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Immobilization of yeast alcohol dehydrogenase on polyaniline coated silver nanoparticles formed by green synthesis

Md. Fazle Alam[a], Amaj Ahmed Laskar[a], Mohd. Zubair[a], Umair Baig[b,c], Hina Younus[a,*]

[a] Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh 202002, India
[b] Center of Excellence for Scientific Research Collaboration with MIT, King Fahd University of Petroleum and Minerals, Dhahran 31261, Saudi Arabia
[c] Laser Research Group, Physics Department & Center of Excellence in Nanotechnology King Fahd University of Petroleum and Minerals, Dhahran 31261, Saudi Arabia

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ABSTRACT

In this study, silver nanoparticles (AgNPs) have been synthesized by green synthesis using *Ziziphus mauritiana* fruit extract and then yeast alcohol dehydrogenase (YADH) has been immobilized on polyaniline coated AgNPs. The AgNPs were characterized using UV–Vis spectroscopy, TEM and FT-IR. These NPs consisted of almost mono-dispersed spherical NPs of average diameter of ~30 nm and exhibited high storage stability at 4 °C for many days. YADH immobilized on polyaniline coated AgNPs exhibited good activity with effectiveness factor (η) value of 0.73. Immobilization did not alter the optimum pH (pH 8.0) of YADH, however the optimum temperature increased by 5 °C. The immobilized enzyme retained greater fraction of activity both in the acidic and alkaline pH range and at higher temperatures as compared to the soluble enzyme. At 70 °C, immobilized YADH retained about 78% activity as compared to 26% retained by the soluble enzyme. The immobilized enzyme also exhibited high thermal stability at 50 °C. Therefore, inexpensive and eco-friendly green synthesis of NPs and excellent activity and stability of YADH immobilized on polyaniline coated AgNPs provides a cost-effective industrial application of the enzyme.

1. Introduction

AgNPs have various diverse applications such as electrical conductivity, catalysis, sensors, biomedical devices, antibacterial, antiplasmodial, anti-inflammatory, anticancer activities and protein aggregation [1–5]. The synthesis of NPs by chemical methods employs the use of toxic chemicals and has the tendency to release harmful by-products. This can be overcome by using green synthesis approach which is inexpensive and eco-friendly. A number of reports have demonstrated the use of plant extracts for the synthesis of AgNPs [3,5–7].

ADH catalyzes the reversible oxidation of alcohols to their corresponding carbonyl compounds [8]. It has great application in the chemical industry for the production of various starting materials and intermediates, the synthesis of chiral compounds, the regeneration of coenzymes NAD(P) and NAD(P)H, and biosensors [9–11]. However, it has low stability which limits its industrial application [12–14]. Immobilization of enzymes on solid support is one of the most important strategies to improve operational stability and product recovery [15]. Immobilization of industrially useful enzymes onto nanomaterials which results in improved performance has paved the way to application-based commercialization [16]. Immobilizing enzymes on NPs has various benefits i.e., large surface to volume ratio for binding a large quantity of the enzyme, lower mass transfer resistance, high catalytic efficiency and hence activity, and enhanced stability [17,18]. The immobilization of ADH on magnetic NPs (Fe_3O_4) has been reported to increase its stability [19,20].

Polyaniline is an environmentally stable polymer with many applications [21]. It has been shown to exhibit high stability at extreme temperature and pH, biocompatibility, and resistance to microbial attacks [22,23]. Ag and Au NPs have good electronic properties, and larger and stable surface area for enzyme immobilization [24]. They easily absorbed protein molecules on their surface without much activity loss and conformational changes [25,26]. Because of their good electrical conductivity, Ag and Au NPs assist in the electron transfer and thus help in the redox reactions catalyzed by

Abbreviations: AgNPs, silver nanoparticles; YADH, yeast alcohol dehydrogenase; TEM, transmission electron microscopy; FT-IR, Fourier transform infrared spectroscopy; η, effectiveness factor.

* Corresponding author. Tel.: +91 571 2720388; fax: +91 571 2721776.
E-mail address: hinayounus@rediffmail.com (H. Younus).

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oxido-reductases [27]. Ag has the highest electrical conductivity among all metals and is much cheaper than Au [28,29]. Therefore, in this study Ag was used for the green synthesis of NPs for the immobilization of YADH. There are many reports in which AgNPs have been used for the immobilization of enzymes i.e., urease [30], glucose oxidase [31], ADH [24], lipase [32], etc.

Recently it has been reported that α-amylase immobilized on polyaniline-assisted AgNPs exhibited better tolerance to changes in pH and temperature, and was catalytically more efficient than the soluble enzyme [21]. In the present study, AgNPs have been prepared using the green chemistry approach i.e., aqueous extract of *Ziziphus mauritiana* has been used to reduce $AgNO_3$ to Ag colloids. We have then synthesized polyaniline AgNPs and have coupled YADH onto them with the aim of increasing its catalytic efficiency and stability for its potential application in the chemical industry.

2. Experimental

2.1. Chemicals

YADH and glutaraldehyde were purchased from Sigma (St. Louis, MO, USA). Silver nitrate and aniline were the products of Qualigens Fine Chemicals, India. NAD^+ and ammonium persulphate were obtained from SRL Chemicals (Mumbai, India). The other chemicals and reagents used were of analytical grade.

2.2. Green synthesis of AgNPs

Ziziphus mauritiana was purchased from Aligarh (India) local market. The fruit (250 g) was chopped and boiled in 500 ml of water for 30 min and then cooled. It was then filtered and the filtrate was collected as the stock. $AgNO_3$ (10 mM) was added drop wise to the stock in a volumetric ratio of 1:1, 1:3, 1:5 or 1:7 with continuous stirring for about 30–45 min. The absorbance was measured at a regular interval till a sharp peak was observed around 390–420 nm. The solution was then centrifuged at 13,000 rpm for 30 min and the pellet was collected and lyophilized to obtain the AgNPs in powder form. The AgNPs obtained were then characterized.

2.3. Coating of green synthesized AgNPs with polyaniline

Coating of green synthesized AgNPs with polyaniline was achieved following the procedure described with some modifications [33,34]. 30 mg of AgNPs (dried at 50 °C for 2 h before use) were dispersed in approximately 100 ml of distilled water under ultrasonic vibrations (SC-I, Chengdu Jiuzhou Ultrasonic Technology Co.) at room temperature for 1 h. This dispersed solution was then diverted into a 500 ml single-necked, round-bottom flask equipped with a magnetic, Teflon coated stirrer and 2 ml of 99.5% aniline (monomer) was added. The mixture was stirred for 30 min for the adsorption of aniline on the surface of AgNPs. 100 ml of 1 M ammonium persulphate was added to the dispersion and later sonicated for 1 h. This reaction mixture was stirred for an additional 24 h under the same condition. The resultant dark green polyaniline coated AgNPs were filtered with a Buchner funnel, and then washed with distilled water till the pH of the solution reached to neutral. The obtained powders were dried completely at 80 °C for further studies.

2.4. Immobilization of YADH on polyaniline coated AgNPs

Polyaniline coated AgNPs were used to couple YADH. For this purpose, the polyaniline coated AgNPs were treated with glutaraldehyde (2.5% v/v) in sodium phosphate buffer, pH 6.0, and then the mixture was constantly stirred at 4 °C for 2 h. Glutaraldehyde, a cross-linking agent activates the NH_2 group of aniline. This activated nano-support was then dialyzed to remove the unbound glutaraldehyde and then lyophilized to powder form. The activated nano-support (10 mg) was suspended in 2.5 ml of 100 mM Tris–HCl buffer (pH 8.0) and then 2.5 ml of 1 mg/ml of YADH was added to it. It was then continuously stirred at 4 °C for overnight and then centrifuged at 8000 rpm for 10 min to get the pellet which was washed thrice with the Tris–HCl buffer to remove the unbound enzyme. The η value was calculated by taking the ratio of theoretically bound YADH to actual bound YADH [35]. The process of covalent conjugation of YADH with polyaniline coated AgNPs has been shown schematically in Fig. 1.

2.5. Characterization of AgNPs

The absorbance spectra (350–600 nm) of AgNPs were monitored using double beam Perkin Elmer Spectrophotometer (Lambda 25). Fourier transform infrared (FTIR) spectroscopy of the AgNPs was done using a Perkin Elmer 1725 FTIR spectrophotometer. The spectra were recorded in a range of 400–4000 cm^{-1} with an average of over 15 scans. The surface morphology and size of AgNPs was studied by transmission electron microscopy (TEM) which was performed on a JEOL Model JEM-1200EX instrument operated at an accelerating voltage of 80 kV.

2.6. Activity assay of soluble and immobilized YADH

The activity of YADH was measured spectrophotometrically using ethanol as the substrate. The standard reaction mixture in a total volume of 1 ml contained 100 μg of YADH, 0.3 mM NAD^+ and 0.1 M ethanol in 100 mM Tris–HCl buffer, pH 8.0. The reaction was initiated by the addition of ethanol, followed by incubation at 40 °C for 3 min and subsequently the increase in absorbance at 340 nm was measured which is due to formation of NADH [36]. Appropriate reaction blanks were used for both the soluble and immobilized YADH, that is for the soluble enzyme all components of the reaction mixture were present in the blank except the enzyme, and for the immobilized enzyme all components of the reaction mixture including polyaniline coated AgNPs were present in the blank expect the immobilized enzyme.

2.7. Stability studies of soluble and immobilized YADH

To study the effect of pH on the activity of YADH, the activity of both soluble and immobilized enzyme was determined by standard assay conditions as described above using 100 mM buffer of different pH values. The buffers used were glycine–HCl (pH 2.0 and 3.0), sodium acetate (pH 5.0), sodium phosphate (pH 7.0), Tris–HCl (pH 8.0 and 9.0) and glycine–NaOH (pH 10.0 and 12.0). The pH at which the enzyme expressed highest activity was taken as the control (100% activity) for the calculation of the remaining percent activity for both the soluble and immobilized YADH. To determine the effect of temperature on the activity of soluble and immobilized YADH, both preparations were assayed using the standard reaction conditions except that the reaction was performed at different temperatures (25–70 °C). The temperature at which the enzyme exhibited highest activity was taken as control to calculate the remaining percent activity for both the soluble and immobilized YADH preparations.

To determine the thermal stability of soluble and immobilized YADH, both preparations were incubated at 50 °C for varying time period, cooled over ice for 2 min, and then the activity was determined under standard assay conditions. The storage stability of both soluble and immobilized YADH was determined for 20 days. Both soluble and immobilized YADH were stored at 4 °C and aliquots were removed at each day for activity measurements.