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Preparation and characterization of a green nano-support for the covalent immobilization of *glucoamylase* from *Neurospora sitophila*

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ABSTRACT

The preparation of green nano supports for the covalent immobilization of enzymes is of special interest both from the economic and environmental point of view. In this contribution, we report on the synthesis of phytochemicals coated silver nanoparticles, which were used as a novel green support for the covalent immobilization of *glucoamylase* isolated from *Neurospora sitophila*. The aqueous extract of *Fagonia indica* was used as a source of reducing and capping agents for the reduction of silver ions into silver nanoparticles. The prepared nanoparticles were characterized by various analytical techniques. UV–visible spectroscopy was used to detect the characteristic surface plasmon resonance bands (426, 438 nm) of the silver nanoparticles. The biosynthesized silver nanoparticles were mostly spherical in shapes with an average particle size of 30–40 nm (TEM and DLS measurements). X-ray diffraction and energy dispersive X-ray studies confirmed the face centered cubic crystalline form and elemental composition of the biogenic silver nanoparticles respectively. FTIR study revealed that plant polyphenolics and protein were mainly involved in the reduction and capping of silver ions. *Glucoamylase* from *Neurospora sitophila* was covalently immobilized to these nanoparticles via EDC (1-(3-(dimethylamino) propyl) 3-ethylcarbodiimidehydrochloride) coupling reaction. The immobilized enzyme exhibited higher pH and thermal stabilities as compared to the free enzyme. The kinetic constant (K_M) value for the immobilized *glucoamylase* was higher (0.73 mg/mL) than its free counterpart (0.44 mg/mL), whereas the V_{max} value was slightly higher for the immobilized *glucoamylase*. The findings of this study conclude that the newly developed green method for the synthesis of green nano-support is simple, cost effective and could be successfully used for the immobilization of various enzymes and other macromolecules.

1. Introduction

Various industrial processes exploit the vital role of biological catalysts in the manufacturing of commercially important products [1]. The industrial applications of amylolytic (alpha-amylase, EC 3.2.1.1 and *glucoamylase*, EC 3.2.1.3) enzymes have been extensively exploited in the fields of food processing, brewing, pharmaceuticals, paper, sugar, detergents, textiles and environmental waste decomposition etc. [2,3]. Nowadays, the overall global production of *glucoamylase* is around 26% of the total enzymes production after proteases [4]. *Glucoamylase* hydrolyzes both starch and glycogen in a stepwise mechanism releasing a single glucose unit and therefore, has widespread uses in industry for the preparation of glucose syrup or crystalline glucose.

A large number of microorganisms including bacteria, fungi and yeast have been reported for *glucoamylase* production under solid-state fermentation [5,6]. However, filamentous fungi are the most important and widely used microbe for protein production. *Neurospora* is considered as a model of model microorganisms for biochemical genetics and molecular biology, and therefore, is of significance importance from the biotechnological point of view [7]. *Neurospora sitophila* has a great potential of growing on a large number of agro wastes products under the ambient conditions [8], and could be an ideal microbe for the production of industrially important enzymes. Furthermore, because of its great cellulose hydrolyzing potential, *Neurospora sitophila* is one of the fastest growing fungi. Based on the above-mentioned properties, *Neurospora sitophila* could be a valuable microbe for *glucoamylase* production using low-cost agricultural byproducts. The industrial viability of enzymes is majorly dependent on their physiochemical stabilities, and enzymes that are stable at a wide range of pH and temperature are very important from the biotechnological point of view. To enhance the biological activity, lifetime, pH and thermal stabilities of enzymes, these biological catalysts are immobilized on suitable solid supports that provide mechanical strength against pH and heat inactivation for a long time uses [9–11]. Various supports that include mesoporous silica, MCM-41 molecular sieve, functionalized glass beads, calcium alginate

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gel beads, polyaniline polymer and epoxy functionalized beads have been utilized for enzymes immobilization [12–17].

In recent years, nanoscale materials have attracted significant attention for their great potential to serve as superior solid supports for enzyme immobilization [11,18]. These nanoscale materials have large surface to volume ratios as compared to the conventional macroscale materials, and would present a large surface area for binding a larger amount of enzyme. Furthermore, these conjugated nanocatalysts can be easily recovered from the reaction mixture for recycling purposes. Among the metal nanoparticles, silver-based nanomaterials have a wide range of applications in various scientific disciplines such as catalysis, electronics, and pharmaceuticals [19–21]. In addition, these nanoparticles could be successfully utilized as a biocompatible solid support for the immobilization of industrially important enzymes. Biological synthesis of metal nanoparticles would provide a versatile source of nano-supports for the covalent immobilization of enzymes. Biogenic silver nanoparticles possess various functionalities, which could be utilized for the covalent immobilization of enzymes via some coupling reagents. Among the bioactive macromolecules, phenolic compounds are the most active surface functional groups that can be linked with the carboxylic groups of enzymes by an ester bond [22]. Plants produce a significant amount of flavonoids and other phenolic compounds, which they use as antimicrobial and antioxidant agents. These bioactive phytochemicals can be successfully utilized for the preparation of metal nanoparticles [23].

In the present study, silver nanoparticles (AgNPs) were prepared by a green method using the aqueous extract of *Fagonia indica* as reducing and capping agents. The prepared nanoparticles were characterized by various analytical techniques and were used as a green support for the covalent immobilization of *glucoamylase* from *Neurospora sitophila*. 1-(3-(dimethylamino) propyl) 3-ethylcarbodiimidehydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were used as coupling agents to establish an ester bond between the hydroxyl groups present on the surface of nanoparticles and carboxylic group of *glucoamylase*. This is the first report describing the covalent immobilization of enzyme on the surface of biologically synthesized silver nanoparticles.

2. Materials and Methods

2.1. Preparation of Plant Extract

A known amount (20 g) of powdered plant material (*Fagonia indica*) was extracted with 250 mL of milli-Q water. The aqueous suspension of *Fagonia indica* was initially heated at 60 °C for 45 min and then magnetically stirred (400 rpm) for 1 h at room temperature (26 °C). The resulting phytochemicals from the extracted biomass were recovered by vacuum filtration (Whatman filter paper No.1). The water extract of *Fagonia indica* thus obtained was stored at 4 °C for further use.

2.2. Synthesis of Biogenic Silver Nanoparticles

The *Fagonia indica* mediated synthesis of biogenic silver nanoparticles was carried out by the method as described in our previous work [23]. Briefly, 15 mL of *Fagonia indica* aqueous extract was treated with 3 mM silver nitrate solution (50 mL) and magnetically stirred (450 rpm) at room temperature (26 °C). The appearance of a dark green color indicated the formation of silver nanoparticles. The reaction mixture was stirred for 6 h in the dark for aging of the growing nanoparticles. After 6 h of incubation, the nanoparticles suspension was centrifuged at 10,000 rpm for 10 min and the resulting pellet (AgNPs) was washed thrice with Milli-Q water to remove un-reacted silver and plant phytochemicals. The purified nanoparticles were freeze dried and stored at room temperature for further uses.

2.3. Characterization

Various analytical techniques were used to study the physical characteristics of the prepared silver nanoparticles. The initial identification of the nanoparticles synthesis was confirmed by UV–visible spectroscopy (Spectrophotometer Shimadzu 2450). Silver nanoparticles reveal a specific surface plasmon resonance (SPR) peaks that are identified by UV–visible spectroscopy. The crystalline structure of AgNPs was studied using powder X-ray diffractometer (Powder X-ray-D8 advanced diffractometer, BRUKER). The energy dispersive X-ray (JEOL-JEM 3010) technique was employed to investigate the elemental composition of the as-synthesized green silver nanoparticles. Transmission electron microscopy (FEI-Tecnai G^2 20 transmission electron microscope) and dynamic light scattering techniques (HORIBA Zetasizer SZ100) were used to determine the particles size and morphology. The surface charge of the prepared nanoparticles was determined by measuring the zeta potential value (HORIBA Zetasizer SZ100). The possible phytochemicals that are involved in the reduction of silver and capping were identified by FTIR spectroscopy (BRUKER 3000 Hyperion Microscope).

2.4. Immobilization of Glucoamylase on Biogenic Silver Nanoparticles

For the immobilization of *glucoamylase* on the surface of biogenic silver nanoparticles, 50 mg of biogenic silver nanoparticles (*Fagonia indica*) was sonicated in 3 mL acetate buffer (0.05 M, pH 5) for 15 min. Then 5 mL (1 mg/mL) of enzyme solution, 2 mL (1 mg/mL) 1-(3-(dimethylamino) propyl) 3-ethylcarbodiimidehydrochloride (EDC) and 2 mL (1 mg/mL) *N*-hydroxysuccinimide (NHS) in acetate buffer were mixed with the silver nanoparticles [22]. The reaction mixture was magnetically stirred (140 rpm) at room temperature for 8 h in dark condition. After 8 h of incubation, the reaction mixture was centrifuged at 14,000 rpm (4 °C) for 15 min and the pellet (enzyme-conjugate) obtained was washed three times with acetate buffer to remove unreacted moieties. The supernatant after each washing step was collected and analyzed for unbound enzyme.

2.5. Determination of Glucoamylase Loading

The total amount of loaded *glucoamylase* on the biosynthesized silver nanoparticles was determined by the following formula.

$\%\text{loading} = \frac{m - C_1 V_1}{m} \times 100$ where m (mg) is the total amount of *glucoamylase* added to the solution, C_1 (mg/mL) is the concentration of *glucoamylase* in supernatant and V_1 (mL) is the total volume of the supernatant.

After the enzyme immobilization, the unreacted portion (supernatant) was collected for calculating the loaded amount of the immobilized enzyme. The total protein content before and after immobilization was determined by a standard protein assay (Bradford protein assay) [24].

2.6. Enzyme Activity Measurement

The enzymatic activities of free and immobilized *glucoamylase* were determined by 3,5-dinitrosalicylic acid method using soluble starch as a substrate [25]. Briefly, 0.1 g of starch was boiled in 100 mL acetate buffer (0.1 M, pH 5) for 15 min to obtain a homogeneous starch solution. The enzyme activity of free and immobilized *glucoamylase* was assayed at 45 °C in a reaction mixture containing 1 mL of diluted enzyme and 0.5 mL of substrate solution in sodium acetate buffer pH 5. The reaction mixture was incubated at 45 °C for 15 min. The reaction was stopped by the addition of 0.5 mL 3,5-dinitrosalicylic acid reagent (DNS) and then boiled for 5 min. The amount of glucose released was determined at an absorbance of 540 nm using a spectrophotometer. One unit of enzymatic activity (U) was defined as the amount of enzyme that releases one μmol of glucose per minute per mL of reaction at 45 °C.