

Isolation and identification of gold nanoparticles synthesizing fungi from Indian Kolar Gold Field mine soil

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Abstract

An indigenous fungal strain was isolated from Indian Kolar Gold Field mine soil. The isolate was heterothallic, branched septate, deeply floccose, fast-growing, dull green with white background conidial columnar mycelium from *Aspergillus* section *Fumigati*. Diverse metabolic patterns of the isolate exhibit high metal, thermal resistance which grows well from $28 \pm 1^\circ\text{C}$ to 37°C and pH concentration was significant on the growth of isolate. Phylogenetic analysis of 16srRNA β -Tubulin gene sequence established relationship among isolate and other taxa. Molecular identification and morphological features of fungal isolate were consistent with those of *Neosartorya udagawae*. Heterothallic *N. udagawae* FJ830683 strain was closely related to homothallic *N. aureola* EF661890. Fungal isolate extract synthesized narrow sized stable Gold nanoparticles (AuNPs).

Key words

Fungal strain, Gold nanoparticles, Kolar Gold Field, *Neosartorya udagawae*

Introduction

Studies of microorganisms at mine sites provide in-depth insight into the microbial diversity (Fabienne *et al.*, 2003). Microorganisms isolated from mine sites are suitable bio-resource for synthesis of metallic nanoparticles (Moharrer *et al.*, 2012). Fungus is ideal for synthesis of gold nanoparticle amongst the microorganisms (Musarrat *et al.*, 2011), due to more extracellular secretions of reductive proteins (Kathiresan *et al.*, 2009), easy handling, swift growth in bulk quantities, mycelial mesh that withstand severe stress (Silver, 1996), convolutions, agitations and other circumstances that generate in bioreactors (Musarrat *et al.*, 2011). Green synthesis of AuNPs draws wider attention in biomedical applications (Mohanpuria *et al.*, 2008) and is most favoured in diagnostic and drug delivery applications (Bartneck *et al.*, 2010).

Neosartorya udagawae is highly metal and thermal resistant (Chaerun *et al.*, 2012) ubiquitous heterothallic

species with columnar conidial head, habitat on soil and human. Sexual *Neosartorya udagawae* and asexual *Aspergillus fumigatus* are two diverse mating categories subsists in equally high temperatures (Horie *et al.*, 1995a) with similar anamorph but differ in growth temperatures. *N. udagawae* grows minimally at 10°C , well between 30°C to 35°C but does not grow above 42°C , whereas *Aspergillus* grows at 45°C but does not grow at 10°C . *N. udagawae* ascospores are nonviable, less virulent, requires less germination period and grew slowly for initial 2 days and reach similar size with those of *A. fumigatus* in 5 days (Sugui *et al.*, 2010).

Phylogenetic analyses and ascospore morphology of the isolate is compatible with *Neosartorya udagawae*. A combination of identification methods was used to identify the genus to avoid inconsistency in phenotypic characteristics (Klich, 2006) overlapping (Balajeet *et al.*, 2006) and identical morphological features (Araujo *et al.*, 2012) of *Aspergillus* and *Neosartorya* species.

In the light of the above, the present study was carried out to isolate a novel metal resistant fungal strain from KGF mines for intra and extra cellular biosynthesis of gold nanoparticles for drug delivery applications.

Materials and Methods

Isolation of fungal strain and growth conditions : Three different soil samples (sample-A from floor level; sample-B from 6 inches deep; and sample-C from 3000 feet depth) were collected from Kolar Gold Field mines. Dissolved the samples in distilled water 1 % (W/V), then they were serially diluted in sterile distilled water, these samples of initial microbial population of 10^2 to 10^6 were inoculated on Czapek Dox Agar media (pH 7.3 ± 0.2) supplemented with Chloroauric acid (HAuCl_4) of 1mM, 2mM, 3mM, 4mM and 5mM concentration (Nangia *et al.*, 2009). The plates were then incubated at $28 \pm 1^\circ\text{C}$ for 5-10 days to check the fungal isolate ability to reduce HAuCl_4 to AuNPs. Soil samples were stored at 4°C for further experiments. Fungal growth was frequently sub-cultured until pure culture was obtained. Pure isolate were characterized by morphological and microscopic observations for identification. Pure isolate was transferred from Czapek Dox Agar media to Czapek Dox Broth supplemented with different concentrations of gold salt (3 mM to 10 mM). They were incubated on a rotary shaker with 180 rpm for 5-10 days at 37°C in the presence of ambient light and ventilation. The culture was then filtered through Whatman filter paper of 15 cm and 22-25 μ pore size, and washed thrice with distilled water to remove media components. Biomass were weighed and noted. pH range for growth assay was determined from pH 2 to pH 11 at $28 \pm 1^\circ\text{C}$ and also on a rotary shaker at 37°C at 180 rpm for 5 to 15 days. Growth temperature ranged between $28 \pm 1^\circ\text{C}$ to 37°C in Czapek Dox Agar (Sugui *et al.*, 2010; Brunella *et al.*, 2011).

Morphological analysis : Fifteen days old culture was used to perceive the structure of the isolate. Pure colonies were used to test with KOH solution and LPCB staining (Guan *et al.*, 2007). 15 % of KOH solution prepared by adding 15 g of KOH and 20 ml glycerol in 80 ml distilled water. On a glass slide, a drop of 15 % KOH solution was added to the fungus and mixed well and placed a cover glass over the preparation and observed under a trinocular microscope for mycelium and ascospores ornamentation. LPCB staining was performed by placing a drop of stain on a glass slide and a small portion of the colony was added with a microbial loop and mixed well. The mixture was allowed to stain for 15 minutes and examined under a trinocular microscope for mycelium, conidial structure and filamental fungi (Samson *et al.*, 2007).

15 days old isolate subculture was prepared to perceive teleomorphic species and measurement of spores

and hyphae by SEM (Samson *et al.*, 1996). The sample was expanded on double-sided conductive carbon tap fixed on the stub and placed in the chamber of ESEM (Environmental Scanning Electron Microscope). After attaining high vacuum, the filament was adjusted to various parameters and then the images were captured and depicted.

Phenotypic analysis : Macro-morphological observations and phenotypic characteristics of the strain examined to perceive the structure, to evaluate the response and production of biochemicals using standardized agar and broth media. 100 ml of Czapek Dox Agar (CDA), Czapek Dox Broth (CDB), Sabourds dextrose Agar (SDA), Sabourds Dextrose Broth (SDB), Oat Meal Agar (OMA), Potato Dextrose Agar (PDA), Potato Dextrose Broth (PDB), Czapek Yeast Extract Agar (CYEA), Malt Extract Agar (MEA) media prepared. All media were prepared by dissolving in deionized distilled water and sterilized at 15 lbs pressure (121°C) for 15 minutes (medium compositions Horie *et al.*, 1995a; Samson *et al.*, 2007). After sterilization the media were allowed to cool up to $28 \pm 1^\circ\text{C}$, antibacterial agent chloramphenicol was added to avoid contamination. Then media were inoculated with seven days old culture in natural ventilation for seven to fourteen days. The presence or absence of growth at the end of the 7 day incubation period was recorded. Colony morphology, sporulation, and microscopic characteristics were examined on the CDA, CDB, SDA, SDB, OMA, PDA, PDB, CYEA, and MEA.

Phylogenetic analysis of 16srRNA and β -tubulin gene sequence analysis : Phylogenetic analysis performed with 16srRNA fungal sequences of genus encoding β -Tubulin Gene to find the phylogenetic relationship between *Aspergillus sp.* and *Neosartorya sp.* (Kimet *et al.*, 2013; John Ravinder *et al.*, 1994; Janos Varga *et al.*, 2000). PCR-amplified 16SrRNA was sequenced to determine the 16SrRNA primary sequence (Van *et al.*, 2013). The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura *et al.*, 1980). Tree with highest log likelihood (-1241.8201) is shown in Fig. 4. The percentage of trees in which the associated taxa were clustered together is shown next to the branches. Initial trees (s) for the heuristic search were obtained automatically. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. A discrete Gamma distribution was used to model evolutionary rate differences among the sites (5 categories (+G, parameter = 0.2082)). Tree was drawn to scale based on genetic distances. All positions with less than 0% site coverage were eliminated. *i.e.*, fewer than 100% alignment gaps, missing data, and ambiguous bases were allowed in any position. Evolutionary analyses were conducted in MEGA5 (Tamura

et al., 2011).

Synthesis of gold nanoparticles : Seven-days-old culture of pH 3 was filtered through Whatman filter paper (15 cm and 22-25 μm pore size) and used for extracellular synthesis of gold nanoparticles. The filtrate was washed thrice with distilled water to remove the media particles. Five grams of pure fungal biomass were suspended in 100 ml of sterile Milli Q water and kept for 3 days on a rotary shaker at 180 rpm. Then, it was centrifuged at 6000 rpm for 10 min. An equal amount of 2 mM, 4 mM, 6 mM, 8 mM and 10 mM gold solution was mixed with supernatant and incubated for 24 hr at 37°C (Anitha and Palanivelu, 2011).

Results and Discussion

Morphological identification of isolate was carried out by micro- and macro-morphology analyses. Initially, ten fungal colonies grown in CDA supplemented with HAuCl_4 , number of colonies decreased with increasing concentration of HAuCl_4 . After ten days only one colony in 2 mM concentration gold solution in CDA found growing strongly due to high metal resistance. Isolate colonies were fast-growing, deeply floccose with loose cottony white mycelium, sporulating structures with bluish green, and vesicles globose to subglobose, sterigmata in a single series, conidia globose 2-3 μm diameter and smooth to slightly coarse. Growth of gold metal resistant fungi on CDA plate of 10^{-4} floor soil supplemented with 2 mM gold solution changed the colour of media from white to pink (Fig. 1a) in 10 days in agreement with the previous study of Nangia *et al.* (2009). The effect of pH change on the isolate was significant growth was observed between pH 3 to pH 7 at $28 \pm 1^\circ\text{C}$. Growth assay was carried out between $28 \pm 1^\circ\text{C}$ to 60°C . The isolate grew between $28 \pm 1^\circ$ to 37°C , no growth was noted at temperature

higher than 40°C and the strain was lifeless at 60°C is in agreement with *N. udagawae* of corneal ulcer which grew minimally at 10°C and did not grow at temperature higher than 42°C (Brunella *et al.*, 2011).

Fungal growth of CDA plates was transferred to different CDB plates supplemented with gold salt from 3 mM to 10 mM concentrations and incubated on a rotary shaker with 180 rpm for 5-10 days at 37°C in the presence of ambient light and ventilation. Flasks containing 4 mM and 5 mM gold salt in CDB reduced auric ions to gold nanoparticles, visual change of colour was noted from colorless solution to burgundy red on 8th day. Other concentrations ranging from 6 mM to 10 mM, remained unchanged up to 15th day. The weight of biomass in 4 mM concentration gold solution was 24.25 g (wet mass) with significant dry mass of 13.47g, and in 5 mM concentration the wet weight was 23.00 g and dry weight was 11.25 g, respectively. The isolate grew well on CDA and CDB containing high level of sucrose and sodium nitrate. The physiological analysis using Lacto phenol cotton blue (Fig.1b) and potassium hydroxide (Fig.1c) staining showed stripe with medulla and long hyphae with bulged vesicle phialides, which were arranged one above the other to form biseriate on which spores formed column on the vesicles.

On CDA, the colonies grew up to 60 mm in seven days and 95 mm in 14 days at $28 \pm 1^\circ\text{C}$ to 37°C . The growth of fungus was mycelium with smooth walled hyphae with branched septate and conidial columnar head appeared with grayish green to dull green spores. On PDA, the colonies grew up to 30 mm in 7 days and 80 mm in 14 days at $28 \pm 1^\circ\text{C}$ to 37°C . The growth of fungus was like flower petals, white cottony thick mycelium, the hyphae was thick in dull green

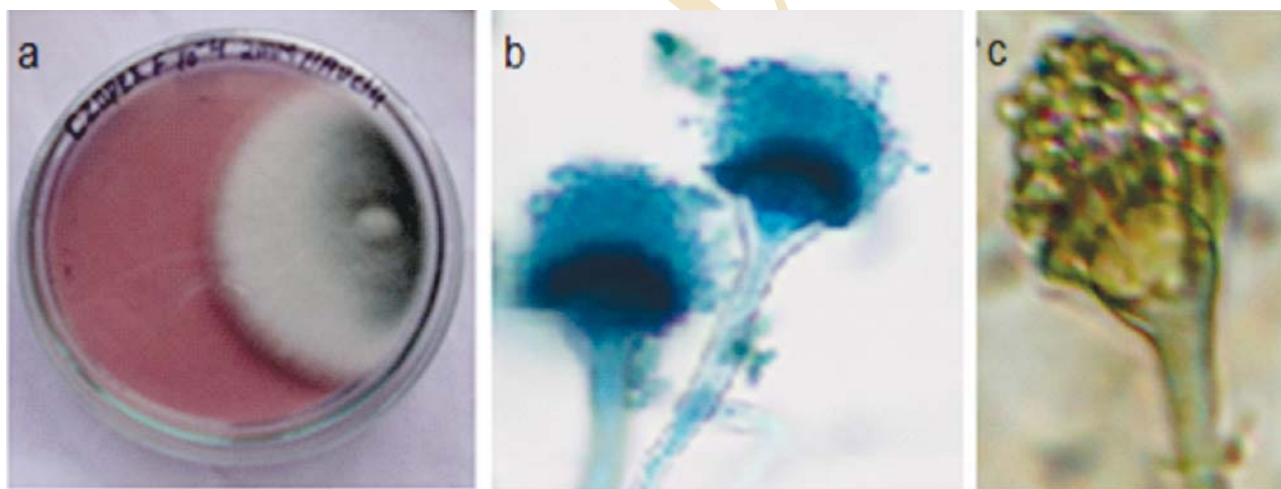


Fig. 1 : (a) Growth characteristics of *N. udagawae* on CDA with 2 mM concentration of HAuCl_4 with 10^{-4} floor soil; (b) Trinocular micrograph of *N. udagawae* on the LPCB stain; (c) Trinocular micrograph of *N. udagawae* on KOH stain

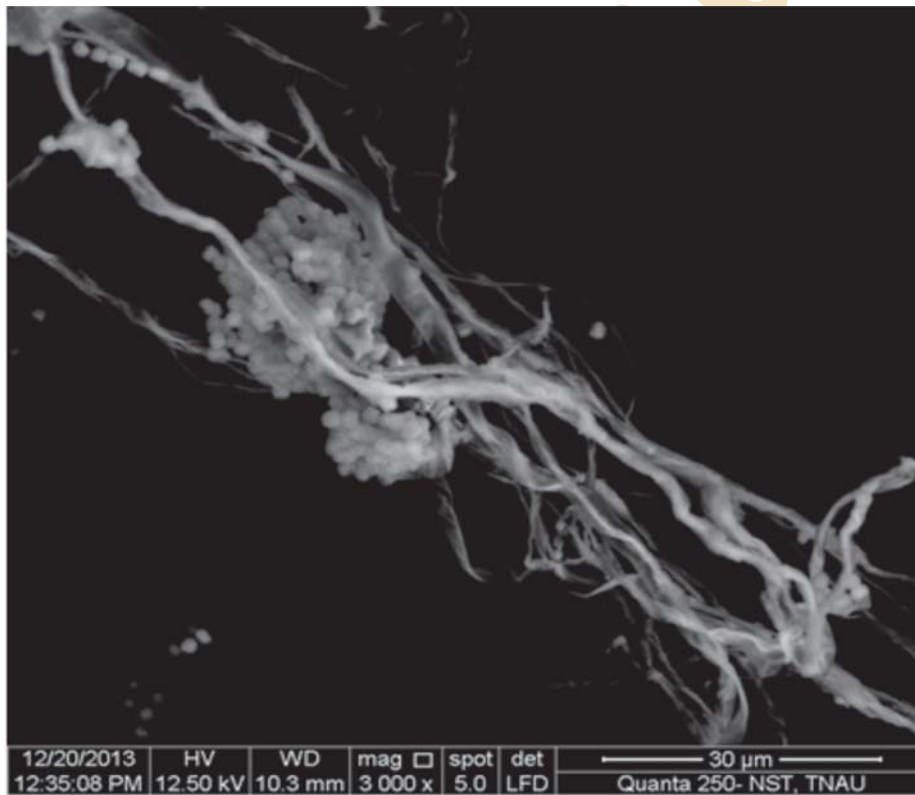
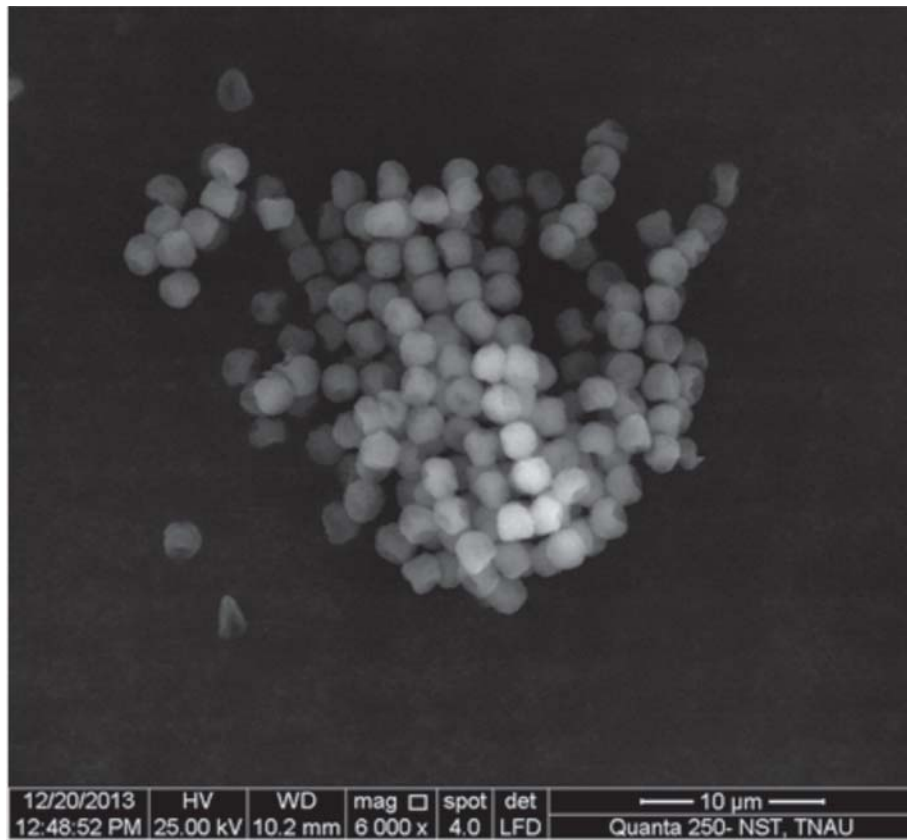


Fig. 2 : (a) Ascospores of *Neosartorya udagawae*. SEM micrograph (scale 10 μ) (b) hyphae along with ascospores of *Neosartorya udagawae* (scale 30 μ)

ATGCCTCGACCTCAATGCTTGAATGATGGGAGATTAGGACCTGTCATCCTAGCAGGCTGTCCTC
 CATGGTTTCAGCGTCGCTGTGATGGGTATCAGCTAACAAATTTACAGGCAGACCATCTCTGGTG
 AGCACGGCCTTGACGGCTCTGGCCAGTAAGTACGAACCTTTATCCTCCCGATTGAGAAAGCGG
 GAAACACGAAAGGCAAGCAGGAAGAGGGGCGCGTGTCTGATGAGGACAATAGCTACAATGGCTC
 CTCCGATCTCCAGCTGGAGCGTATGAACGTCTATTTCAACGAGGTGCGTGGATGAAACTCGACT
 CTACACTACTTCGGCAACATCTCACGATCTGACTCGCTACTAGGCCAACGGCGACAAATATGTT
 CCTCGTGCCGTTCTAGTCGATCTCGAGCCCGGTACCATGGACGCTGTCCGTGCCGGTCCCTTCG
 GCGAGCTCTTCCGTCCCGACAACCTTCGTCTTCGGCCAGTCTGGTGCTGGTAACAACACTGGGCCAA
 GGGT

Fig. 3 : Beta tubulin gene sequence of isolated strain *Neosartorya udagawae*

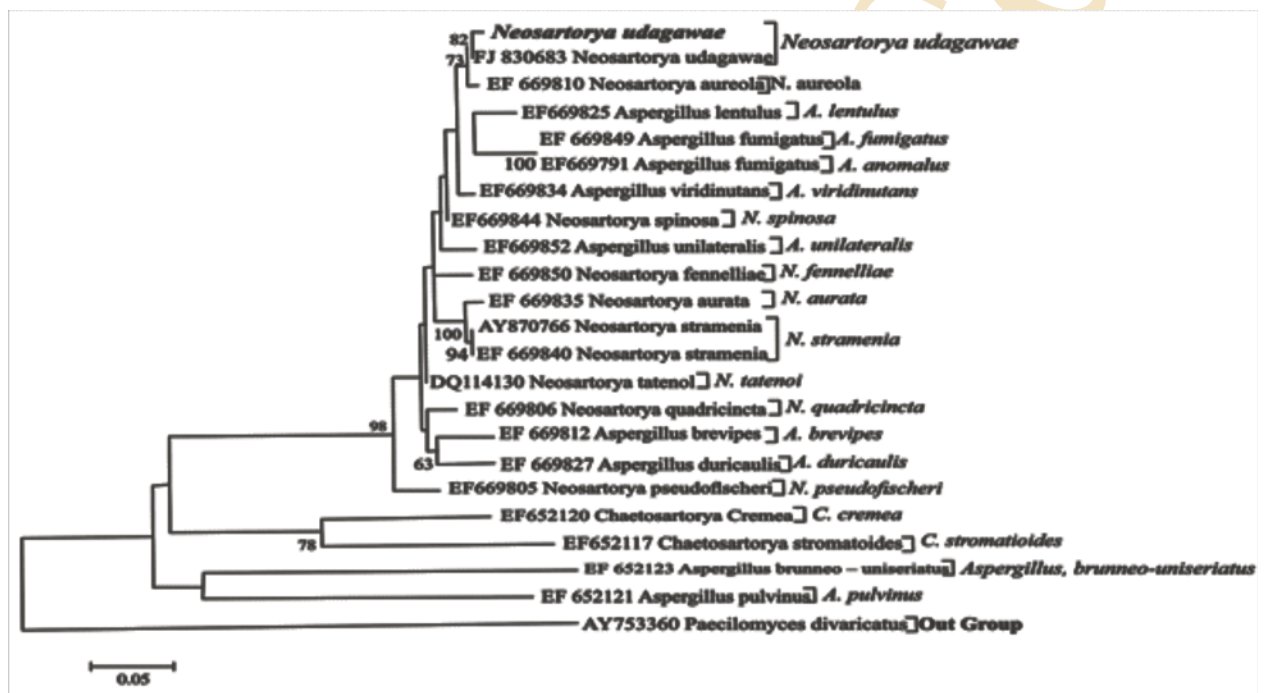


Fig. 4 : Phylogenetic tree based on homology sequence of 16S rRNA analysis. Each number on a branch indicates the bootstrap confidence values (100 replicates). The scale bar indicates 0.05 substitution per nucleotide position

columnar head with conidial spores. On SDA, the colonies grew up to 30 mm in 7 days and 90mm in 14 days at 28±1 °C to 37 °C. The growth of fungus was in hexagonal shape with white cottony mycelium at base with thick dull green columnar conidial spores.

On CYEA the colonies grew up to 45mm in 7 days and 90 mm in 14 days at 28±1°C to 37 °C. The growth of fungus was like a mat with lines on all the sides, flower like appearance with smooth white cottony thick mycelium. On OMA, the colonies grew up to 50mm in 7 days and 90mm in 14 days at 28±1 °C to 37 °C. The growth of fungus was like yeast budding shape white mycelium with loose cottony

thick hyphae with dull green columnar conidial spores. The colonies grew up to 60mm on MEA in seven days and 80mm in 14 days at 28±1 °C to 37 °C. The growth of fungus was oval shape mycelium with thick white cottony hyphae, dull green columnar conidial ascospores. On CDB, white thin plane fungal mat with dull green spots were observed after seven days. On SDB white thin fungal mat with bulges was formed after seven days. On PDB, white plane thick fungal mat with dull green spots was observed after seven days.

SEM was used to examine the spores and hyphae. The SEM micrograph of 15 days old culture is shown in Fig. 2. The size of spores were 1.797 µm and that of hyphae was 1.588

µm. The data described above presented the morphological features of the isolate on CDA, PDA, SDA, CYEA and MEA plates. Especially, the isolate had a characteristic dull green color colony on the above medias. The hyphae, conidia, conidiophores and morphological ascospores ornamentation under microscopic analysis suggest the structure of *Neosartorya udagawae* (Brunella *et al.*, 2011). Colored extrolite secretion like fumigatin, fumagillin, tryptoquivaline and tryptoquivaline alone (Samson *et al.*, 2007), growth temperatures range from 28±1 °C to 37 °C and inability to grow at temperature higher than 40 °C serve as a marker to distinguish strains from *Aspergillus* section fumigati (Sugui *et al.*, 2010). *N. udagawae* and *A. fumigatus* resembles identical morphological characteristics (Vinh *et al.*, 2009; Araujo *et al.*, 2012). However, the isolate differs from *A. fumigatus* by oval shape mycelium with thick white cottony hyphae and dull green columnar conidial ascospores, longer periods for ascospores formation and growth temperature from 28 ± 1°C to 37 °C. The isolate secreted a pink extrolite in the CDA plate with 2 mM concentration with 10⁻⁴ floor soil sample dilution. But microorganism did not produce the same metabolites consistently; therefore it was difficult to determine the isolate based on biochemical analysis method (Klich, 2006). Hence, phylogenetic and gene sequence analysis was carried out for clarity and accurate identification of the isolated fungal strain.

Examined the genetic relatedness of isolated section *Neosartorya* and previously known other taxa. The neighbour joining tree based on partial β-tubulin gene sequence is shown in Fig.4. Identification of fungal isolate *Neosartorya udagawae* was confirmed by β-tubulin gene sequence (Fig.3). The analysis involved 14 nucleotide sequences and there were a total of 417 positions in the final dataset. *Paecilomyces divaricatus* AY753360 was used as an out group in this experiment. Besides, the evolutionary relationship the following conclusions could be drawn from the phylogenetic tree. *Neosartorya udagawae* was found to be different clade well separated from all other species in the section Fumigati and not closely associated with other *Neosartorya* species. *Neosartorya udagawae* originated from asexual *Aspergillus fumigatus* EF669791. The isolate *N. udagawae* established relationship among 10 *Neosartorya* and 9 *Aspergillus* species namely *N. aureola*, *N. lentulus*, *A. fumigatus*, *A. anomalus*, *A. viridinutans*, *N. spinosa*, *A. unilateralis*, *N. fennelliae*, *N. aurata*, *N. stramenia*, *N. tatenoi*, *N. quadricincta*, *A. brevipes*, *A. duricaulis*, *N. pseudofisherii*, *C. cremea*, *C. stromatioides*, *A. brunnea*, *A. pulvinus*. In agreement with Hong *et al.* (2007) the sequences of *A. fumigatus* and *A. anomalus*, *N. stramenia* and *N. aurata*, *A. brevipes* and *A. duricaulis*, *C. cremea* and *C. stromatioides*, *A. brunnea* and *A. pulvinus*, respectively were identical indicating their close relationship. Isolate *Neosartorya udagawae* FJ830683 positioned in *Neosartorya*

group and strain showed highest sequence similarity to *Neosartorya aureola* EF661890 in agreement with Hong *et al.* (2007). Interestingly, *Neosartorya aureola* was the only homothallic species (Alena *et al.*, 2013) in this group and *Neosartorya* sp. formed homogeneous monophyletic clusters.

When fungal extract was added to equal volume of 2mM concentration gold solution, immediate change in colour was observed from golden yellow to beet root within seconds. This indicates that proteins or enzymes of fungal cells develop their own defence system of detoxification mechanism to protect themselves from gold metal and other environmental stresses. Synthesis of AuNPs attributed to the surface binding of stabilizing agents. These enzymes reduced HAuCl₄ into gold nanoparticles. The surface plasmon vibrations in conical flasks were similar to gold nanoparticles by cell filtrates of *Fusarium solani* (Gopinath and Arumugam, 2014) and *Trichoderma koningii* (Maliszewska *et al.*, 2009). Gold nanoparticle peak at 500 nm was parallel to the results of Gopinath and Arumugam, (2014). Strong surface plasmon resonance centered at 500-600 nm even after 24 hr of reaction indicate the stability of nanoparticles synthesised by fungal isolate.

The fungal isolate *Neosartorya udagawae* exhibited high metal and thermal resistance, produced high biomass, exceptional growth in optimal conditions and capable of rapid synthesis of narrow sized stable AuNPs. The green synthesis of AuNPs attributed to stabilizing enzymes secreted by fungal strain. This eco-friendly and cost effective protocol will have an impending role in biomedical applications.

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