEA of glucose isomerase, the Mag-CLEA was washed with sodium phosphate buffer after each cycle of 30 min reaction time at 70°C and then suspended again in a fresh reaction mixture to measure enzyme activity. The residual activity was calculated by taking the enzyme activity of the first cycle as 100%.

Results and Discussion

XRD analysis of Particle

The *XRD* spectrum of the particles contains 8 major peaks, which are clearly distinguishable. These peaks are consistent with the database in the JCPDS file No. 24-0072. This confirms the existence of iron oxide particles (αFe_2O_3) with a rhombohedral structure, which has magnetic properties and can be used for the magnetic separation. The spectrum contains some other minor peaks which may be due to presence of other phase.

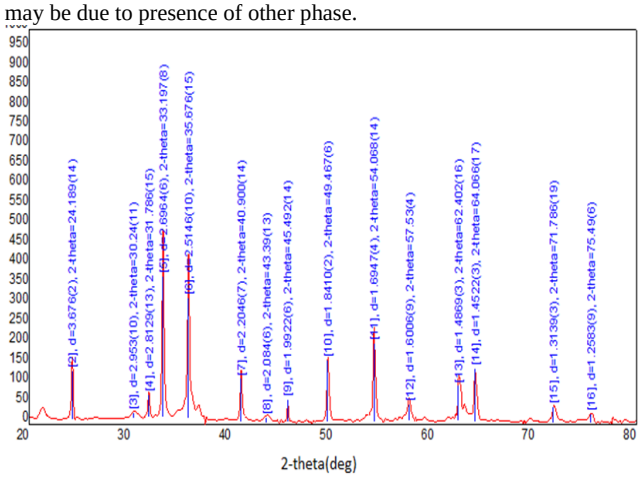


Figure 3. XRD pattern of Magnetic particle prepared by co-precipitation method.

The particle size was obtained by Debye-Scherrer Equation:

$$D = k\,\lambda/\beta \cos\theta$$

Where D is the average diameter in nanometer, λ is the wavelength of X-ray radiation (1.54 A), θ is the diffraction angle, $k = 0.94$ (shape parameter) and β is the full width at half maximum of the X-ray diffraction peaks. The size of the particle is calculated from high intensity peak. The crystal size has been found to be close to 47.84 nm

FTIR study

The coating of the particles was confirmed through FTIR spectra (Figure 4). The band at 543cm^{-1} is due to Fe-0 stretching vibration of Fe_20_3. When the particles of hematite are coated with aminosilane, the characteristic peak of primary amine $-NH_2$ appear at 3383 and 1635cm^{-1}. The band at 2928 cm-1 is due to the stretching of C-H from methyl group (-CH2, -CH3). The band at 2330 and 2364cm^{-1} is due to stretching of C(triple bond)N. Coated hematite show a band at 1053cm-1 which is due to the Si-O bond.

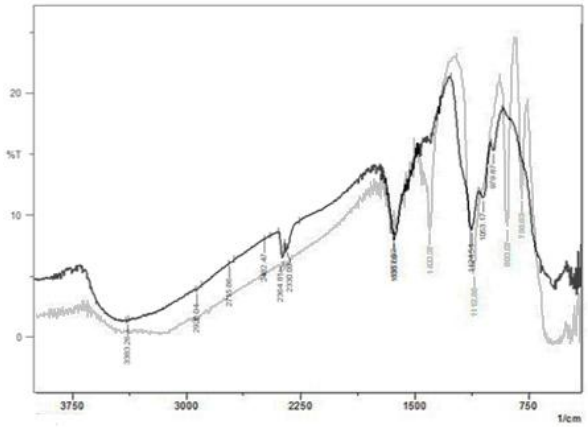
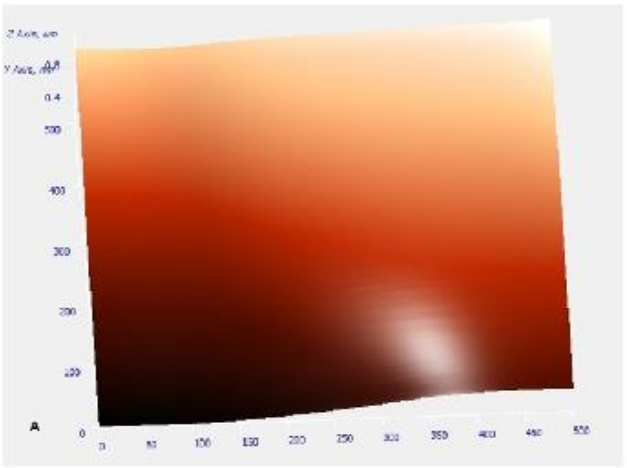


Figure 4. FTIR of Hematite particle without coating (A) and after aminosilane coating (B)

SPM study



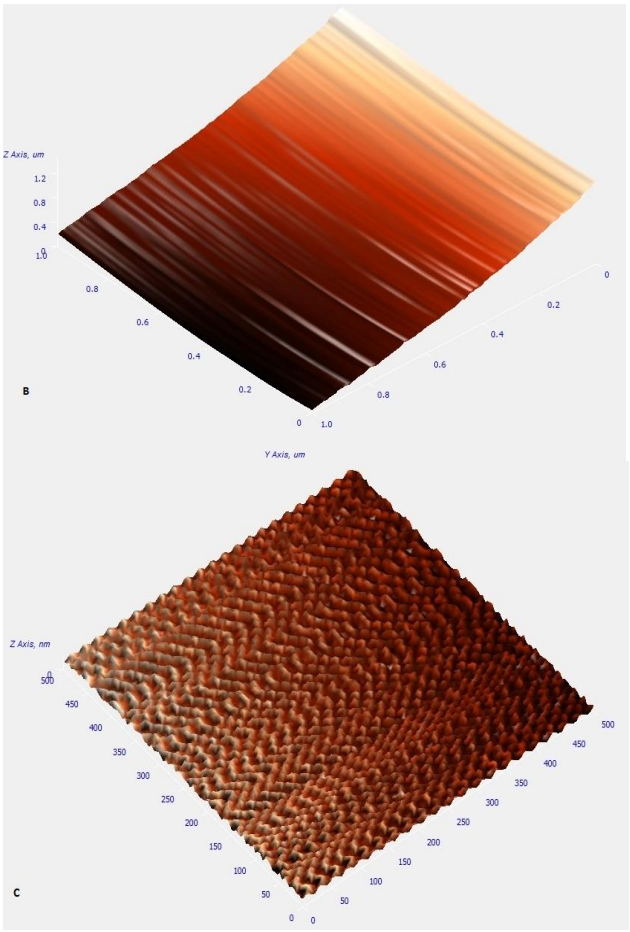


Figure 5. (A) SPM image of PVA membrane without any coating. (B) SPM image of PVA membrane coated with magnetic particles on its surface. (C) SPM image of PVA membrane having enzyme coated particles on its surface.

Scanning probe microscopy (SPM) was used to confirm the presence of protein on magnetic particle. For that, polyvinyl alcohol (PVA) membrane was used as a background material for embedding of magnetic particle. 0.5µm x 0.5µm was scanned to see the change on the surface of PVA membrane after coating. It can be concluded from figure 5(B) that magnetic particle has smooth surface. Only after the enzyme coating the roughness of surface was increased.

Optimal conditions for enzyme activity

The activity of enzyme is strongly dependent on temperature. The isomerase activity of free enzyme increased gradually with temperature and a maximum activity was found at 70^0C. The optimum temperature of glucose isomerase was shifted to 80^0C after immobilization. From the graph (Figure 6), it can be concluded that Magnetic CLEA is more temperature resistance than free enzyme. This shift in optimal temperature could be due to covalent bond formation between proteins caused by glutaraldehyde during CLEAs preparation which might reduce the conformational flexibility of enzyme, requiring higher activation energy and temperature for the molecule to reorganize the proper configuration for binding to the substrate [Aytar and Bakir2008; Talekar, 2012; Sangeetha and Abraham 2008]. The immobilization procedure seems to protect the enzymatic configuration from distortion and damage by heat exchange.

pH is one of the major factor that can affect enzyme activity in aqueous solution. Immobilization of enzyme results in conformational change of enzyme which cause shift in optimum pH. The optimum activity of free enzyme was found at pH 7 whereas the optimum pH of immobilized enzyme was shifted to 0.5 units to the acidic region (pH 6.5). This shift may be caused by change in charge of surface amino acid because of cross-linking with glutaraldehyde, as has been previously described for α-amylase [Talekar, 2012], esterase[Jae and Kim, 2010], subtilisin[sangeetha, 2008] and tyrosinase [Aytar and bakir, 2008].

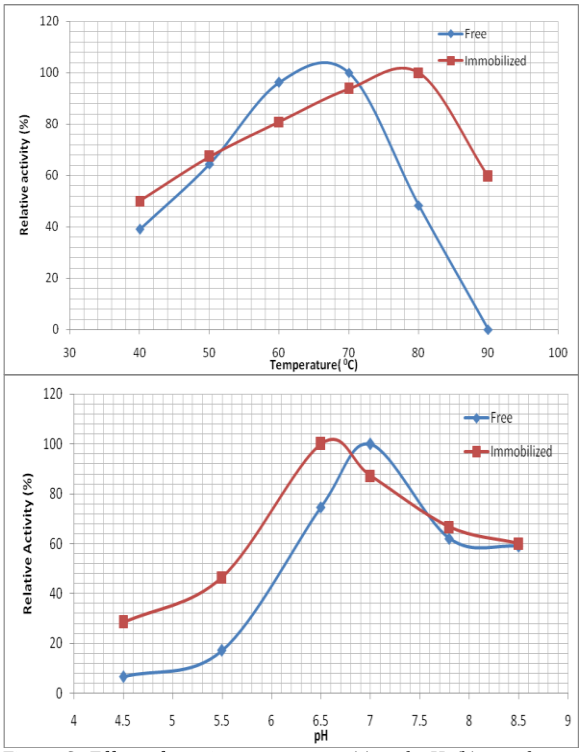


Figure 6: Effect of varying temperature (a) and pH (b) on glucose isomerase activity

Reusability of Magnetic CLEA of Glucose Isomerase

Magnetic–CLEA offers the advantage of reusability which is essential for cost effective industrial use either in repeated batch or in continuous process. The reusability of Magnetic

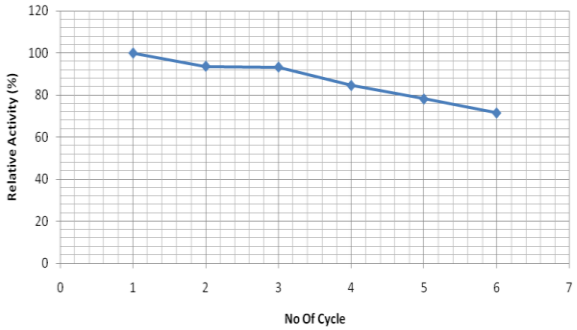


Figure 7. Reusability of Magnetic CLEA of Glucose isomerase.

CLEA of glucose isomerase was studied up to 6 cycles. After each cycle, Magnetic –CLEA was separated from the reaction mixture using magnetic field, washed and used again in the next cycle. The Magnetic CLEA had better reusability, only 20% activity lost after 6 cycles. This loss in activity with increase in cycles may be related to enzyme inactivation.

Conclusions

The Magnetic-CLEAs of glucose isomerase were successfully prepared by cross-linking of aggregated enzyme and amino functionalized magnetic particles. Temperature and pH optima of enzyme shifted after immobilization; this could increase the applicability of the enzyme. Moreover, after immobilization, significant increase in enzyme activity was observed as compare to free enzyme. The result of reusability experiment demonstrated that the Magnetic- CLEAs retained 80 % of activity even after six cycles of use. Hence, we conclude that Magnetic-CLEA of glucose isomerase clearly have practical utility in the HFCS production.

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