Biosynthesis of Silver Nanoparticles from Marine Yeast and Their Antimicrobial Activity Against Multidrug Resistant Pathogens

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Summary

In this particular work, the extra cellular biosynthesis of silver nanoparticles was performed by using marine yeast isolated from Nicobar Islands, India. Production of silver nanoparticles is confirmed by the absorption peak at 430 nm in UV-Vis spectroscopy due to the surface Plasmon resonance of silver nanoparticles. It is also characterized by atomic force microscopy (AFM), Fourier transform infra red spectroscopy (FT-IR) and X-ray diffraction (XRD). The silver nanoparticles around 87 nm were formed. The marine yeast was identified as *Candida* sp. VITDKGB by 28s rDNA sequencing technique. Biologically synthesized silver nanoparticles were further examined for antimicrobial activity against multi drug resistant *Staphylococcus aureus, Klebsiella pneumoniae*. The antimicrobial activity was performed by agar well diffusion method on Muller Hinton agar plates. *S. aureus* formed 14.66±1.52 mm zone of inhibition with MIC value of 20µg/ml, whereas *K. pneumoniae* formed 12.33±0.57 mm zone of inhibition with MIC value of 40µg/ml.

Keywords: *Candida* sp VITDKGB, Biosynthesis, Silver nanoparticles, Atomic force microscopy.

Introduction

In the present situation nanotechnology is a vastly developing field and has the considerable attention with various applications in day today life. Currently, a variety of metal nanomaterials are being produced using copper, zinc, titanium, magnesium, gold, alginate and silver. The metal nanoparticles have several applications in various fields such as optical devices [1], catalytic [2], bactericidal [3], electronic [4], sensor technology [5], biological labelling [6] and treatment of some cancers [7].
Classically the metal nanoparticles are being synthesized by various physical and chemical methods. These methods have several drawbacks as they are complex, costly, toxic and non eco friendly techniques. In last 2 decades, scientists are looking forward to develop low cost, non toxic, eco friendly technique for the production of nanoparticles. [8,9]. Emergence of biological methods using plants and microorganism for the synthesis of nanoparticles has created a lot of interest in nanoparticles research. The microbial mediated biosynthesis of nanomaterials has recently been recognized as a promising source for mining nanomaterials.[10] Biosynthesis of nanoparticles using bacteria and fungi are already well reported.[11, 12] This method has emerged as a simple and viable alternative to more complex physical and chemical synthetic procedures to obtain nanomaterials.

Silver nanoparticles are undoubtedly the most widely used nanomaterials among all nanoparticles with several applications in antimicrobial agents, textile industries, water treatment, sunscreen lotions etc. [3, 13] Some examples for biologically synthesized nanoparticle using Microorganisms are *Aspergillus flavus* [10], *Cladosporium cladosporioides* [14], *Fusarium oxysporum* [15], *Pseudomonas aeruginosa* [12] and *Phaenerochaete crysosporium* [11].

Most recently the microbial drug resistant is emerged as a major problem in health care industry as microbes involve in the change of their metabolism and genetic structure to acquire resistant against the drugs used in the treatment of common infectious disease. These drug resistant pathogens are more pathogenic with high mortality rate than that of wild strain. To overcome microbial drug resistant, scientists are looking forward for the development of alternative and novel drugs. Silver nanoparticles have been well known for its strong inhibitory and bactericidal effects and can effectively used for the treatment of various infectious diseases [4].

This study involves the biological synthesis of silver nanoparticles was carried out by novel marine yeast *Candida* sp VITDKGB. Yeast possess several advantage over bacteria for the bulk production of nanoparticles as the yeast are rapid grower, producing high amount of enzymes and easy to handle in laboratory conditions and required simple nutrients for growth. Characterization of the synthesized silver nanoparticles by performed by UV - Visible spectroscopy, XRD analysis and Fourier Transform Infrared Spectroscopy (FTIR) analysis, Atomic Force Microscopic (AFM) analysis, Scaning Electron Microscopy (SEM),. Mechanism of silver nanoparticles synthesis was characterized by nitrate reduction test. Synthesized silver nanoparticles were further screened for its antimicrobial activity against multi drug resistant organisms. Future studies can be conducted to purify the silver nanoparticles.

Materials and Methods

Chemicals

Potato Dextrose agar (PDA), Silver nitrate, Lactophenol Cotton Blue Stain, Potassium bromide (FTIR grade), Potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), Magnesium Sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), Ammonium Sulphate $(NH_4)_2SO_4$, yeast extract and glucose from Himedia and SRL Company.

Sample

Soil sediments samples were collected from coastal areas of Nicobar Islands, India during November 2009. Samples were collected in sterile plastic containers and brought to the Molecular and Microbiology Research Laboratory, VIT University. Sample was dried in the hot air oven for overnight at 45ºC. The dried samples were collected in the sterile plastic bags, leveled and stored up to further use. [6]

Isolation of the silver tolerant marine yeast

Isolation of marine yeast was performed by serial dilution and spread plate method on the sabauroud's dextrose agar plate (prepared in 50% marine water). One gram of soil sample was serially diluted in sterilized distilled water to get a concentration range from 10^{-1} to 10^{-6}. A volume of 0.1 ml of each dilution was transferred aseptically to SDA plates. The sample was spreaded uniformly using a glass rod. The plates were incubated at 35ºC for 48 hours.

The yeast isolates were further streaked on SDA plates (enriched with different levels of silver nitrate). Plates were incubated at 35ºC for 48 hours. The cultures growing on the plate were considered as silver tolerant strain and subcultured on SDA plates in order to obtain pure culture. Pure isolates were maintained at 4ºC in refrigerator for further studies.

Biosynthesis of Silver Nanoparticles

The marine yeast was grown in 100 ml sabauroud's dextrose broth (prepared in 50% marine water) in 250 ml Erlenmeyer flask. The flask was incubated in a shaker incubator at a speed of 120 rpm for 48 hours at 35ºC. The broth was harvested and centrifuged at 10,000 rpm for 10 minutes in a cooling centrifuge (4ºC), the supernatant was transferred to another tube the pellet was discarded.

For the synthesis of silver nanoparticles, 100 ml of supernatant was taken in a 250 ml Erlenmeyer flask and challenged with 1 mM silver nitrate. The flask was incubated on orbital shaker at a speed of 120 rpm for 48 hours at 35ºC in dark condition in a rotary shaker incubator. Control (without the silver nitrate, only cell supernatant) was also run along with the experimental flask

Characterization of synthesized silver nanoparticles

The reduction of silver ions was confirmed by qualitative testing of supernatant by UV–visible spectrophotometer. 1 ml of sample supernatant were withdrawn at 1, 3, 24, 48 hrs and absorbance was measured by using UV–visible spectrophotometer (U-2800, Japan) between 400-600 nm. The lyophilized sample was subjected to FTIR Spectroscopy analysis (Thermo Nicolet, Avatar 330 model). Two milligrams of the sample was mixed

with 200 mg KBr (FT-IR grade) and pressed into a pellet. The sample pellet was placed into the sample holder and FT-IR spectra were recorded in the range 4000-450 cm^{-1} in FT-IR spectroscopy at a resolution of 4 cm^{-1}.A thin film of the sample was prepared on a glass slide by dropping 100 µl of the sample on the slide, and was allowed to dry for 5 min. The slides were then scanned with the AFM (Nanosurf Easyscan 2, Switzerland). The lyophilized sample was further characterized by XRD (Bruker, D8 advance, Germany) to know the crystalline nature of the sample. The diffracted intensities were recorded from 10º to 90º 2 Θ angles.

Nitrate reductase assay

Nitrate reductase is an enzyme that converts nitrate to nitrite. The activity was measured by putting in the substrate for the enzyme (nitrate) and then measuring the amount of nitrite after 1 h. The net increase in nitrite at 1 h is the amount of nitrate reductase activity.

Polyphasic taxonomy

The fungal isolates were observed using hand lens and the colony morphology was recorded with respect to color, shape, size and nature of colony. The isolated yeast was microscopically characterized by Gram staining, lactophenol cotton blue mounting, germ tube test and capsule staining,

The strain was screened based on the above traits and the efficient isolate was sent for molecular characterization based on 28s rDNA sequencing Chromous Biotech, Chennai, India. 28s rDNA fragment was amplified using PCR polymerase. The PCR product was sequenced bi-directionally using the forward (TCCGTAGGTGAACCTGCGG) and reverse (TCCTCCGCTTATTGATATGC) primers. The sequence was analysed by ABI3730XL capillary DNA sequencer (ABI Prism 310 Genetic Analyzer, Tokyo, Japan). The phylogenetic tree was constructed by using Tree view 4.5 and the genus and species were successfully identified.

Antimicrobial activity of the silver nanoparticles

Test organisms

Multi Drug Resistant Bacterial (MDRB) strains of *Staphylococcus aureus* and *Klebsiella pneumoniae* cultures were collected from Sri Narayani Hospital, Sripuram, Vellore, TN, India. Both organisms were maintained on nutrient agar medium and stored at 4ºC. Both organisms were inoculated in MHB and incubated overnight at 37°C to make a uniform suspension.

Antibiogram

Both MDR organisms were screened for their sensitivity towards ten standard antibiotics. Antibiotics included ampicillin (10 mcg/disc), Cepodoxime (10 mcg/disc), Chloramphenicol (30 mcg/disc), Ciprofloxacin (5 mcg/disc), Co-trimoxazole (23.75 mcg/disc), Gentamycin (10 mcg/disc), imipenem (10 mcg/disc), nalidixic acid (30 mcg/disc), rifampicin (5 mcg/disc). Drug sensitivity test was performed by disc diffusion method on Muller hinton agar (MHA) plates. Bacterial isolates were inoculated in to nutrient broth for 8 hours. The concentration of the suspensions was adjusted to 0.5 using a spectrophotometer. Isolates were seeded on Mueller Hinton agar plates by using sterilize cotton swabs. The standard antibiotic discs were placed on the agar surface using

a sterilize forceps. Plates were incubated at 37°C for 48 hours. Plates were observed for zone of inhibition. The experiment was performed in triplicates. [11]

Antibacterial assay

Antimicrobial activity of the silver nanoparticles was checked by agar well diffusion method on MHA plates. The concentrations of both suspensions were adjusted to 0.5 using a spectrophotometer and were lawn cultured on MHA plates by using sterilised cotton swabs. In each of these plates, three wells were cut out using a standard cork borer (7 mm diameter). Using a micropipette, 100 µl of silver nitrate solution (100µg/ml), 100µl of silver nanoparticle (100µg/ml) and 100µl of distilled water was added to separate wells. Plates were incubated for 24 hours at 37°C. Anti-bacterial activity was evaluated by measuring the zone of inhibition. Experiment was performed in triplicates. [13]

Minimum Inhibitory Concentration (MIC)

MIC of silver nanoparticles against MDRB strains were checked by modified agar well diffusion method .[8] Synthesized silver nanoparticles were dissolved in distilled water to get concentration range of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100µg/ml. The concentration of test cultures was adjusted to 0.5 using a spectrophotometer and test organisms were lawn cultured on MHA plates. Agar surface was bored by using a sterilize cork borer of 7 mm diameter. A 100 µl of each dilution was poured in to wells. All test plates were incubated at 37°C for 24 hours. The minimum concentration of silver nanoparticle showing a clear zone of inhibition was considered to be MIC. Experiment was performed in triplicates.

Statistical analysis

The results of the antimicrobial activity of biologically synthesized silver nanoparticles are expressed as mean ± standard deviation of the response of 3 replicates determinations per sample. Level of significance was assessed by the Student *t* test at $P>0.05$. Results were analyzed statically by using Microsoft Excel 2007 (Roselle, IL, USA).

Results

There is an increase in need to produce the nanoparticles in a biolological mean that does not use the toxic chemicals in any steps of production. Microorganisms are considered as potential biofactory for the synthesis of metallic nanoparticles.

Isolation of the silver tolerant yeast

The cultures were grown on the SDA plates enriched with different concentrations of silver nitrate. A total of three different yeast colonies were appeared on the plates. These isolates were primarily screened for nanoparticle synthesis by colour change method.

Characterization of silver nanoparticle

Colour change

Culture supernatant of *Candida* sp VITDKGB was mixed with 1 mM silver nitrate solution and incubated in dark in rotary shaker. Samples showed changed in colour from almost light pale yellow to brown, this is a clear indication of the formation of silver nanoparticles in the reaction mixture. The intensity of the colour was increased during the period of incubation. The appearance of brown colour was due to the excitation of

surface plasmon vibrations.[15]Control showed no change in colour of the mixture when incubated in the same conditions (Figure 1).

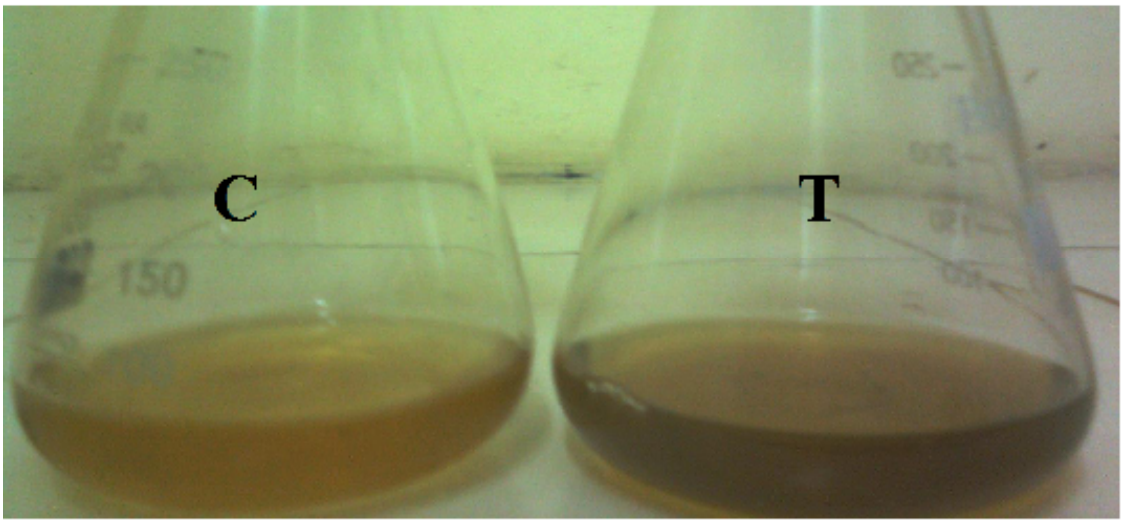


Fig.1. Biosynthesis of silver nanoparticles- colour change reaction: conical flasks containing the culture supernatant of the *Candida* sp. *VITDKGB* (C) and conical flasks containing the culture supernatant of the *Candida* sp. *VITDKGB* after exposure to $AgNO_3$ solution for 24 h (T)

UV-Vis analysis

Synthesis of colloidal silver nanoparticles was initially performed by UV - Visible spectroscopic analysis. Samples were collected at 6th, 24th, 48th and 72nd hour and the UV – Visible spectrum was recorded, a strong peak was observed at 430 nm, indicate the presence of silver nanoparticles. UV – visible spectra is reported in Figure 2.

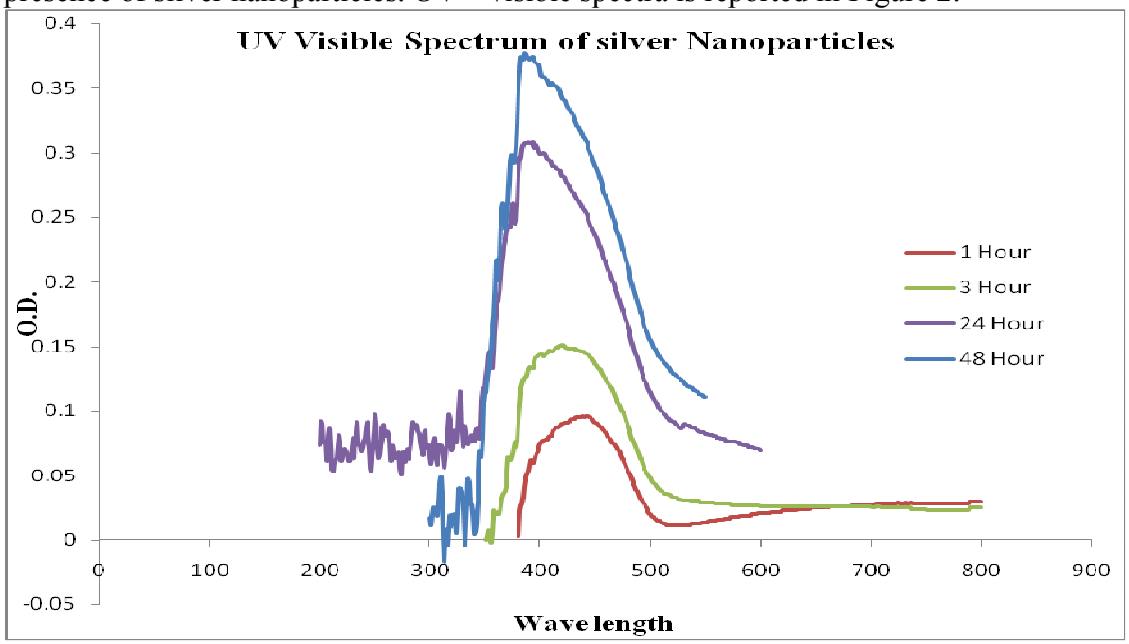
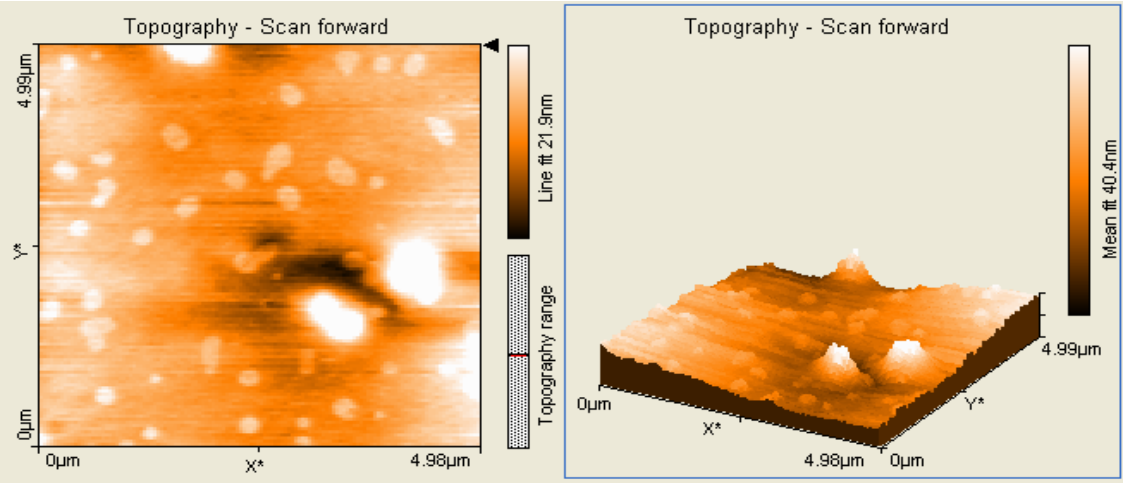


Fig. 2. UV-Visible spectrum of yeast cell supernatant containing silver nanoparticles at different time intervals.

AFM analysis

The specified morphological features of the synthesized NPs were investigated by AFM analysis. The surface morphology of the sample can be better visualized and understood by their 3D topographic view (Figure 3). It was noticed that the silver nanoparticles were formed and it was around 87 nm in size.[11]



5. FTIR analysis

The lyophilized nanoparticle samples were analyzed in FTIR to identify the possible biomolecules responsible for the reduction of the Ag+ ions by the cell filtrate. The FTIR spectrum is presented in Figure 4. The representative spectra of nanoparticles obtained manifests absorption peaks located at about 3442.97 cm^{-1} was assigned to the stretching vibration of primary amines. Another band seen at 1383.16 cm^{-1} corresponds to the C-N stretching of amines. This proves the presence of protein in the sample.[12] Few other bands also observed as 2927.34 cm^{-1} (Aliphatic – CH_3 and CH_2 Stretching), 1631.31 cm^{-1} (-NHCO of amide), 1224.76 cm^{-1} (Ester carbonyl group, phenol), 1062.49 cm^{-1} (C-O Stretching of polysaccharides, Si-O asymmetric stretch) and 643.72 cm^{-1} (CH out of plane bending of carbohydrade).

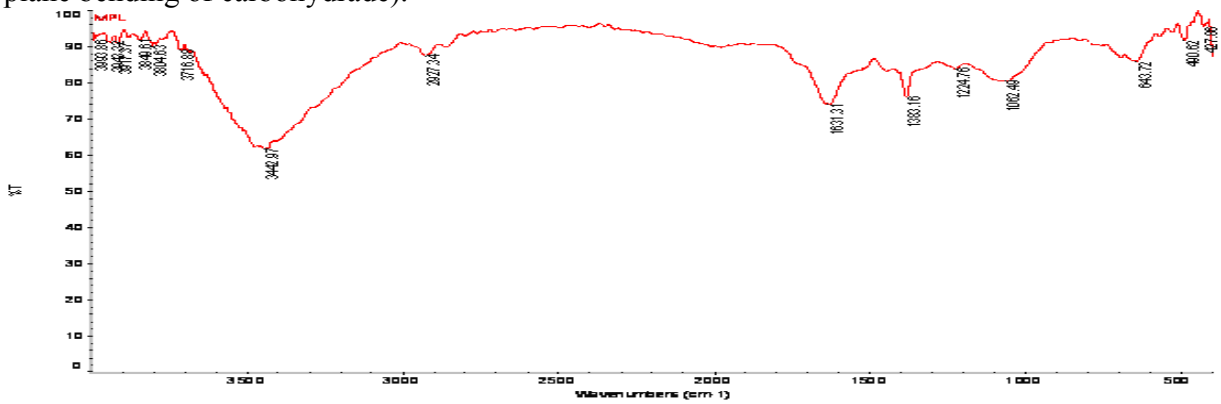


Fig. 4. FTIR Spectrum of lyophilized cell supernatant with silver nanoparticles after 48hours incubation.

XRD analysis

The pattern of the sample corresponds to the silver nanoparticles. The 2theta values were taken in the range of 10-30 and compared with XRD spectrum of pure crystalline structure was published by the joint committee of powder diffraction standards file no. 040783. The presence of 2-THETA values of 38.4°, 46.47°, 64.79°,77.58° corresponds to (111), (200), (220) and (311) planes of silver respectively (Figure 5) . The data conformed presence of silver nanoparticles in the sample.[12]

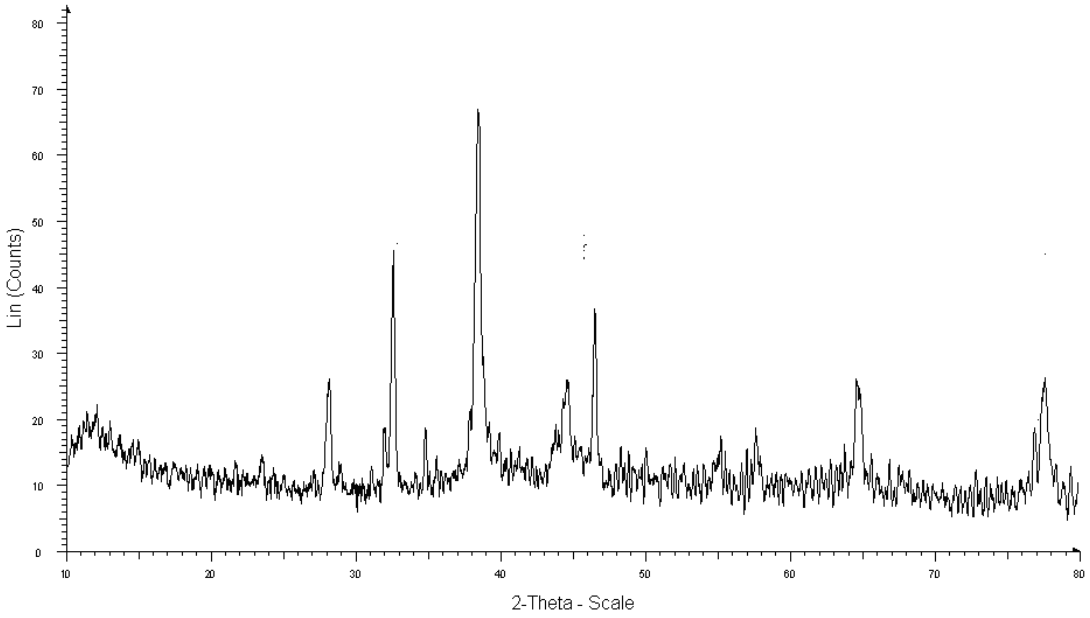


Fig. 5. XRD spectrum of lyophilized cell supernatant with silver nanoparticles

7. Antibiogram

Antibacterial activity of biologically synthesized silver nanoparticles was analyzed against two multidrug resistant organisms included *S. aureus* and *K. pneumonia*e. These two strains showed resistance against all drugs. The nanopatrticles exhibited significantly high antimicrobial activity than that of the pure silver nitrate. The results are expressed as mean ± standard deviation of the three replicates (Table 1). Silver nanoparticles formed 7.33±0.57 and 5.66±0.57 mm zone of inhibition against multidrug resistant *S. aureus* and *K. pneumoniae* respectively. The biologically synthesized silver nanoparticles exhibited very low MIC value, which conclude the higher activity of silver nanoparticles. Nanoparticles showed 20, 40 µg/ml MIC values against *S. aureus* and *K. pneumoniae* respectively.

Table 1. Antimicrobial activity of silver nanoparticles against MDRB

Name of pathogens	Zone of Inhibition (mm)	
	AgNP	**$AgNO_3$**
Staphylococcus aureus	14.66±1.52	7.33±0.57
Klebsiella pneumoniae	12.33±0.57	5.66±0.57

Characterization of isolated yeast

The yeast isolates was characterized on the basis of colony characteristics, microscopic appearance and molecular characterstics. Considering the colony characteristics and microscopic appearance (Table 2) the isolate was identified as *Candida* sp. Taxonomical identification of the bacterial isolate was performed by 28s rDNA analysis. The 28s rDNA sequence of the isolate was blasted using online tool blast of NCBI gene bank and the phylogenetic tree was constructed with other homologous sequences (Figure 6). The sequence was submitted to NCBI gene bank (Accession number: HM194888). Based on the morphological, physiological and molecular identification, the isolate was identified as *Candida* sp VITDKGB.

Table 2. Characterization of the isolated yeast strain

Characteristics		Results
Morphology	Growth on SDA	Moist, white and slimy colonies
	Growth on corn meal agar	Large amount of long branched pseudohyphae were seen. Oval shaped blastoconidia were budding off from the pseudohyphae
Microscopy	Gram staining	Gram positive, spherical to sub spherical budding yeast cells were observed, the cells were arranged in group
	LPCB	Spherical to sub spherical budding yeast cells were observed, the cells were arranged in group.
	Germ tube test	Negative
	Capsule staining	Negative

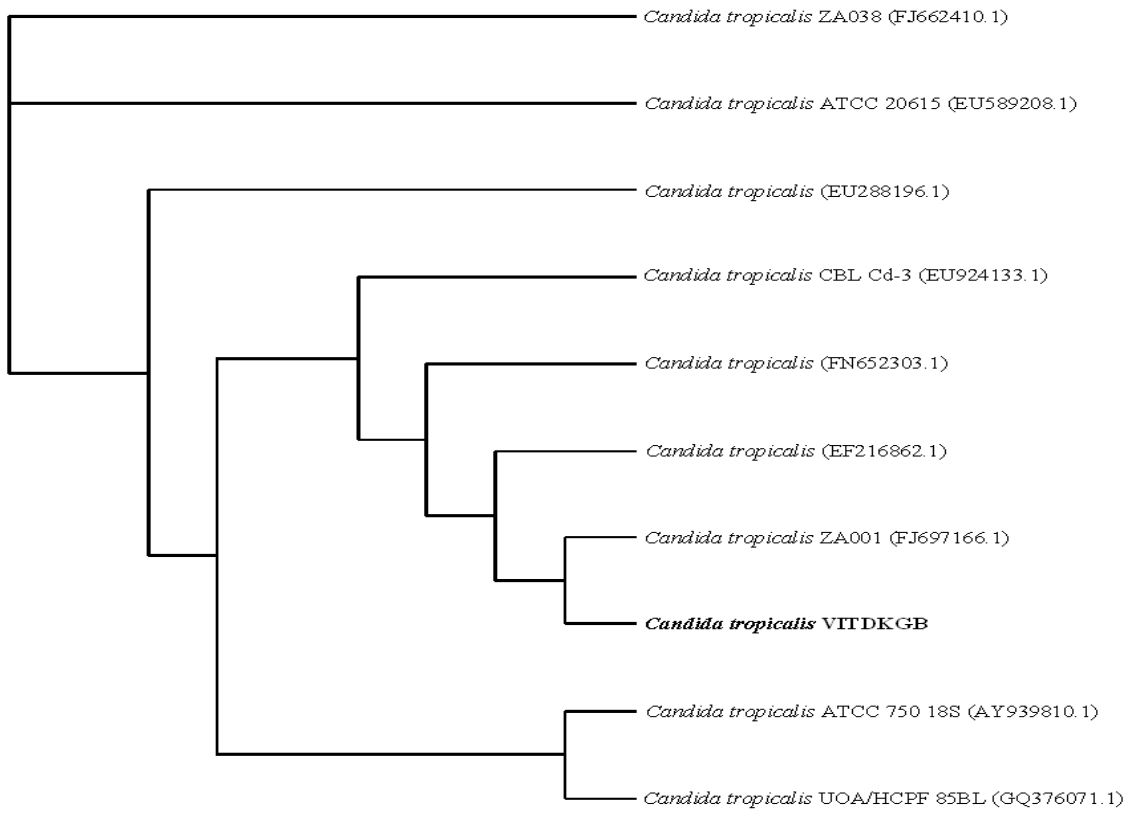


Fig. 6. Phylogenetic tree of *Candida* sp VITDKGB

Nitrate reductase test

The test organism *Candida sp VITDKGB* was found positive for nitrate reductase activity. The nitrate reductase activity of the culture supernatant was found to be 320 nmol/ h/ml. Nitrate reductase activity of the isolate indicates the possible mechanism of the reduction of silver nitrate in to silver nanoparticles.[16]

Discussion

Multi drug resistance is a condition enabling the microorganism to resist distinct drug or chemical of a wide variety of structure and function targeted at eradicating the organism. These multi drug resistance organisms are highly infectious with high mortality rate and severity of infection by these multi drug resistant organism is very high in immunocompromised patients especially who are suffering with AIDS.[15] To combat the multi drug resistance organism, discovery and development of new antimicrobial compounds is very essential, therefore scientist are looking forward to discover novel antimicrobial compounds from alternative sources and here silver nanoparticles provides an important option for the discovery of new antimicrobial compounds. *C. tropicalis* is unicellular yeast found throughout the world. Several studies documented *C. tropicalis* is in the marine environment, *C. tropicalis* has been isolated from the Indian Ocean water, intestines of marine animals in Pacific and Atlantic Ocean, bathing beaches in South

Florida and from valve shell fish from long island sand USA. [13, 17, 18] A total of 45 isolates of *C .tropicalis* were isolated from coastal waters of north eastern Taiwan.[19] Above cited literature representes *C. tropicalis* as a native flora of marine environment.The appearance of brown colour was due to the excitation of surface plasmon vibrations.[15] Compare with the earlier, study of extracellular synthesis of silver nanoparticles by yeast species *MKY3,* the particle size was 1-5 nm in size, which is much smaller than that of the current study.[10] *Candida sp VITDKGB* was producing both spherical and rod shape nanoparticles. Pal et al. (2007) reported like truncated triangular nanoparticles show bacterial inhibition with silver content of 1 μg. While, in case of spherical nanoparticles total silver content of 12.5 μg is needed. The rod shaped particles need a total of 50 to 100 μg of silver content.[19] The silver nanoparticles from *Candida* sp. VITDKGB are new antimicrobial compounds against multidrug resistant pathogens. The future silver nanoparticles from *Candida sp* become a very good alternative therapy for evolutionary microorganisms.

Conclusions

In this study silver nanoparticles were biologically synthesized using yeast isolates. The yeast was isolated from marine sediments and characterized by molecular techniques (28s rDNA) as *Candida sp VITDKGB* (Acc No: HM194888). Results conclude that the isolate is a prominent producer of silver nanoparticles. These silver nanoparticles found to be effective to inhibit multi drug resistant organisms such as *Staphylococcus aureus* and *Klebsiella pneumoniae.*

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