Non Hazardous synthesis of graphene and its application in the preparation of L-Asparginase based biosensor obtained from *Bacillus cereus* strain for the detection of leukemia

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ABSTRACT

High quality graphene oxide was synthesized using expanded graphite following a modified Hummer's method. This method is facile, inexpensive and avoids use of strong acids.To exfoliate, the Graphene Oxide (GO) sheets were thermally exfoliated by rapid heating at 1050^0C for 30 seconds under an Argon atmosphere.Further the antileukemic enzyme Asparaginase was isolated from a bacterial source, Bacillus cereus strain BHU-MBU BC1 (ACCESSION No. KJ472206).

The isolated enzyme was further purified in a single step through DEAE-Sepharose column (2.0 X 10.0 c.m) at 4 °C. Kinetic parameters of the as purified enzyme were thoroughly studied. As purified enzyme was further attached to graphene. It was observed that graphene conjugated enzyme showed the enhanced activity and the reusability of the enzyme was found to be 12 times retaining activity upto 80%. The temperature optima of the immobilized enzyme were also found to be improved from 45 º to 60º C.

Keywords: Asperginase, graphene sheet, expanded graphite, gel filteration.

1. INTRODUCTION

Graphene is a sheet of two-dimensional (2D) single layer sp^2 hybridized carbon atoms with exceptional electrical, mechanical and thermal properties [1,2]. Geim and co-workers discovered graphene, the thinnest known material, in 2004 and received Noble Prize in Physics for this breakthrough. Since its finding, research on graphene has amplified exponentially exploring different properties and applications ranging from electronic, optoelectronic devices and photoconductive materials in solar cells to medical imaging, drug delivery and tissue engineering.

Graphene derivatives, including pristine graphene, GO, chemically reduced GO (rGO) [3] and doped graphene [4] have been intensively studied for their widespread applications in biosensing and detection of biomolecules such as thrombin [5], ATP [6], oligonucleotide [7], amino acid [8], and dopa-mine [9]. Several types of GO-based biosensors have been built, which include: (1) Making use of super efficient fluorescence quenching ability of graphene, some novel fluorescence resonance energy transfer (FRET) based biosensors have been developed ; (2) Based on the unique electronic property of graphene, FET type biosensors have been made [10]; (3) Controllable self-assembling of graphene-biomolecules allows to build highly ultrasensi-tive biosensors for detection of DNA and other molecules [33]; and (4) GO-based novel biosensors via electrochemical principle have been constructed by taking use of its huge surface area, good electrical conductivity, and excellent capability of loading various biomolecules via chemical or physical interactions [9].There are various routes of synthesizing graphene like chemical method, electrochemical exfoliation, thermal exfoliation etc. The most widely used method is thermal exfoliation following Hummer method synthesis protocol.In the present study we have synthesized graphene sheet using expanded graphite following a modified Hummer's method. This method is facile, inexpensive and avoids use of strong acids. After the synthesis, this carbon nanostructure is used for the immobilization of asperginase enzyme for biosensing application. L-asparginase (EC 3.5.1.1), a medically important enzyme, hydrolyze L-asparagine (essential amino acid) to aspartic acid and ammonia. Since several types of tumour cells require L-asparagine for protein synthesis, they are deprived of an essential growth factor in the presence of L-asparaginase, thus, resulting in cytotoxicity of leukaemic cells. L-asparaginase is a relatively wide spread enzyme, found in many microorganisms. L asperginase was purified from a bacterial source, Bacillus cereus strain BHU-MBU BC1 (ACCESSION No. KJ472206) in the lab. Enzyme kinetics has been studied to obtain best catalytic performance and enhanced reusability with greater storage ability.

Also, the attachment was characterized by Transmission Electron Microscopy (TEM).

2. MATERIAL AND METHODS

The (L- Asparaginase) produced by Bacillus cereus strain BHU-MBU BC1 (ACCESSION No. KJ472206) was considered as crude enzyme. The chemicals for buffers preparation were of analytical or electrophoretic grade from Merck Eurolab GmbHDamstadt, Germany. All other chemicals and reagents were purchased from

SigmaAldrich(St. Louis, MO). Milli Q (Millipore, Bedford, MA) water with a resistance of higher than 18 MΩ was used all throughout the experiments.

2.1 Graphene Synthesis

Section Graphene oxide was synthesized using expanded graphite following a modified Hummer's method[11]. This method is facile, inexpensive and avoids use of strong acids. Expanded graphite was pressed initially in a roller followed by liquid nitrogen treatment. A sheet like structure is obtained which is further grinded to form a powder and any threads or granules present in the powder is sieved. As obtained graphite powder was treated with potassium permanganate and H_2SO_4 in an ice water bath under continuous stirring until a uniform liquid paste was formed with a large volumetric expansion. Deionized water was added, and rapid stirring was restarted to prevent effervescing. After placing the flask at 90° C for about 1 hour a homogeneous suspension of dark yellow in color was obtained. The suspension was then filtered and was subjected to repeated washing and centrifugation (10000 rpm, 20 min per cycle) to remove unwanted impurities. To fully exfoliate, the Graphene Oxide (GO) sheets were thermally exfoliated by rapid heating at 1050^0C for 30 seconds under an Argon atmosphere. The product was isolated by filtration and washed copiously with water and vacuum dried. TEM spectroscopy was employed for the purpose of characterization

2.2 Production of Enzyme (L-Asparaginase)

Crude enzyme was prepared by following two steps:

a. **Preparation of Microbial Culture:** Bacillus cereus strain BHU-MBU BC1 (ACCESSION No. KJ472206) used in the present investigation was obtained from our lab. Colleague Gundampati et al (2014)[12]. The strain was maintained on LB-agar medium (3% LB and 2 % agar) (pH 7.0) at 30 ^{0}C for 24h. The culture slants with grown culture were stored at 4ºC and was sub-cultured at every month interval and stored at 4°C.

b. **Production of L-asparaginase in Shake Flask Cultures :** The production of L-asparaginase was performed in the optimized semisynthetic medium (modified- Czapekdox medium) containing (g/L): glucose, 2.0; L-asparagine, yeast extract, 1.0 and peptone etc., 1.0 at initial pH of 6.5. After 48 h fermentation, the culture was centrifuged at 10000 rpm for 10 min at 4 ± 1ºC and supernatant was used as crude enzyme.

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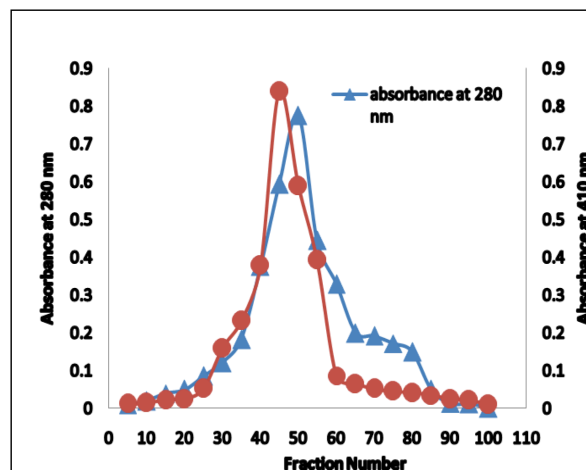
This crude enzyme was subjected to estimate the asparaginase activity and total protein was estimated by Bradford methods.

2.3 Assay of Asparaginase activity

The enzyme of different preparation was assayed by nesslerization method of Wriston and Yellin (1973)[13] with some modifications. L-Asparaginase activity was measured in a reaction mixture containing 0.5 mL of 10 mM L-asparagine prepared in 50 mM Sodium phosphate buffer (pH 7.9), 0.2 mL of the enzyme and 1.8 mL of assay buffer at 37 °C for 30 min. Subsequently, the reaction was terminated by adding 1 mL of trichloroacetic acid (15 %w/v). The ammonia liberated in the supernatant was estimated spectrophotometrically at 410 nm by adding 0.5 mL Nessler's reagent

2.4 Asparaginase Purification and Quantification



The enzyme (L- Asparaginase) produced by Bacillus cereus strain BHU-MBU BC1 (ACCESSION No. KJ472206) was considered as crude

Fig.1 Elution profile of Asparaginase

enzyme. The isolated enzyme was purified in a single step through Ion Exchange Chromatography (DEAE-Sepharose column) at 4 °C to apparent homogeneity .

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Ion-Exchange Chromatography: The crude enzyme solution was loaded onto DEAE-Sepharose column (2.0x10.0 c.m) which was pre-equilibrated with 50 mM Sodium phosphate buffer pH 7.9 and was eluted with a linear gradient of 0 – 1M NaCl in the same buffer. The fraction having Asparaginase activity were pooled as revealed in **Fig. 1** and concentrated by Amicon membrane

Dialysed enzyme solution was loaded onto DEAE-Sepharose column (2.0 x10.0 c.m) and eluted with a linear gradient of 0 – 1M NaCl. Various fractions were assayed for the protein (at 280 nm) and activity (at 410 nm).

Immobilization of l-Asparaginase

Method of Immobilization: Opted two method of Immobilization i.e. Direct and Indirect Immobilization.

Direct Immobilization: Functionalized Graphene was first equilibrated with50 mM Sodium phosphate buffer (pH 7.9) at 4 °C for overnight then centrifuged and precipitated graphene was dissolved in 2 ml of same buffer with 200 µl of purified enzyme (.104 mg / ml) and kept it 4 °C for overnight. Next day sample was centrifuged and precipitate showing Asparaginase activity was considered as immobilized Enzyme.

Indirect Immobilization: Functionalized Graphene was first equilibrated with50 mM Sodium phosphate buffer (pH 7.9) at 4 °C for overnight then centrifuged and precipitated graphene was treated with Cystein and Glutarldehyde then proceeds the same process as in case of Direct immobilization which also shows the Asparaginase activity but not better than Direct Immobilzed Asparaginase.

Reusability of Immobilized Enzyme: The immobilized Asparaginase was stored in 50 mM sodium phosphate buffer pH 7.9 at 4 ^{o}C. The activities of consecutive samples were assayed at interval of 24 hr, using standard assay conditions. The immobilized enzyme shows 80 % of activity even after 12 times use.

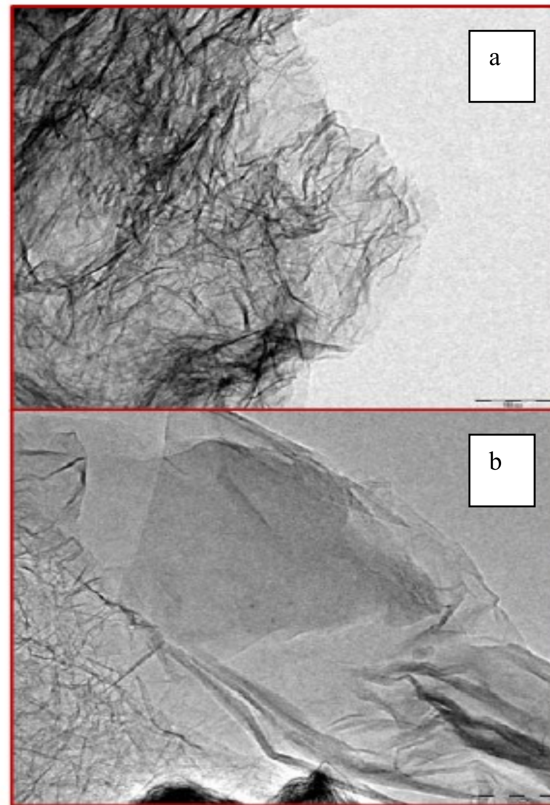

Fig 2 TEM micrograph of. (a) Graphene sheet and(b)Asperginase attached Graphene sheet

3. MICROSTRUCTURAL CHARACTERIZATION OF GS AND ASPERGINASE-GS

Functionalized graphene sheets (both native and coupled) were characterized using transmission electron microscope (TEM; Technai 20 G^2, 200 kV), .For TEM studies, a drop of sample was placed on electron microscope 200 mesh copper grid and allowed to evaporate the water to complete dryness of the sample, followed by loading into the machine.

The morphology and microstructure of the as prepared GS and Asperginase-GS samples were characterized by TEM. A representative TEM image of GS sample is shown in Fig 2 (a,b) .Graphene sheet show a typical fluffy morphology but when it is attached to enzyme Asperginase it showed reduction in wrinkles .

It is assumed that asperginase is adhered non covalently using pi-pi bonding available on the surface of the graphene sheet.Further this was confirmed by enzyme assay. As immobilized enzyme will further be utilized for the quantification of asperagine present in the blood samples of Leukaemia patients.

4. CONCLUSIONS

Graphene was successfully synthesized from expanded graphite. As synthesized Graphene sheet was used for the attachment of Asperginase enzyme which was purified from the bacterial source. Its kinetic studies and reusability was studied The immobilized enzyme shows 80 % of activity even after 12 times use. The temperature optima of the immobilized enzyme was also found to be improved from 45 º to 55º C.

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