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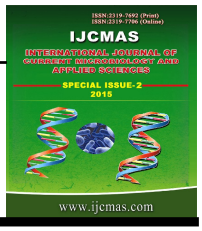


Original Research Article

Biosynthesis and Characterization of Silver Nanoparticles Produced By Microorganisms Isolated From *Agaricus bisporus*

Bhavna P. Nath, Snehal R. Niture, Savita D. Jadhav, Shradha O. Boid*

Department of Microbiology, Modern College of Arts, Science and Commerce, Shivajinagar, Pune-5, India

**Corresponding author*

Keywords

Nanoparticles, *Agaricus bisporus*, UV-Visible Spectroscopy, FTIR, FE-SEM, Therapeutic activity

A B S T R A C T

Nanotechnology is the engineering of functional systems at atomic and molecular level and thus the field of nanotechnology has gained great importance because of its applications in various areas such as chemicals, textile industries, drug and gene delivery and electronics, diagnosis, artificial implants, tissue engineering, computing, biosensors, etc. Mushrooms have been a vital part of the normal human diet and are known to show therapeutic activities. In addition mushrooms are found to synthesize silver and gold nanoparticles which also show therapeutic activities. In the present study silver nanoparticles have been synthesized successfully by hot water extract of mushroom. Simultaneously microorganisms have been successfully isolated from Button mushroom (*Agaricus bisporus*). In all, fourteen isolates have been obtained and their biochemical characterization was done. Silver nanoparticles have been synthesized by using these isolates and nanoparticles have been characterized by FTIR analysis and FE-SEM. The isolates were also checked for production of bioemulsifier. Future prospect includes identification of the isolates and studying the effect of different reducing agents on the synthesis of nanoparticles. This work is significant as it studies nanoparticle synthesis from microorganisms isolated from mushrooms. Till date, nanoparticle synthesis has been studied from mushroom extract.

Introduction

Nanotechnology is the engineering of functional systems at atomic and molecular level. It helps to modify and develop the important properties of metals in the form of nanoparticles which shows various applications in cell labeling, biomarkers, antimicrobial agents and nano-drugs in treatment of various diseases. In nanotechnology, a particle is defined as a small object that behaves as a whole unit in terms of its structure and characteristics. Nanoparticles are sized between 1 and 500 nanometers. Nanoparticles may or may not exhibit size-related properties that differ significantly from those observed in fine particles or bulk materials (Abhilash, 2010). Although the size of most molecules would fit into the above outline, individual

molecules are usually not referred to as nanoparticles (Abhilash, 2010). The field of nanotechnology has gained great importance because of its applications in various areas such as chemicals, textile industries, drug and gene delivery, electronics, diagnosis, artificial implants, tissues engineering, computing, biosensors, etc (Abhilash, 2010).

Mushrooms have been a promising source of nutrients and are an inevitable part of human diet. The carbohydrate content of mushrooms represents the bulk of fruiting bodies accounting for 50 to 65% on dry weight basis. Mushrooms generally have more protein content than any other vegetable (Wani *et al.,* 2010). Lintzel (1941) reported the digestibility of mushroom protein to be as high as 72 to 83%. In mushrooms, the fat content is very low as compared to the carbohydrate and protein contents. The fats present in mushroom fruiting bodies dominantly have unsaturated fatty acids. Also mushrooms are known to possess great medicinal importance (Wani, *et al.,* 2010).

Sujatha *et al.* (2013) reported synthesis of silver nanoparticles from edible mushrooms using hot-water extract which is in accordance to our study. Some microorganisms present in mushrooms might be thermoliable and thus would not survive at high temperature during boiling. Therefore, the effect of these microorganisms in synthesis of silver nanoparticles by mushrooms might be negligible.

Study on the role of these microorganisms in synthesis of silver nanoparticles has not been reported yet. The present study aims at using these microorganisms as a system for synthesis of silver nanoparticles.

Nanoparticles can probably have an impact on crude oil biodegradation. Nanoparticles have gained tremendous interest due to their unique properties compared to their bulk counterparts. Potential impact of commercial nanoparticles on crude oil biodegradation has not been investigated so far (Ismail *et al.,* 2010). Accordingly, it is likely that engineered nanoparticles may interfere with the biodegradation of environmental pollutants as some of them are also known to have antimicrobial activity. The present work aims at correlating nanoparticles with the production of bioemulsifier.

Materials and Methods

Isolation of microorganism from edible button mushrooms (*Agaricus bisporus*)

The edible button mushrooms were surface sterilized by washing it with double glass distilled water followed by washing with 0.001 N mercuric chloride. The mushrooms were then rinsed again twice by double glass distilled water. The mushrooms were aseptically introduced into sterile saline and were crushed with the help of sterile glass rod and vortexed. This suspension was then inoculated (Spread plate technique) onto sterile media containing plates in duplicates. Brain Heart Infusion, Nutrient Agar, Cetrimide Agar, Eosin Methylene Blue Agar, Mannitol Salt Agar, Urea Indole Agar, Sabouraud Agar, Fluid Thioglycolate Agar, Mac Conkey's Agar (SRL Chemicals) Tryptone Soya Agar (Himedia Laboratories) were used for this purpose. One set of plates was incubated in anaerobic jar to check for growth of facultative anaerobes and other set was incubated at 37°C for 48 h.

Morphological and biochemical characterization of isolates

Morphological features including colony shape, size, color, margin, elevation, opacity

and consistency were determined. Gram staining, Motility, Catalase test, Oxidase test, Oxidative-Fermentative test and Sugar Fermentation test (1g% Glucose, 1g% Lactose and 1g% Mannose) were performed.

Synthesis of Silver Nanoparticles from the isolates obtained from *Agaricus bisporus*

Isolated colonies were inoculated into sterile Fluid Thioglycolate medium and incubated on orbital shaker incubator at 37°C for 24 h for preparing the inoculum. For synthesis of Silver Nanoparticles, inoculum and 10 mM Silver nitrate ($AgNO_3$) solution were added in equal proportion in an acid-washed amber colored bottle which was incubated at 37°C for 24 h on orbital shaker incubator at 100 rpm.

Synthesis of Silver Nanoparticles was estimated by Spectrophotometric (Bioera's Elite UV-Visble Spectrophotometer) analysis between 400 nm -500 nm (peak at 441 nm) (Prakash *et al.,* 2010).

Fourier transmission infrared spectroscopy (FTIR) analysis

FTIR analysis is performed to determine the functional groups present in a sample which reduces silver nitrate to metallic silver nanoparticles. For FTIR analysis, crude sample containing nanoparticles was used. FTIR analysis was performed at Department of Physics, Savitribai Phule University of Pune.

Field emission scanning electron microscopic analysis

For FE–SEM, crude sample containing nanoparticles was oven dried at 60°C for 3 h. This dried sample was then filled in sterile vials.

Antibacterial activity of silver nanoparticles

Antibacterial activity of silver nanoparticles was tested against *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris* and *Pseudomonas aeruginosa* by well-diffusion method. Sterile Muller Hinton Agar (Himedia Laboratories) plates were used for determining anti-microbial activity of silver nanoparticles synthesized by Isolate 1 and Isolate 2 respectively. Bacterial colonies (*Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris* and *Pseudomonas aeruginosa*) were inoculated in sterile Luria Broth for enrichment. The optical densities of these cultures were adjusted to 0.05 at 460 nm by adding sterile Luria Broth and this was then used as suspension for checking anti-microbial activity. The bacterial suspension was spread-plated on sterile Muller Hinton Agar plates. 50 µl of crude silver nanoparticle sample was added in each well and the plates were kept in the refrigerator for 1 h for pre-diffusion. The plates were then incubated at 37°C for 24 h. Diameter of zone of inhibition was measured (Sujatha *et al.,* 2010).

Bioemulsifier production by the isolates obtained from *Agaricus bisporus*

Bioemulsifier production by Isolate 1 and Isolate 2 were checked by determining the emulsion index and emulsion assay. For measuring the emulsion index and the emulsion assay, the cultures were inoculated in flask containing sterile Fluid Thioglycollate Medium and were incubated at 37°C on orbital shaker incubator at 100 rpm for 24 h. Sterile, uninoculated media was kept as a control. 10 ml of broth was removed and centrifuged at 8000 rpm at 4°C for 15 minutes and the cell free supernatant

was used for measuring emulsion index and emulsion assay.

A) Emulsion index (E_{24}): For measuring emulsion index, 2 ml oil (groundnut oil, soyabean oil) was added to 2 ml of supernatant and was vortexed for 2 min. Then the tubes were allowed to stand for 24 h. After 24 h, the height of emulsion formed was measured.

$$Emulsion\ Index\ (\%\ E_{24}) = \frac{Height\ of\ emulsion\ layer \times 100}{Total\ height\ of\ mixture\ in\ cm}$$

B) Emulsion assay: For measuring emulsion assay, 2 ml oil (groundnut oil, soyabean oil) was added to 3 ml of supernatant and it was vortexed for 2 min. Then the tubes were incubated at room temperature for 1 h. After incubation, the aqueous layers formed were removed carefully. The absorbance of these aqueous phases was taken at 400 nm (Ilori *et al.*, 2010).

Results and Discussion

Fourteen bacterial isolates (five isolates were facultative anaerobes and nine isolates were aerobes) were successfully isolated from edible white button mushrooms (*Agaricus bisporus*). Out of these, ten isolates were Gram Positive Rods, one isolate was Gram Positive Cocci and three isolates were Gram Negative Rods. All the isolates fermented glucose and mannose sugar, and out of fourteen isolates, while none of the isolates fermented lactose sugar. All fourteen isolates showed positive results for oxidative and fermentative tests (Table 1 & 2).

Silver nanoparticles were successfully synthesized from isolate 1 and isolate 2. The other isolates obtained from mushroom did not show synthesis of silver nanoparticle at standard condition and thus may require optimization of parameters for synthesis of silver nanoparticles. UV-Visible Spectrophotometric analysis was performed between 400 nm to 500 nm. Surface plasmon peak was obtained at 441 nm for the silver nanoparticles synthesized by both the isolates (Figure 1).

FTIR analysis of the silver nanoparticles synthesized by isolate 1 and isolate 2 was performed at the Department of Physics, Savitribai Phule University of Pune. For silver nanoparticles synthesized from isolate 1, the peak appeared at 3342.79 cm^{-1} shows the stretching of bonded hydroxyl (-OH) group and H-bonded. The band seen at 1635.44 cm^{-1} is characteristics of -C=O carbonyl groups and -C=C- stretching. For silver nanoparticles synthesized by isolate 2, the peak appeared at 3342.99 cm^{-1} shows that the stretching of bonded hydroxyl (-OH) group and H-bonded. The band seen at 1640.16 cm^{-1} is characteristics of -C=O carbonyl groups and -C=C- stretching (Figure 2 & 3).

Field Emission Scanning Electron Microscopy was performed to determine the structure of the nanoparticles produced. The SEM images of silver nanoparticles produced by isolate 1 were showing nanoparticles of irregular shape having a diameter in the range of 500 nm- 10 µm. The FE-SEM images of silver nanoparticles produced by isolate 2 were showing nanoparticles of diamond shape having a diameter in the range of 5µm - 50 µm (Figure 4 & 5).

Antibacterial activity of the crude extract containing silver nanoparticles synthesized by Isolate 1 and Isolate 2 against *Proteus vulgaris, Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were checked by using the well diffusion method (Figure 6 & Table 3).

Isolate 1 and Isolate 2 were checked for synthesis of bioemulsifier. Emulsion assay and Emulsion Index were calculated for both the isolates at 24 h, 48 h and 72 h respectively. Maximum emulsification assay (1.47 EU/ml) was observed for Isolate 1 after 72 h using groundnut oil and emulsification assay (2.93 EU/ml) was observed for Isolate 2 after 72 h using soyabean oil. Maximum emulsion index (31.30 %) was observed for Isolate 1 after 72 h using groundnut oil and emulsion index (42.80 %) was observed for Isolate 2 after 72 h using soyabean oil (Figure 7; Table 4, 5, 6 & 7).

Fourteen isolates were successfully obtained from *Agaricus bisporus.* All the isolates were found capable of fermenting glucose and mannose sugar but all the isolates were not capable of fermenting lactose sugar. According to Wani *et al.* (2010), mushrooms contain glucose and mannose sugar but do not contain lactose sugar. This is in accordance with our study and may be reason behind the isolates being non-lactose fermenters. Biosynthesis of silver nanoparticles from isolates obtained from *Agaricus bisporus* was observed successfully. Out of fourteen isolates, two isolates showed synthesis of silver nanoparticles. Remaining twelve isolates may produce silver nanoparticles but this may require optimization in the procotol. Plasmon peak for both the isolates were observed at 441 nm. Prakash et al. (2010) observed the spectrophotmetric plasmon peak to be at 435 nm which is in accordance to our results. The shift in plasmon peak may me because of increase in the diameter of the silver nanoparticle causing "Redshift" (plasmon resonance shifts to lower energies).

Table.1 Results of sugar fermentation test

Isolate No.	Glucose	Lactose	Mannose
Isolate 1	+	-	+
Isolate 2	+	-	+
Isolate 3	+	-	+
Isolate 4	+	-	+
Isolate 5	+	-	+
Isolate 6	+	-	+
Isolate 7	+	-	+
Isolate 8	+	-	+
Isolate 9	+	-	+
Isolate 10	+	-	+
Isolate 11	+	-	+
Isolate 12	+	-	+
Isolate 13	+	-	+
Isolate 14	+	-	+

*Note: + = Acid Production; - = No Acid Production

Table.2 Results of oxidative-fermentative test

Isolate No.	Oxidative Test	Fermentative Test
Isolate 1	+	+
Isolate 2	+	+
Isolate 3	+	+
Isolate 4	-	+
Isolate 5	+	+
Isolate 6	+	+
Isolate 7	-	+
Isolate 8	-	+
Isolate 9	+	+
Isolate 10	-	+
Isolate 11	+	+
Isolate 12	+	+
Isolate 13	+	+
Isolate 14	+	+

* Note: + = Positive; - = Negative

Table.3 The diameters of the zones of inhibition due to antibacterial activity of silver nanoparticles

Organism	Diameter of Zone of Inhibition (mm)	
	Isolate 1	Isolate 2
Proteus vulgaris	12	11
Escherichia coli	11	10
Pseudomonas aeruginosa	13	12
Klebsiella pneumoniae	10	11

Table.4 Emulsification Assay of isolate 1 and isolate 2 are as follows (Groundnut oil)

Isolate	24 hours	48 hours	72 hours
Isolate 1	0.220 EU/ml	0.545 EU/ml	1.47 EU/ml
Isolate 2	0.137 EU/ml	0.443 EU/ml	1.23 EU/ml
Control	-	-	-

Table.5 Emulsification Assay of isolate 1 and isolate 2 are as follows (Soyabean oil)

Isolate	24 hours	48 hours	72 hours
Isolate 1	0.879 EU/ml	1.43 EU/ml	2.62 EU/ml
Isolate 2	0.916 EU/ml	1.78 EU/ml	2.93 EU/ml
Control	-	-	-

Table.6 Emulsification Index (E_{24}) of isolate 1 and isolate 2 are as follows (Groundnut oil)

Isolate	24 hours	48 hours	72 hours
Isolate 1	23.70 %	26.12 %	31.30 %
Isolate 2	20.13 %	22.40 %	27.00 %
Control	-	-	-

Table.7 Emulsification Assay (E_{24}) of isolate 1 and isolate 2 are as follows (Soyabean oil)

Isolate	24 hours	48 hours	72 hours
Isolate 1	29.91 %	34.60 %	40.00 %
Isolate 2	31.42 %	36.30 %	42.80 %
Control	-	-	-

Figure.1 Synthesis of silver nanoparticles by isolate 1 and isolate 2. A) Color change in the sample due to synthesis of silver nanoparticles by isolate 1 and isolate 2. B) Spectrum of Silver nanoparticles synthesized by isolate 1. C) Spectrum of silver nanoparticles synthesized by isolate 2

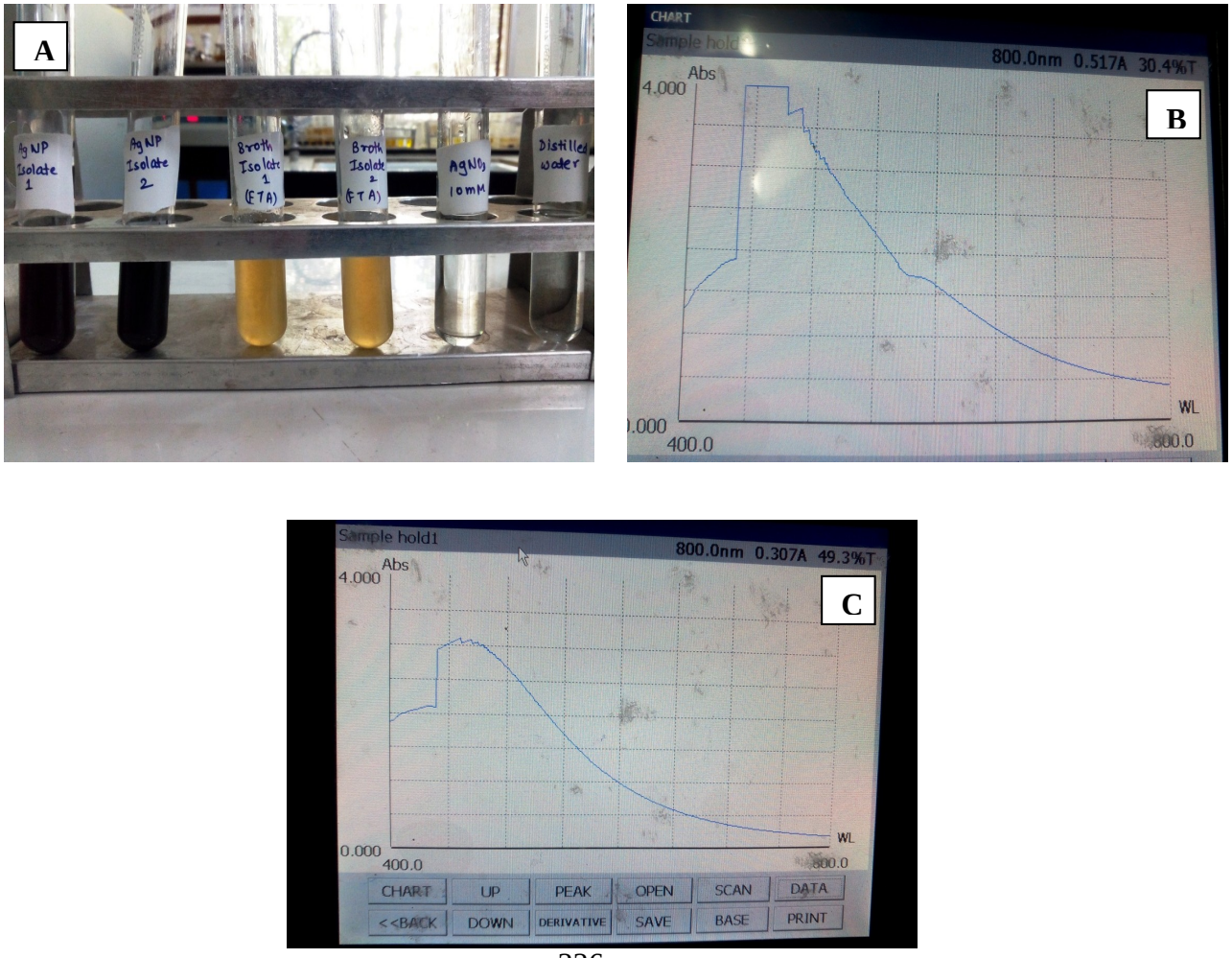


Figure.2 FTIR Analysis of silver nanoparticles synthesized by isolate 1

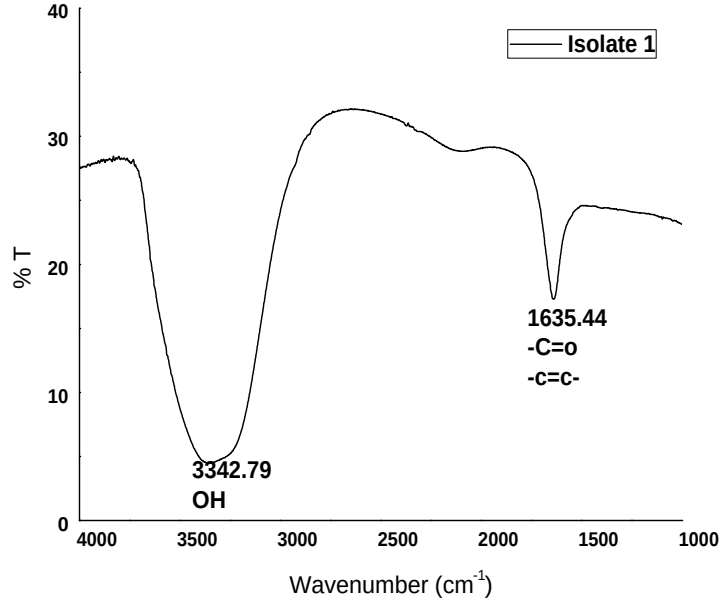


Figure.3 FTIR Analysis of silver nanoparticles synthesized by isolate 2

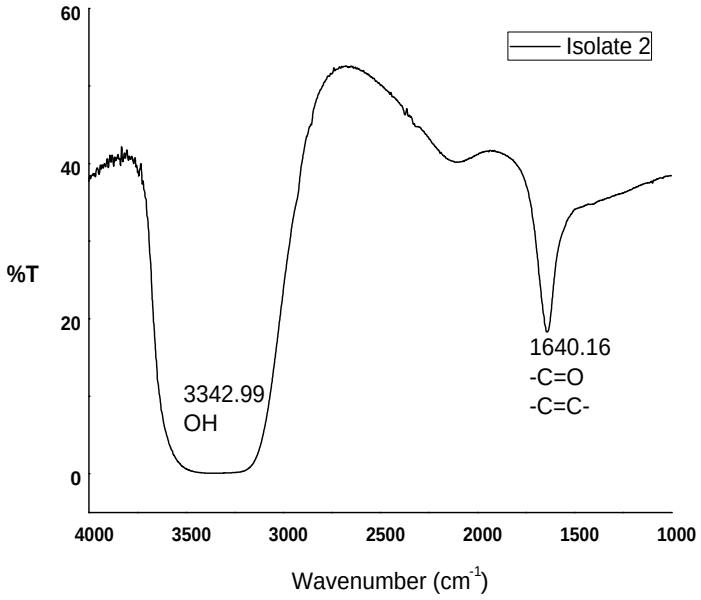


Figure.4 Field emission scanning electron micrograph (A &B) of silver nanoparticles synthesized from isolate 1

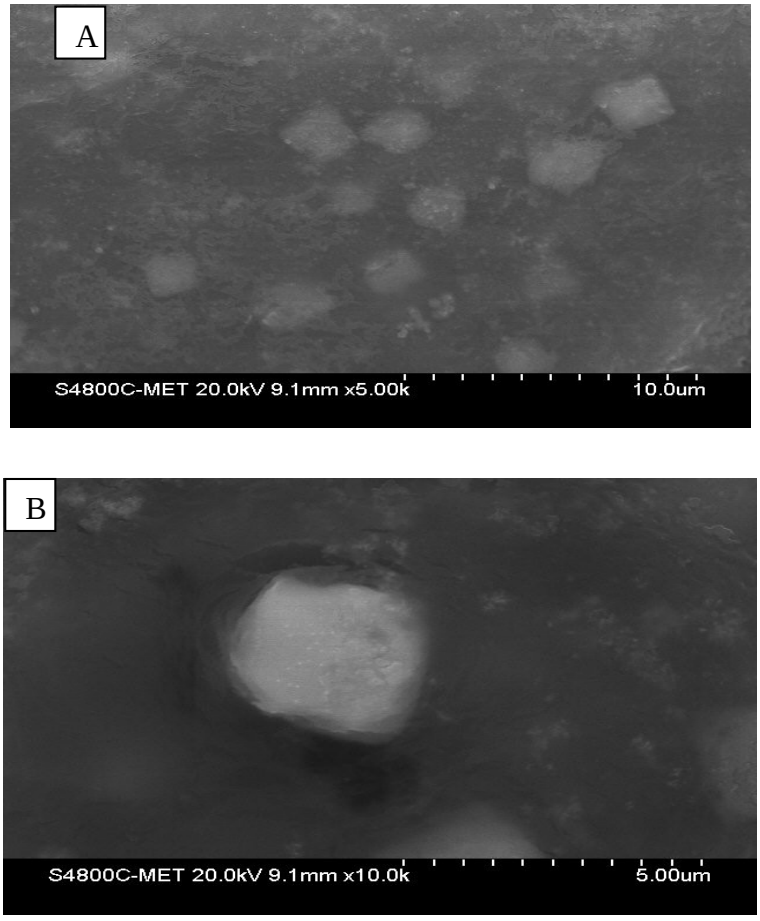
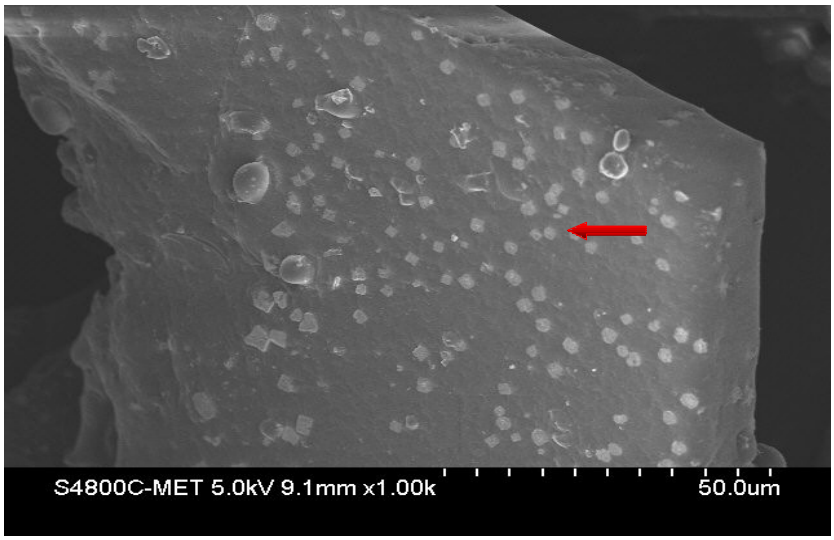


Figure.5 Field emission scanning electron micrograph of silver nanoparticles synthesized from isolate 2



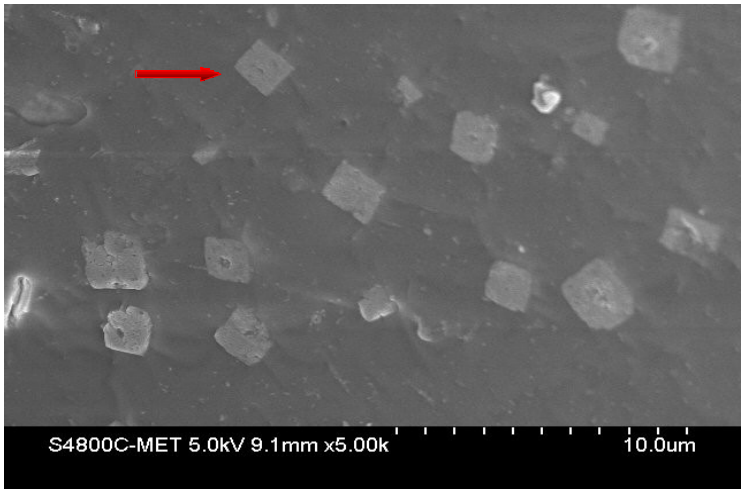


Figure.6 Zone of inhibition is seen due to the antibacterial activity of silver nanoparticles synthesized from the isolates from *Agaricus bisporus*. A) Antibacterial activity of silver nanoparticles against *Proteus vulgaris*. B) Antibacterial activity of silver nanoparticles against *Klebsiella pneumoniae*. C) Antibacterial activity of silver nanoparticles against Escherichia coli. D) Antibacterial activity of silver nanoparticles against *Pseudomonas aeruginosa*

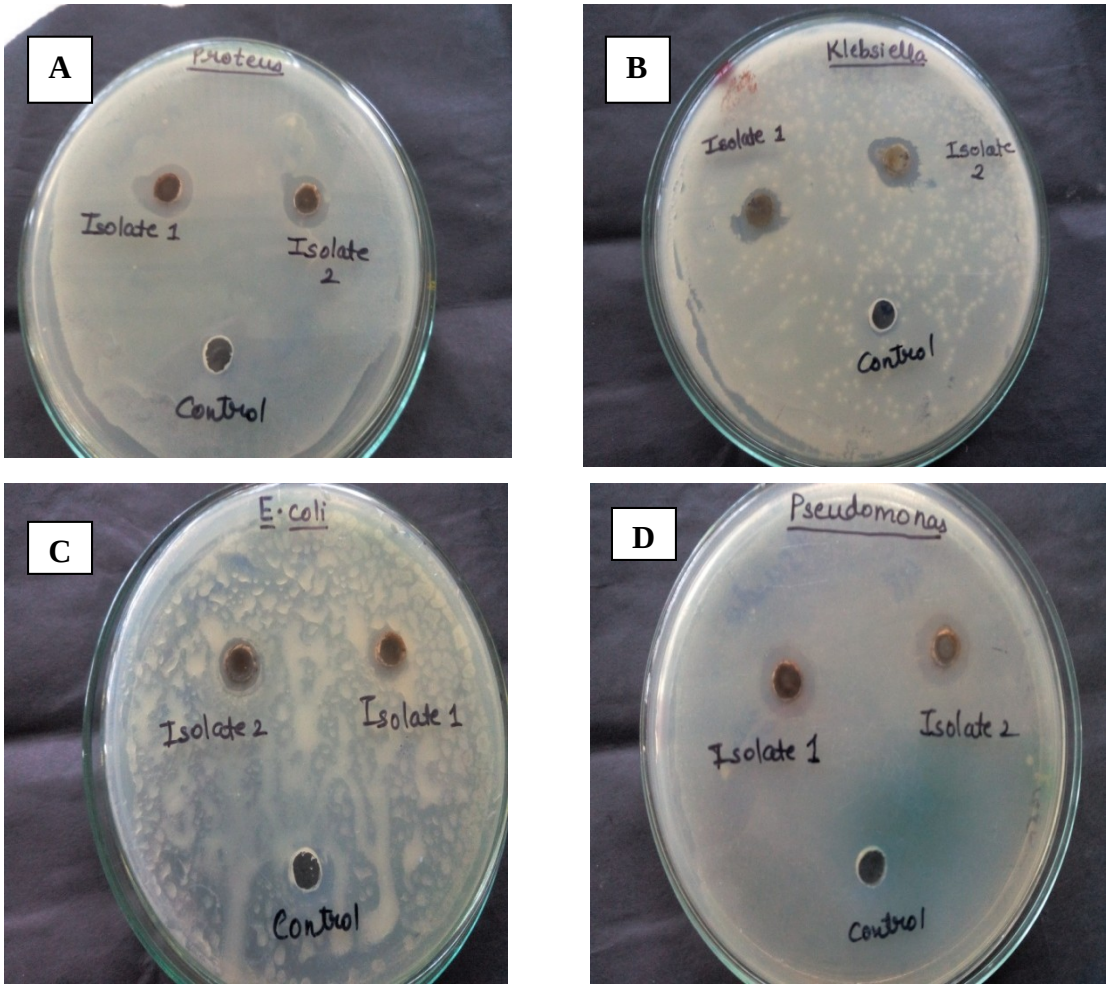
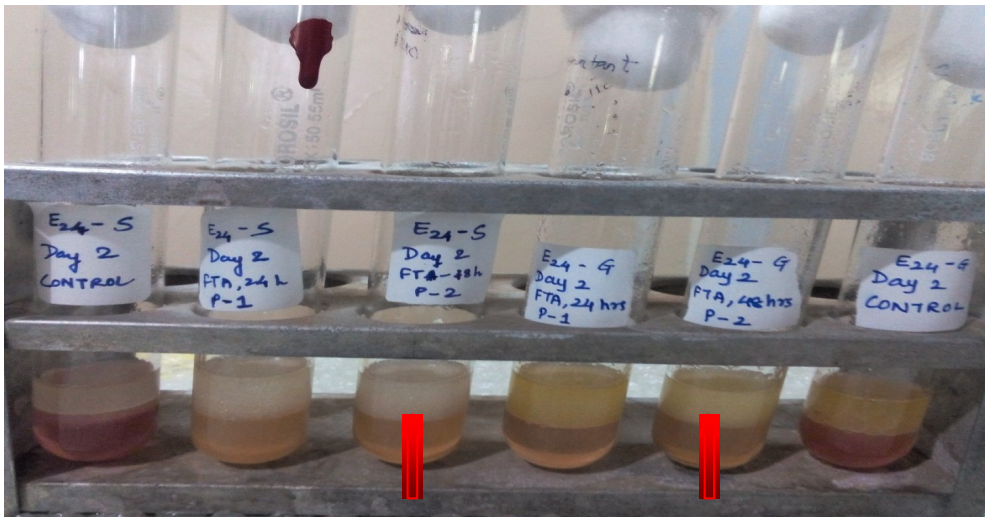


Figure.7 Emulsion layer produced by isolate 1 and isolate 2 after 48 hours.



FTIR analysis of silver nanoparticles obtained from isolate 1 showed peak at 3342.79cm^{-1} which indicates stretching of bonded hydroxyl (-OH) group and H-bonded. The peak seen at 1635.44 cm^{-1} is characteristics of -C=O carbonyl groups and -C=C- stretching. FTIR analysis of silver nanoparticles obtained from isolate 2 showed peak at 3342.99 cm^{-1} shows that the stretching of bonded hydroxyl (-OH) group and H-bonded. The peak seen at 1640.16 cm^{-1} is characteristics of -C=O carbonyl groups and -C=C- stretching. Similar peaks have been observed in the FTIR graph of silver nanoparticles reported by Jeevan *et al.* (2012) and Anarkali *et al.* (2012).

The diameter of the silver nanoparticles produced by isolate 1 and isolate 2 was determined from the field emission scanning electron micrographs. The silver nanoparticles synthesized by isolate 1 were of irregular shape having a diameter in the range of 500 nm - 10 µm. The silver nanoparticles synthesized by isolate 2 were of diamond shape having a diameter in the range of 5µm - 50 µm. In the study reported by Rajeshkumar *et al.* (2013), the size of silver nanoparticles was 50 nm – 100 nm. The silver nanoparticles synthesized by these isolates might be nucleating to form nanoclusters which would be the reason behind increase in the diameter of these nanoparticles. Increase in the diameter of these nanoparticles may cause decrease in the antibacterial activity of the silver nanoparticles which justifies the decrease in diameter of the zone of inhibition produced against the test organisms.

Also, isolate 1 and isolate 2 successfully produced bioemulsifier. Maximum bioemulsifier producing was seen after 72 h (1.47 EU/ml by isolate 1 using groundnut oil and 2.93 EU/ml by isolate 2 using soyabean oil). Maximum emulsion index (31.30 %) was observed for Isolate 1 after 72 h using groundnut oil and emulsion index (42.80 %) was observed for Isolate 2 after 72 h using soyabean oil. According to Maniyar *et al.* (2011) maximum bioemulsifier production was observed between 72 h to 96 h which is in accordance to our results.

In the present study, microorganisms were successfully isolated from mushrooms. Also

silver nanoparticles were synthesized from the bacterial isolates obtained from *Agaricus bisporus*. The bacterial isolates also showed production of bioemulsifier. This study shows that silver nanoparticles synthesized from isolates obtained from mushrooms posses antibacterial activity. Future prospects include identification of the isolates and studying effect of different reducing agents on the synthesis of silver nanoparticles by the isolates obtained from *Agaricus bisporus*.

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