International Journal of Biological Macromolecules 77 (2015) 105–113

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Cellulase assisted synthesis of nano-silver and gold: Application as immobilization matrix for biocatalysis

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ARTICLE INFO

Article history:
Received 28 November 2014
Received in revised form 19 February 2015
Accepted 9 March 2015
Available online 19 March 2015

Keywords:
Cellulase
Biocatalysis
Nanoparticles

ABSTRACT

In the present study, we report in vitro synthesis of silver and gold nanoparticles (NPs) using cellulase enzyme in a single step reaction. Synthesized nanoparticles were characterized by UV–VIS spectroscopy, Dynamic Light Spectroscopy (DLS), Transmission Electron Microscopy (TEM), Energy-dispersive X-ray Spectroscopy (EDX), X-ray Diffraction (XRD), Circular Dichroism (CD) and Fourier Transform Infrared Spectroscopy (FTIR). UV–visible studies shows absorption band at 415 nm and 520 nm for silver and gold NPs respectively due to surface plasmon resonance. Sizes of NPs as shown by TEM are 5–25 nm for silver and 5–20 nm for gold. XRD peaks confirmed about phase purity and crystallinity of silver and gold NPs. FTIR data shows presence of amide I peak on both the NPs. The cellulase assisted synthesized NPs were further exploited as immobilization matrix for cellulase enzyme. Thermal stability analysis reveals that the immobilized cellulase on synthesized NPs retained 77–80% activity as compared to free enzyme. While reusability data suggests immobilized cellulase can be efficiently used up to sixth cycles with minimum loss of enzyme activity. The secondary structural analysis of cellulase enzyme during the synthesis of NPs and also after immobilization of cellulase on these NPs was carried out by CD spectroscopy.

1. Introduction

In recent times, synthesis of noble metal nanoparticles (NPs) like silver and gold is gaining attention due to their unique properties and applications in various areas [1,2]. For the synthesis of metal NPs number of methodologies has been described in the literature [3,4]. Broadly, they can be synthesized either by physical, chemical or biological methods [3–5]. Physical and chemical methods are generally energy consuming and require toxic ingredients/hazardous materials which makes them 'not so favored' methods for synthesis [6,7]. The biological method of nanoparticle synthesis is a relatively simple, cost effective and environment friendly method than the conventional chemical/physical method of synthesis and thus gains an upper hand.

A vast array of biological resources available in nature including plants and plant products, algae, fungi, yeast, bacteria, and viruses could be employed for synthesis of NPs [2,8,9]. Biosynthetic approach has led to the fabrication of several inorganic NPs, including silver, gold, copper, palladium, etc. [10,11]. Till date, the exact mechanism of nanoparticle synthesis by biological resources has not been fully understood. Studies indicate that proteins/enzymes play a major role in a biosynthesis process as they consist of number of amino acids which are reported to interact with the metal ions [12–15].

Xie et al. [16] demonstrated that proteins are the principal biomolecules which are involved in the algal synthesis of gold NPs. Ahmad et al. [17] postulated that a NADPH dependent reductase is involved in silver nanoparticle synthesis by *Fusarium oxysporum*. However, the biochemical mechanism of metal ion reduction and subsequent nanoparticle formation remains unexplored. Eby et al. [18] described that hen egg white lysozyme in the presence of light can act as the sole reducing and capping agent for the formation of silver NPs. Rangnekar et al. [19] described the synthesis of gold NPs using a pure enzyme alpha amylase for the reduction of $AuCl_4^-$, with the retention of enzymatic activity in the complex. They suggested that the presence of free and exposed —SH groups is essential in the reduction of $AuCl_4^-$ to gold NPs. Ravindra [20] reported that lysine from serrapeptase enzyme is involved in the synthesis and stabilization of gold NPs. Gupta et al. [21] describe the synthesis of silver NPs using native BSA and DTNB modified BSA. They found that modified and unmodified BSA are equally good for the biosynthesis of silver NPs [21]. In our lab we have synthesized and characterized gold and silver NPs using an enzyme alpha amylase from *Aspergillus oryzae* [3,22]. Recently, we reported the

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http://dx.doi.org/10.1016/j.ijbiomac.2015.03.014
0141-8130/

synthesis of silver NPs using neem leaf extract containing alpha amylase enzyme [23]. This enzyme activity was retained on the NPs during synthesis. Several studies on immobilization of enzymes have shown that stability and reusability of enzymes increases after immobilization on nanomaterials [24,25]. Therefore, in the present study synthesis of silver and gold NPs using cellulase enzyme has been described. The boiosynthesized NPs were further exploited as immobilization matrix for cellulase enzyme.

2. Experimental

2.1. Materials

Cellulase (from *Aspergillus niger*) and auric chloride were procured from Sisco Research Laboratories (SRL), Mumbai, India. Silver nitrate was purchased from Merck India Ltd. Carboxy methyl cellulose sodium salt (CMC) was obtained from Qualigens Fine Chemicals Pvt., Ltd. All other chemicals and solvents used were of analytical grade and used without further purification.

2.2. Biosynthesis of silver and gold NPs by cellulase enzyme

Biosynthesis of the silver and gold NPs was carried out by incubating 10 ml of cellulase enzyme (1 mg/ml for silver and 2 mg/ml for gold in Tris–HCl buffer, pH 8.0) and 90 ml of freshly prepared aqueous solution of silver nitrate (1 mM)/auric chloride (1 mM) in each case. Solutions were kept at 25 °C and the syntheses of NPs were monitored by UV–VIS spectroscopy. The biosynthesized NPs were purified as described earlier [26].

2.3. Characterization of purified NPs

Dynamic light scattering (DLS) measurements were done by using the Spectroscatter RiNA, GmbH class3B at 20 °C for 10 cycles. Samples for Transmission Electron Microscopy (TEM) were prepared by drop coating purified solution of silver and gold NPs on to carbon-coated copper TEM grids. TEM measurements were performed on a JEOL, F2100 instrument operated at an accelerating voltage at 200 kV. An EDX (Model EVO-40, ZEISS) spectrum was also recorded for elemental analysis of above prepared sample. XRD patterns of both NPs were recorded by X'Pert Pro X-ray diffractometer (PANanlytical BV) by operating X-ray tube at 45 kV and 35 mA and radiation used was Cu-Kα. Fourier transform infrared (FTIR) spectra were recorded with a Shimadzu, FTIR spectrophotometer between 4000 and 400 cm^{-1}, with a resolution of 4 cm^{-1}. Circular Dichroism (CD) measurements of free cellulase, cellulase assisted silver and gold NPs and immobilized cellulase on silver and gold NPs in sodium acetate buffer (pH 4.5, 50 mM) were carried out on applied photophysics circular dichroism spectropolarimeter using 1 nm/10 s signal. The CD instrument was consistently calibrated with D-10 camphorsulfonic acid and N_2 purging was continuously done in the lamp, optics and sample chamber in a ratio of 1:3:1. Each spectrum was corrected for blank contribution.

2.4. Activity measurement of cellulase enzyme

Cellulase activity in free enzyme and immobilized cellulase on silver and gold NPs were measured by using CMC as a substrate [27]. The immobilized cellulase on silver and gold NPs was continuously shaken during the assay. One unit of enzyme is defined as the amount of enzyme required to produce 1 μmol of reducing sugar per min under defined conditions. The amounts of reducing sugar were estimated using the dinitrosalicyclic acid methods [28].

2.5. Protein estimation

Amount of protein was estimated by the dye binding method (Bradford dye), using bovine serum albumin as the standard [29].

2.6. Immobilization of cellulase on silver and gold NPs

Immobilization of cellulase on silver and gold NPs was carried out by physical adsorption method. Cellulase enzyme solutions (containing 680 U for silver and 350 U for gold, each dissolved in 50 mM sodium acetate buffer, pH 4.5) were incubated with 5 mg of silver and gold NPs respectively, suspended in 1 ml of 50 mM sodium acetate buffer, pH 4.5. The mixtures were incubated at 25 °C with constant shaking after 1 h they were centrifuged at 9000 rpm for 5 min at 4 °C. The silver and gold NPs containing the adsorbed enzymes were washed with 50 mM sodium acetate buffer pH 4.5 containing 1 M NaCl and 50% Ethylene glycol to remove the loosely bound enzyme. The enzyme activities on both the NPs were determined in the supernatants and washings. To calculate and estimate the immobilization efficiency on both the NPs, the enzyme loads were varied for silver (220–1200 U), for gold (175–1100 U) and the immobilization was carried as above. All the experiments were conducted in the batch mode.

2.7. Thermal stability of free enzyme and immobilized cellulase on silver and gold NPs

Free enzyme and immobilized cellulase on silver and gold NPs were incubated in 50 mM sodium acetate buffer (pH 4.5) at 75 °C for 60 min and residual activities were assayed using CMC as the substrate in each case. An appropriate aliquot of free enzyme and immobilized cellulase on silver and gold NPs (containing 680 U for silver and 350 U for gold) were withdrawn at various time intervals of incubation, cooled to 25 °C and their activities were determined.

2.8. Reusability of the immobilized cellulase on silver and gold NPs

The immobilized cellulase on silver and gold NPs (680 U for silver and 350 U for gold) was made to 0.5 ml with the assay buffer and incubated with 0.5 ml of the substrate under shaking condition at 50 °C, separately. After 30 min the supernatants were removed by centrifugation at 9000 rpm for 5 min at 4 °C and the enzyme activities were estimated in the supernatants. The immobilized cellulase on silver and gold NPs was washed three times with 1 ml of assay buffer. For second cycle the immobilized cellulase on silver and gold NPs was again incubated with 0.5 ml of fresh substrate and the reaction was carried out as before in both the immobilized enzymes.

3. Results and discussion

In the present study, noble metal NPs like silver and gold were biosynthesized using cellulase enzyme (from *A. niger*). Extracellular biosynthesis of silver [30] and gold NPs [31] has been reported earlier also using *A. niger*. Similarly, synthesis of silver and gold NPs using extracellular secretions of *A. niger* has been reported by Jaidev and Narasimha [32]. They suggested the role of different extracellular enzymes/proteins present in the supernatant catalysis the synthesis, especially the involvement of nitrate reductase has been described by them. Several other investigators also claimed that during the biosynthesis of silver and gold NPs using fungal secretions major players are the proteins/enzymes [30–32]. Cellulase from *A. niger* is also an extracellular enzyme, it catalyzes the multistep hydrolysis of cellulose to glucose [33]. The cellulase