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Green Synthesis and Antibacterial Activities of Silver Nanoparticles Using Extracellular Laccase of *Lentinus edodes*

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Abstract

This study reports the multi-step mutagenesis of *Lentinus edodes* towards optimization of the production of laccase and novel application of laccase in the biosynthesis of silver nanoparticles (AgNPs) which could be used to develop an eco-friendly method for the rapid biosynthesis of AgNPs. The wild strain of *L. edodes* was subjected to UV irradiation at 254 nm and the resultant viable mutant was further treated with acridine orange, a chemical mutagen. The strains were evaluated for the production of laccase and the crude laccase of the UV mutant (UV_{10}) was used for the green synthesis of AgNPs. The particles were characterized by UV-Visible spectroscopy, Fourier transform infrared (FTIR) spectroscopy and scanning electron microscopy (SEM). Laccase activities of wild, UV_{10} and $UV_{10}ACR_8$ strains of *L. edodes* were obtained as 2.6, 10.6 and 2.8 U/ml/min respectively after 7 days of fermentation, showing laccase yield improvement of 4.08-fold for UV_{10}mutant. UV-Visible spectroscopy indicated the formation of AgNPs at absorption band of 430 nm. FTIR result indicated that proteins were responsible for AgNP synthesis, while SEM analysis confirmed the formation of walnut-shaped nanoparticles with size range of 50-100 nm. The biosynthesized nanoparticles revealed effective inhibition against clinical isolates of *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. To the best of the authors' knowledge, this result represents the first report on the biosynthesis of AgNPs using *L. edodes* metabolite. The report adds to the growing relevance of *L. edodes* as potential industrially viable organism, used for diverse biotechnological applications.

Keywords: *Lentinus edodes*, silver nanoparticles, green synthesis, mutagenesis, antibacterial activities, laccase

Introduction

Nanobiotechnology is defined as the aspect of biotechnology that deals with synthesis, design and stabilization of various nanoparticles using biological tools (Shaligram *et al*., 2009; Lateef *et al*., 2015a). It involves manipulation of particles structure with dimension smaller than 100 nm. Nanoparticles have been classically produced by physical and chemical methods (Guzman *et al*., 2012) involving techniques like heating (Huang and Yang, 2004) and irradiation (Abid *et al*., 2002; Gasaymeh *et al*., 2010). However, these methods are costly, toxic and hazardous (Mohamed, 2013; Lateef *et al*., 2015a), hence the need for alternative, eco-friendly approaches, which may be assured by biological methods. Biological methods of synthesis of nanoparticles have shown very promising solutions to those posed by classical approaches. Green synthesis of AgNPs using diverse biological materials such as spider cobweb, Cola nitida (Kolanut) seed, seed shell, pod and cell-free extract of Bacillus safensis with potent biological activities was recently demonstrated (Lateef et al., 2015b,c).

Myconanotechnology is the interface of mycology and nanotechnology, involving the use of fungi in the biosynthesis of metal nanoparticles (Mukherjee, 2002; Lloyd, 2003; Rai *et al*., 2009). Extracts like amino acids, polysaccharides, vitamins and enzymes, as well as whole cells of plants, yeasts, bacteria and fungi have been used to mediate synthesis of nanoparticles (Kalimuthu *et al*., 2008; Acosta, 2009; Philip, 2009; Tripathy *et al*., 2010; Lateef *et al*., 2015c). Fungi are preferred mediators of nanoparticles synthesis because they are easy to handle, require simple nutrients and possess high wall-binding capacity, as well as intracellular metal uptake capabilities (Sanghi and Verma, 2009).

Metals like copper, zinc, titanium (Schabes-Retchkiman *et al*., 2006), magnesium, gold (Gu *et al*., 2003) and silver had come up for synthesis of nanoparticles (Mohamed, 2013). Silver nanoparticles have been reportedly used in treatment of burns, as dental materials, textile fabrics, water treatment and sunscreen lotions (Duran *et al*., 2007). Silver nanoparticles have applications in the production of antimicrobial paints (Kumar *et al*., 2008), non-linear optics, spectrally selective coatings for solar energy adsorption and intercalation materials for electrical batteries, optical receptors, catalyst of chemical reactions, bio-labelling and antibacterial agents (Durán *et al*., 2005). Nanoparticles have also been used in nanoscaffolds to regenerate central nervous system cells (Ellis-Behnke, 2006), bio-separation membranes (Martin and Kohli, 2003), drug delivery to target organs (Uhrich *et al*., 1999), gene transfection (Kneuer *et al*., 2000), medical imaging (Harisinghani *et al*., 2003), nucleic acid sequence and protein detection, nanophase extractors (Martin and Kohli, 2003) and treatment of local

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anaesthetic toxicity. In addition, silver nanoparticles have proved very effective as good antimicrobial agent against bacteria, viruses and other eukaryotic microorganisms (Gong *et al*., 2007; Lara *et al*., 2010).

Lentinus edodes is one of the choice edible mushrooms which can be cultivated in a warm moist climate. It occurs naturally throughout Southeast Asia and has been reported in China, Japan, Korea, Vietnam, Thailand, Burma, North Borneo, Philippines, Taiwan and New Guinea. The geographic distribution of shiitake in nature extends beyond Northeast Asia, but the exact limits are uncertain (Bisen *et al*., 2010). *Lentinus* spp. have been used by people all over the world for their nutritional value, medicinal properties and other beneficial effects (Barros *et al*., 2008; Thillaimaharani *et al*., 2013; Finimundy *et al*., 2014). In this work, a multi-step mutagenesis of *L. edodes* towards optimization of the production of laccase was undertaken, and the novel application of laccase in the biocatalysis of silver nanoparticles synthesis was demonstrated. The ability of the biosynthesized nanoparticles to inhibit pathogenic bacterial strains was also investigated.

Materials and Methods

Fungal strain

A pre-identified wild strain of *L. edodes* was obtained from Mushroom Research Centre, Solan, Himachal Pradesh, India. The culture was maintained by sub-culturing monthly and stored at 4 °C on potato dextrose agar (PDA) slants to maintain viability.

Generation of mutant strains

The process of mutagenesis was carried out by exposing a 14-day actively growing culture of the wild fungus on PDA plate (90 mm) to ultraviolet irradiation (254 nm, 15 cm) for 5, 10, 15, 20, 25, 30, 45, 60, 75 and 90 min. Mutants were immediately sub-cultured and incubated in the dark at 25 ± 2 °C for 14 days (Adebayo *et al*., 2012). Based on improved exopolysaccharide (EPS) productivity (Dubois *et al*., 1956), a viable mutant obtained at 10 min of ultraviolet irradiation (UV_{10}) was selected and subsequently exposed to chemical mutagenesis in 7.81×10^{-5} g/ml of acridine orange (BDH Chemicals Ltd, Poole, England) suspended in potato dextrose broth (PDB) (Toh-e and Wickner, 1981). The culture was incubated at 25 ± 2 °C for 72 h after which the strain was re-introduced onto freshly prepared PDA slant and incubated for 14 days to obtain fully ramified acridine mutant strain $UV_{10}ACR_8$. The wild, UV_{10} and $UV_{10}ACR_8$ strains generated were reassessed for quality improvement and used for the production of laccase.

Fermentation medium and conditions for the production of laccase

Cultures of wild and mutant strains of *L. edodes* were inoculated into 250 ml fermentation medium obtained based on five media factors (Nehad and El-Shammy, 2010) containing: glucose (40.0 g/L), yeast extract (1.1 g/L), peptone (2.0 g/L), KH_2PO_4 (2.0 g/L), $MgSO_4$ (1.7 g/L) at pH 5.5. The cultures were incubated at 25 ± 2 °C at 100 rpm for 7 days. After seven days of cultivation, laccase was obtained by sieving broth cultures of the respective strains through Whatman No 1 filter paper and centrifuged at 4,000 rpm for 15 min at 10 °C. The resultant filtrate was collected as crude enzyme fraction and used for further studies.

Laccase assay

Laccase activity was determined using the method of Ride (1980). Reaction mixtures consisted of 0.5 ml of the enzyme, 2.2 ml of phosphate buffer (0.2 M) and 0.3 ml of 0.01% syringaldehyde (Sigma-Aldrich, Germany) which was dissolved in absolute ethanol. The absorbance of the reaction mixture was determined at 530 nm on a spectrophotometer (Genesys 10 UV, Thermoelectron Corporation, UK) immediately and after 10 min of reaction. The change in absorbance was then obtained and used to calculate the laccase activities of wild, UV_{10} and $UV_{10}ACR_8$ strains of *L. edodes*.

The enzyme activity was obtained as follows:

$$\text{Unit per ml of enzyme} = \frac{\text{Change in A530 nm Per min Test - Change in A530nm Blank}}{0.01(0.5)} \times \text{Dillution Factor}$$

One unit of laccase enzyme will produce a change in absorbance of 0.01 at 530 nm per min at pH of 5.7 and room temperature in 3 ml reaction volume using syringaldehyde as substrate.

Laccase-mediated synthesis and characterization of silver nanoparticles (AgNPs)

AgNPs were synthesized by reacting crude laccase of UV_{10} which had the highest laccase activity with 1 mM silver nitrate solution as described by Lateef *et al*. (2015a). About 12 ml of crude laccase enzyme of respective strain was dispensed into reaction vessel containing 30 ml of 1 mM silver nitrate ($AgNO_3$) for the green synthesis of AgNPs. A control containing 42 ml of silver nitrate in a separate reaction vessel was also set up. The reaction was investigated at room temperature (28 ± 2 °C) with intermittent shaking. The development of colour as a result of formation of AgNPs was monitored visually, while the absorbance spectrum of the reaction mixture was measured using a UV-visible spectrophotometer (Genesys 10 UV, Thermoelectron Corporation, UK). The Fourier transform infrared (FTIR) spectroscopic analysis of the dried nanoparticles was carried out using KBr pellets on a spectrophotometer (BUCK Scientific M530, BUCK Corporation, USA) to identify the possible biomolecules responsible for the synthesis of nanoparticles. The morphology of the biosynthesized nanoparticles was elucidated through scanning electron microscopy. Scanning electron images were collected using an ASPEX 3020 in bright field mode at an accelerating voltage of 15 kV.

Antibacterial activities of the biosynthesized silver nanoparticles

The antibacterial test was carried out using agar disc diffusion method as described by Lateef *et al*. (2015a). A total of ten clinical bacterial isolates including three strains of *Escherichia coli, Klebsiella* spp. and *Pseudomonas aeruginosa* and a strain of *Staphylococcus aureus* (obtained from the culture bank of Microbiology Unit, Department of Pure and Applied Biology, Ladoke Akintola University of Technology, Nigeria) were used in this study. Paper disc of 6 mm diameter was prepared from Whatman No. 1 filter paper. The bacteria suspension used was standardized by picking a loopful of the bacteria and suspending in 10 ml sterile saline water. This was used to make subsequent 10 fold dilutions from which 0.1 ml amount was added to 4 ml amount of nutrient agar at 50 °C. This was poured on plates of basal medium and left alone to solidify. The paper discs were impregnated with nanoparticles solutions (dispersion in water) of

Table 1. Laccase activities of wild and mutant strains of *L. edodes*

Strain	Laccase activity (U/ml)	Fold improvement relative to the wild strain
Wild	2.6	-
UV_{10}	10.6	4.08
$UV_{10}ACR_8$	2.8	1.08

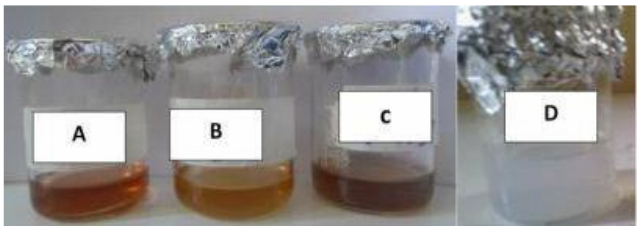

Fig. 1. Colour formation during the synthesis of AgNPs after 1 h of reaction (A- wild; B- UV_{10}; C- $UV_{10}Acr_8$; D- control)

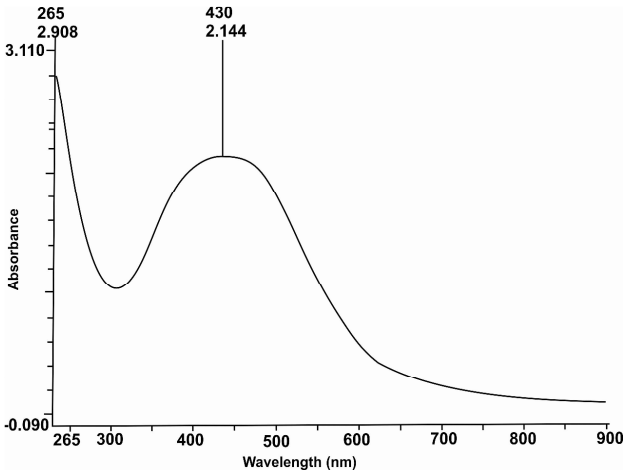

Fig. 2. UV-Vis absorption spectrum of the biosynthesized AgNPs using laccase of UV_{10} strain of *L. edodes*

UV_{10} and placed on bacteria lawn after solidification. Zones of inhibitions were observed and measured after incubation at 37 °C for 18 h.

Results

Effect of mutagenesis on laccase production by the wild, UV_{10} and $UV_{10}ACR_8$ strains of L. edodes

Laccase activities of wild, UV_{10} and $UV_{10}ACR_8$ strains of *L. edodes* were recorded as 2.6, 10.6 and 2.8 U/ml/min respectively, after 7 days of fermentation (Table 1). The enzyme activity was in the following order: UV_{10} > $UV_{10}ACR_8$ > wild, showing improvement in the laccase activities of the generated mutants over the wild strain. The data obtained in the hereby study were higher than that of Kumar and Srikumar (2010), which reported an activity of 2.8×10^{-4} U/ml for laccase from *Opuntia vulgaris* and 1.75 U/ml, as well as than the result of 2.5 U/ml obtained for the wild and mutant strains of *Pleurotus pulmonarius* respectively by Adebayo *et al.* (2012).

The production level achieved in the current study was however similar to the laccase activity of 11.3 U/ml by *L. edodes* when ATBS substrate was used by Makkar *et al.* (2001). Though there is limited information on laccase activities of *L. edodes* (Cavallazzi *et al*., 2004; Morozova *et al*., 2007), laccase productivity reported in this work supports the application of mushrooms in extracellular synthesis of laccases (Leonowicz *et al*., 2001; Makkar *et al*., 2001; Claus, 2004; Strong, 2011; Dhakar and Anita, 2013; Fonseca *et al*., 2013). Several applications of mushrooms and laccases have been reported for degradation of recalcitrant environmental pollutants (Couto and Herrera, 2006), detoxification of industrial effluents of textile and petrochemical industries (Kuhad *et al*., 1997; Abadulla *et al*., 2000; Hou *et al*., 2004; Couto *et al*., 2005), medical diagnostics and cleaning up of pesticide and explosives (Duran and Esposito, 2000). Therefore, laccases have lots of industrial applications in the biodegradation of pollutants, design of biosensors to detect phenols in wastewater and the development of biofuel cells (Fernández-Fernández *et al*., 2013).

Biosynthesis and characterization of silver nanoparticles

There are limited reports on the use of metabolites of *L. edodes* in the production of nanoparticles, as only two reports for its usage in the synthesis of gold nanoparticles were reported till date (Vetchinkina *et al*., 2013, 2014). Some works have demonstrated the formation of gold nanoparticles (Faramarzi and Forootanfar, 2011; Sanghi *et al*., 2011; El-Batal *et al*., 2015) and silver nanoparticles using fungal laccases (Durán *et al*., 2014, 2015). However, in this study, crude laccase from *L. edodes* was used to synthesize AgNPs.

The AgNPs were characterized with typical yellowish-brown colour produced from the bio-reduction of silver ions by the enzyme. The intensity of colour increased as the bio-reduction of silver ions progressed and stabilized when the reaction was completed. Fig. 1 shows the colour development during the biosynthesis of the AgNPs.

Typical AgNPs colouration, previously reported from bacterial exopolysccharides and mushroom culture extracts and mycelia, was yellowish brown for *Lactobacillus rhamnosus* (Kanmani and Lim, 2013), yellow for *Pleurotus ostreatus*, *Agaricus bisporus* and *Ganoderma lucidum*, reddish brown for *Calocybe indica*, light-grey for *Schizophyllum comune* and dark brown for *Stachybotrys chartarum* (Mohamed, 2013). The variation in colour is attributed to the variation in composition of biomolecules involved in the synthesis of nanoparticles and to the excitation of surface plasmon vibrations in metal nanoparticles (Mulvaney, 1996).

The UV-Vis absorption spectrum of the biosynthesized AgNPs of UV_{10} was obtained at 430 nm (Fig. 2). UV-Visible absorption spectroscopy has proven to be a versatile technique in the analysis of AgNPs (Sastry *et al*., 1997) and may provide information about morphology, size and stabilization of AgNPs (Kanmani and Lim, 2013).

The FTIR measurements were carried out to identify the possible biomolecules responsible for the stabilization of the synthesized AgNPs. The FTIR spectrum (Fig. 3) of the biosynthesized AgNPs showed peaks at 1,039; 1,242; 1,394; 1,459 1,546; 1,633 2,923 and 3,354 cm^{-1}. The peak at 3,354 cm^{-1} indicated the presence of primary amine (Shaligram *et al*., 2009), while the band at 1,546 cm^{-1} have been previously reported as a result of secondary amine (Vigneshwaran *et al*., 2007). The bands at 1,459 and 1,394 cm^{-1} are due to C=O and hydroxyl group deformation responsible for the bio-reduction of silver metal ion to silver nanoparticles (Vigneshwaran *et al*., 2007; Philip, 2009; Thirumurugan *et al*., 2011; Gopinath and Velusamy, 2013), while bands at 1,242 and 1,039 cm^{-1} are due

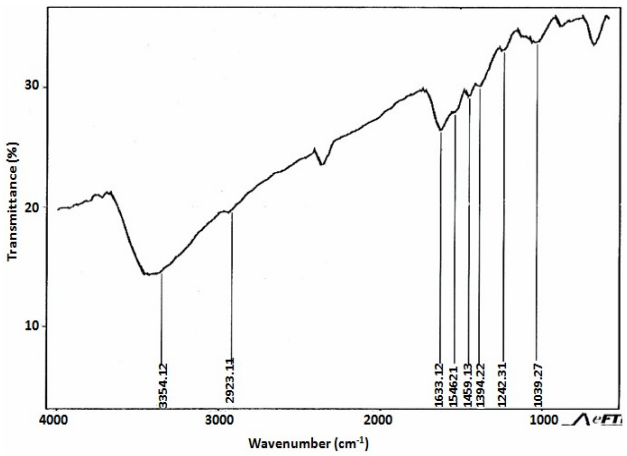

Fig. 3. FTIR spectrum of the biosynthesized AgNPs

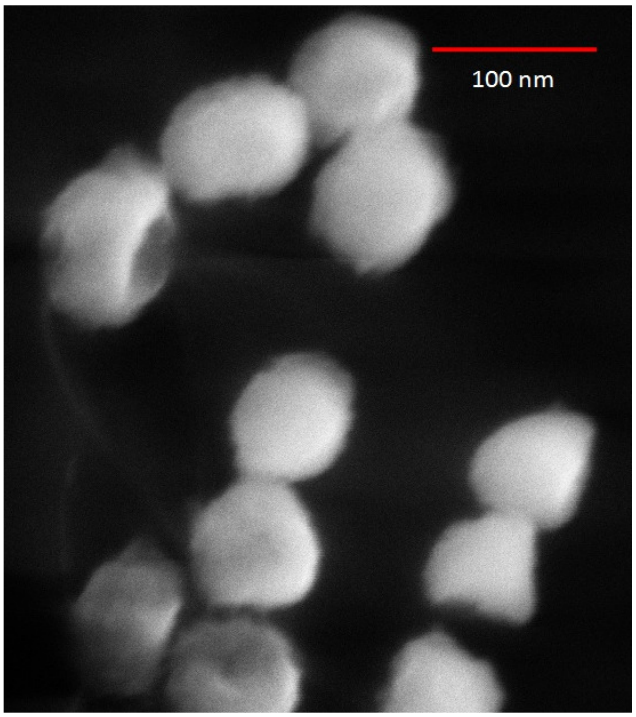

Fig. 4. Scanning electron micrograph of the biosynthesized AgNPs

to OH and aliphatic amine deformation (Philip, 2009; Gopinath and Velusamy, 2013). It has been reported that proteins can bind to nanoparticles through free amine groups or cysteine residues in the proteins (Gong *et al*., 2004), hence it can be inferred that these biomolecules of *L. edodes* were responsible for capping and stabilization of AgNPs. Thus far, this is the first report on the biosynthesis of AgNPs using the metabolite of *L. edodes*.

The scanning electron micrograph of AgNPs produced is shown in Fig. 4. The AgNPs were walnut-shaped and the size ranged between 50-100 nm. Spherical shaped AgNPs of varying sizes have been previously reported (Kannan *et al*., 2013; Lateef *et al*., 2015c). This study has therefore revealed the capability of laccase of *L. edodes* at forming nanoparticles, which may add to the growing applications of laccases and *L. edodes*.

Table 2. Antibacterial activity of the laccase biosynthesized AgNPs using UV_{10} strain of *L. edodes*

Bacterial isolates	Zone of inhibition (mm)
E. coli (Strain A)	15.0
E. coli (Strain B)	-
E. coli (Strain C)	20.0
P. aeruginosa (Strain A)	12.0
P. aeruginosa (Strain B)	14.0
P. aeruginosa (Strain C)	-
K. pneumoniae (Strain A)	-
K. pneumoniae (Strain B)	12.0
K. pneumoniae (Strain C)	11.0
S. aureus	-
Percentage antimicrobial activity (%)	60

Strains A, B and C were obtained from urine, high viginal swab and wound respectively

Antibacterial activities of the biosynthesized AgNPs

The AgNPs showed selective antimicrobial activities against the ten clinical bacterial isolates tested. AgNPs synthesized induced a maximum inhibitory zone of 20 mm at a concentration of 141 µg/ml for UV_{10} AgNPs (Table 2). The efficacies of AgNPs have been reported against different strains of bacteria (Morones *et al*., 2005; Kim *et al*., 2007; Priyadarshini *et al*., 2013; Lateef *et al*., 2015a, c) and reported to have high surface to volume ratio, which enhanced antimicrobial activity as compared with bulk silver metal (Rai *et al*., 2009). The antibacterial activity has been reported to be size dependent (Bhat *et al*., 2011), while interaction between AgNPs, silver and phosphorus containing biomolecules of the bacterial cell may aid the entrance of the particles into the bacterial cell, thereby resulting in cell-killing through the attack of the respiratory chain and cell division. As indicated in Table 2, the UV_{10} AgNPs had an activity of 60% against total bacterial strains tested. Therefore, the appreciable antibacterial property demonstrated by the biosynthesized AgNPs has shown that it can be incorporated as antibacterial agent for some applications.

Conclusions

This study has led to the development of new strains of *L. edodes* with improved laccase production through physical mutation. The application of laccase of *L. edodes* in the green synthesis of AgNPs was demonstrated, leading to the formation of walnut-shaped AgNPs. The FTIR analysis revealed that proteins were responsible for the AgNPs formation with the AgNPs synthesized, demonstrating remarkable antimicrobial activity against clinical isolates of *E. coli, P. aeruginosa* and *Klebsiella pneumoniae*. Therefore, the technique presented in this study could be used in the development of novel fungal strain for application in the production of enzyme and green synthesis of nanoparticles for biotechnological applications.

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