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Biosynthesis and antimicrobial potential of silver and zinc oxide nanoparticles using *Candida diversa* strain JA1

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ABSTRACT

In the present investigation, extracellular biosynthesis of silver and zinc oxide nanoparticles was investigated using Candida diversa strain JA1 isolated from waste water of milk processing unit in Vellore, Tamil Nadu, India. The biological synthesized metal nanoparticles were characterized through several analytical techniques including UV-visible spectrophotometer, X-ray diffraction pattern analysis (XRD), FE-Scanning electron microscope (SEM) with EDX-analysis (EDXA). The antimicrobial properties of biosynthesized metallic nanoparticles were tested against medically important Gram positive (Enterococcus sp. and Staphylococcus aureus), Gram negative (Escherichia coli, Salmonella sp., Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella pneumonia, Shigella sp.) and fungal pathogenic microorganisms (Aspergillus terreus strain JAS1, Ganoderma sp. JAS4, Scedosporium sp. JAS1, Candida tropicalis and Fusarium sp.). The combined effect of antibiotics and biologically synthesized metal nanoparticles were also evaluated against clinical pathogens.

Keywords: Biosynthesis; *Candida diversa* Strain JA1; Characterization; Antimicrobial and Synergistic effect; Clinical pathogens

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INTRODUCTION

Nanobiotechnology deals with developing biosynthetic and environmental-friendly technology for synthesis of nanomaterials. The noble metals have been extensively used to undergo reduction of metal ions by various physical, chemical and biological sources. The recovery of the precious metals by microbial sources such as bacteria, fungi with the formation of their nanoparticles is an eco-friendly alternative to the conventional methods [1]. The scientific community has turned to biological systems for synthesis and assembly of nanomaterials instead of toxic chemicals used in chemical protocols [2]. The major drawback of chemical reduction method is the use of toxic reducing agents which possesses serious health effects to human health and environment [3]. To overcome the disadvantages of chemical reduction method, the biological reduction method attempts to obtain eco-friendly nanoparticles. Nanoparticles possess unique properties like magnetic, electrical, catalytic, optical, piezoelectric, pyroelectric and photoconducting properties [4]. Nanomaterials have also found potential applications in the fields of medical and pharmaceutical nanoengineering. They are employed as therapeutic agents [5], in chronic disease diagnostics, and in sensors [6]. The metal nonaparticles including silver and zinc oxide nanoparticles have gained considerable attention due to their unique antibacterial, antifungal, and UV filtering properties [7]. The microbial biosynthesis of nanomaterials using bacteria [8] and fungi [9] has been exhaustively studied by various researchers. There are very few reports related to yeast mediated biosynthesis of nanomaterials. The extracellular synthesis [10]

of silver nanoparticles by silver tolerant yeast strain MKY3 with soluble silver in log phase of growth which could precipitate majority of silver extracellular as elemental naoparticles [11]. The increasing antibiotic resistance is a major threat to our society. The development of new antibiotics and drug targets encourages the scientific community to look for other alternatives for antibiotic production. As metallic nanoparticles demonstrate well known antimicrobial property, the development of novel applications in this field makes them an attractive alternative to antibiotics [12]. In the present study, silver and zinc oxide nanoparticles have been successfully synthesized by *Candida diversa* strain JA1. The sophisticated analytical techniques including UV-absorption spectroscopy, X-ray diffraction spectroscopy and Scanning electron microscopy with EDXA was employed to characterize biosynthesized metal nanoparticles. The antimicrobial and synergistic effect of biological synthesized silver and zinc oxide nanoparticles was determined against various clinical pathogens.

MATERIALS AND METHODS

Sample Collection and Isolation

The samples for isolation of yeast were collected from the milk processing unit in Vellore in sterile container and transported to laboratory for further processing. The isolation of *Candida diversa* strain JA1 was carried out by standard serial dilution method [13] and spread plate was performed on Malt yeast peptone glucose agar (yeast extract 0.3%, malt extract 0.3%, peptone 0.5%, glucose 1% and agar 1.5%). The colonies appeared were further purified by repeatedly streaking on Malt yeast peptone glucose agar (MYPGA).

Molecular Characterization of Strain JA1

The genomic DNA of *Candida diversa* strain JA1 was extracted using Rainey [14] protocol. The 18S rRNA gene was amplified with the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The amplified DNA fragment was separated on 1% agarose gel. The purified PCR product was sequenced using the Big-Dye terminator kit ABI 310 Genetic Analyzer (Applied Biosystems, USA). The phylogenetic position of the isolated strain (*Candida diversa* strain JA1) was assessed by performing a nucleotide sequence database search using the BLAST program from NCBI GenBank. The nucleotide sequencing result was submitted to the GenBank national Centre for Biotechnology Information (NCBI) and accession number obtained is KC509573.

Biosynthesis of Nanoparticles

The biological synthesis of silver nanoparticles was carried with *Candida diversa* strain JA1, the active culture of the isolate was inoculated into potato dextrose broth and the flasks were incubated at 28 °C and 200 rpm for 3 d. The freshly prepared culture of *Candida diversa* strain JA1 was centrifuged at 4000 rpm for 30 min and the supernatant was used for the biosynthesis of silver nanoparticles (AgNPs). The collected supernatant (1%) was added to deionized water treated with 1 mM silver nitrate ($AgNO_3$) with (≥ 99.0% purity, Sigma Aldrich) and was further incubated for 96 h at 28 °C. The deionized water was used as a solvent in the synthesis of AgNPs [3].

The isolate *Candida diversa* strain JA1 was allowed to grow in Malt-yeast peptone glucose broth medium for 3 d. After incubation period was complete, the freshly prepared *Candida diversa* strain JA1 culture was centrifuged at 4000 rpm for 30 mins and supernatant was used for the biosynthesis of zinc oxide nanoparticles (ZnO NPs). 0.1 g of Zinc Oxide (ZnO) with (≥ 99.0% purity, Sigma Aldrich) was added to de-ionized water followed by 1% supernatant of *Candida diversa* strain JA1 culture. The biosynthesis of ZnO NPs was carried out in Erlenmeyer flask containing de-ionized water treated with ZnO and was incubated at 37 °C under agitation (200 rpm) for 24–48 h until the white deposition appears at the bottom of flask [15].

Characterization of Synthesized Nanoparticles

The UV-absorption spectrum of $AgNO_3$ and ZnO treated supernatant was monitored by taking small aliquots periodically at 1 h, 24 h and 48 h and 24 h, 48 h and 72 h respectively. The metal ion reduction was monitored by measuring UV-spectrum treated supernatant periodically. The aliquots of this solution were monitored for UV-spectra after 1 h, 24 h and 48 h. The UV-Vis spectroscopy measurements were recorded on HITACHI, Model U-2800 spectrophotometer from 300 nm to 600 nm. The AgNPs and ZnO NPs dispersed in deionized water were observed for their surface plasmon resonance at 420 nm and 374 nm respectively. In this analysis, the supernatant and $AgNO_3$ and ZnO were used separately as control 1 and 2 respectively. The biologically synthesized AgNPs and ZnO NPs were freeze-dried, powdered and used for X-ray diffraction (XRD) spectroscopy [16]. The phase formation, purity and crystalline structure of metallic naoparticles were recorded using powder X-ray diffractometer

(Model-D8 Advance, made in BRUKER Germany) at 40 kV/20mA using continuous scanning 2Θ mode. The biologically synthesized metallic nanoparticles were mounted on specimen stubs with double-sided adhesive tape coated with platinum in a sputter coater and examined under field emission scanning electron microscopy (HITACH, Model E-1010 Ion sputter) to avoid charging and examined under SEM (HITACH, Model S-3400N). In addition, the presence of silver and zinc oxide metals in the sample was analyzed by energy dispersive x-ray analysis (EDXA) combined with Field Emission Scanning Electron Microscopy.

Biological Assay

The antimicrobial potential of biosynthesized AgNPs and ZnO NPs against clinical and plant pathogens was determined by Kirby-Bauer method. The test organisms (clinical and plant pathogens) were acquired from Microbial Biotechnology Laboratory, SBST, VIT University, Vellore, India. The Gram negative organisms (*Escherichia coli*, *Salmonella* sp., *Shigella* sp., *Proteus mirabilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*), Gram positive organisms (*Enterococcus* sp. and *Staphylococcus aureus*), and fungal strains (*Candida tropicalis*, *Fusarium* sp., *Scedosporium* sp. JAS1, *Ganoderma* sp. JAS4, *Aspergillus terreus* strain JAS1) were used to study the antimicrobial properties of biologically synthesized metallic nanoparticles. The multi drug resistance of clinical pathogens was screened against standard antibiotic discs ciprofloxacin (30 μg/disc), ofloxacin (5 μg/disc), vancomycin (30 μg/disc), erythromycin (15 μg/disc), penicillin (10 μg/disc), tigecycline (15 μg/disc) and fungal isolates were screened against flucanazole (25 μg/disc) and voriconazole (5 μg/disc).

The antibiotic sensitivity test was performed by Kirby-Bauer Method on Muller-Hinton agar plates [17]. The bacterial test pathogens were lawn cultured on Muller-Hinton agar plates using sterile cotton swabs. The standard antibiotic discs were placed on it using sterile forceps. Plates were incubated at 37 °C for 24-48 h and were observed for zone of inhibition around the disc. Fungal test pathogens were seeded into Potato Dextrose Agar petridishes, antibiotics disc was placed on it using sterile forceps. Plates were incubated at 30 °C for 5 d and were observed for zone of inhibition. The combined effect of antibiotic with biosynthesized metallic nanoparticles defines the synergistic effect against clinical pathogens. A disk diffusion method was used to evaluate the synergistic effect of antibiotics with biosynthesized AgNPs and ZnO NPs against Gram negative and positive test strains on Muller-Hinton agar plates. To determine the synergistic effects, each standard antibiotic disk was further impregnated with 10 μL of freshly prepared AgNPs and ZnO NPs. Muller-Hinton agar medium plates were seeded with 100 μL of test organisms and antibiotic disk impregnated with AgNPs and ZnO NPs were placed onto agar plates. After incubation at 37 °C for 24 h the zones of inhibition were measured. For fungal test organisms, potato dextrose agar plates were seeded with test strains and antibiotic discs were placed onto agar plates for 5d. The zone of inhibition was measured after the incubation period was completed.

RESULTS AND DISCUSSION

The pure colonies of isolate were obtained on Malt yeast peptone glucose agar and the isolate was identified and characterized as *Candida diversa* strain JA1 based on the molecular characterization through 18S rRNA sequencing studies shown in Fig. 1. The metallic nanoparticles were successfully synthesized by the culture supernatant of *Candida diversa* strain JA1. The appearance of a yellowish-brown color in the silver nitrate treated flask indicated the formation of silver nanoparticles, whereas no color change was observed in supernatant without silver nitrate in both controls. The deposition of white clusters at the bottom of flask treated with zinc oxide indicated the formation of ZnO NPs by the culture supernatant of *Candida diversa* strain JA1. The synthesis of metallic nanoparticles is mediated by enzymes present in the microorganisms such as nitrate reductase [18]. The nitrate reductase enzyme is induced by nitrate ions and reduces silver ions to metallic silver. The mechanism behind reduction of silver ions is due to electron shuttle enzymatic reduction process [19] and microorganisms produce cofactors like NADH and NADH-dependent nitrate enzymes that are important factor in bioreduction of metal ions [20].

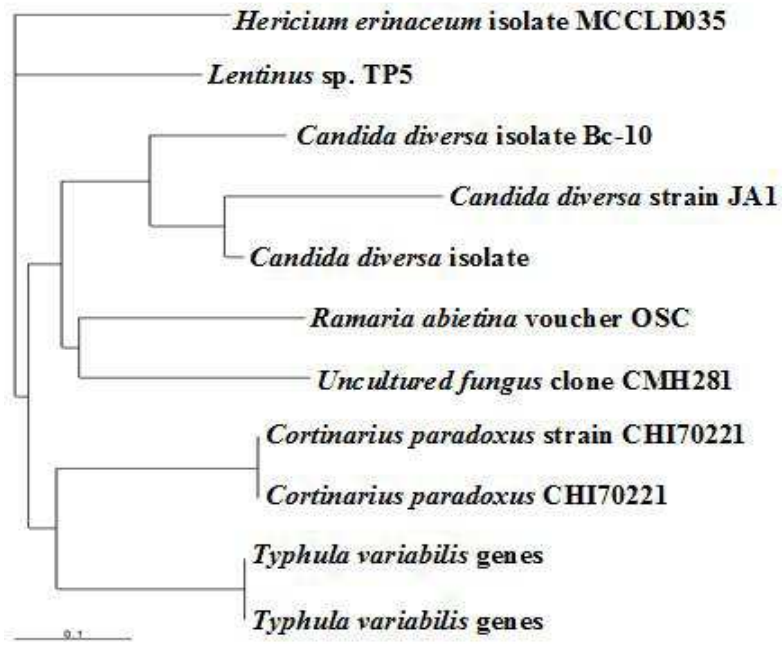

Fig. 1 Phylogenetic relationship based on 18S rRNA gene nucleotide sequences between the *Candida diversa* strain JA1 and reference sequences retrieved from NCBI Gen Bank constructed through the neighbor joining method

The aqueous metal ions were reduced to silver and zinc oxide nanoparticles in $AgNO_3$ and ZnO treated flask. The formation and stability of the reduced metallic nanoparticles in the colloidal solution was monitored by UV–Vis absorption spectrum. The UV absorption spectrum from *Candida diversa* strain JA1 reaction vessel at different time of reaction is plotted in Fig. 2 and 3 which indicates the presence of AgNPs and ZnO NPs respectively. A strong and broad peak observed between 420 nm and 425 nm confirms the formation of AgNPs. According to Henglein [21], the observation of this particular peak represents the nanoparticles ranging from 2 nm to 100 nm. An absorption peak observed at 374 nm indicates the successful biosynthesis of ZnO NPs. The XRD pattern of silver nitrate treated sample shows four intense peak in whole spectrum 2θ ranging from 10 to 90.

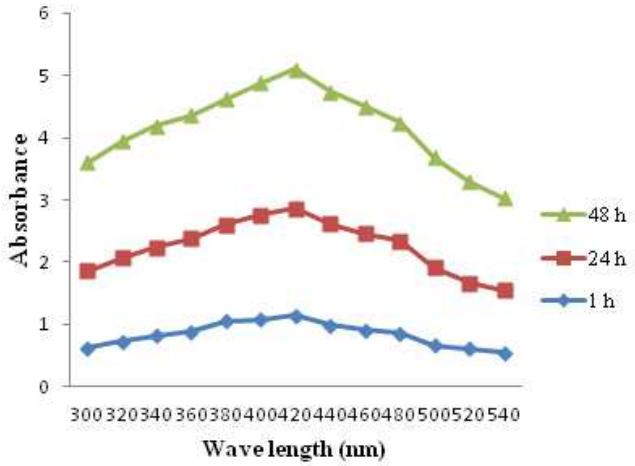

Fig. 2 UV-visible absorption spectrum of AgNPs synthesized by extracellular components of *Candida diversa* strain JA1

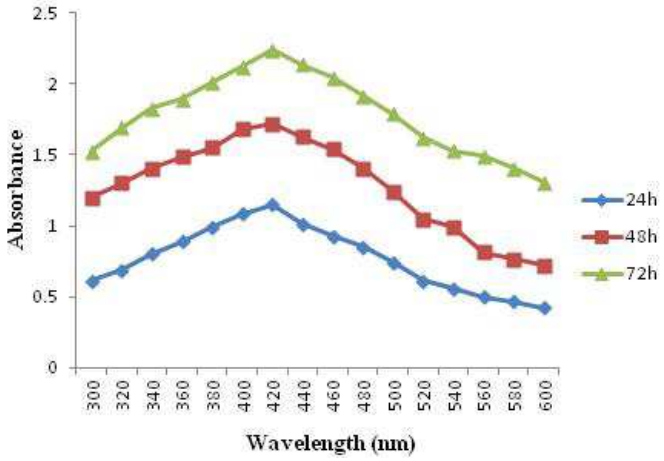

Fig. 3 UV-visible absorption spectrum of ZnO NPs synthesized by extracellular components of *Candida diversa* strain JA1

The characteristic XRD peaks were centered at 38°, 45° and 65°, which is similar to the following crystalline planes of silver; (1 1 1), (2 0 0) and (2 2 0), respectively. The strong and narrow diffraction peaks in the XRD pattern indicate that the product has well crystalline structure (Fig. 4). The XRD peaks at 31°, 47°, 56° and 75° were identified as (1 0 0), (1 0 1), (1 0 2), (1 1 0) and (2 0 2) reflections, respectively (Fig. 5).

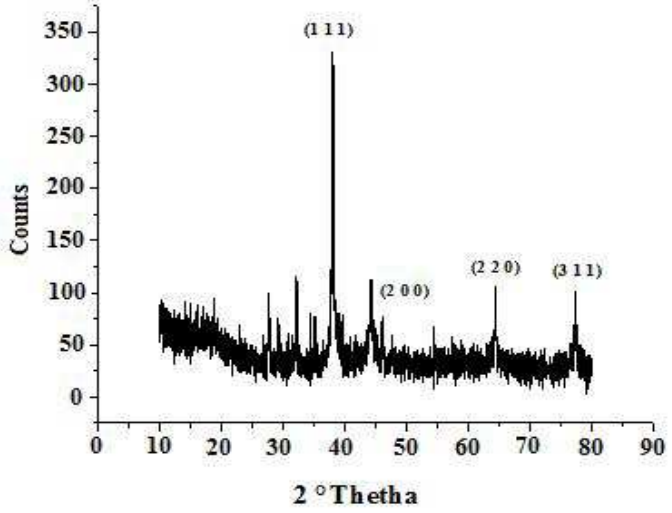

Fig. 4 XRD pattern of biosynthesized AgNPs by extracellular components of *Candida diversa* strain JA1

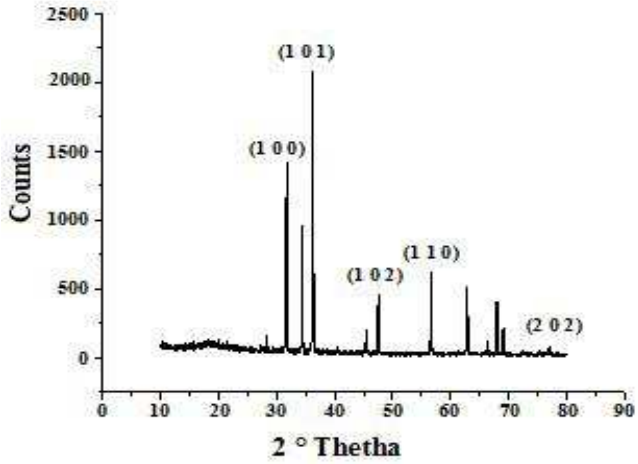

Fig. 5 XRD pattern of biosynthesized ZnO NPs by extracellular components of *Candida diversa* strain JA1

These peaks indicate the synthesis and well crystalline structure of zinc nanoparticles. The formation of the biosynthesized AgNPs and ZnO NPs was examined by EDXA combined with FE-SEM. The EDXA spectrum revealed a strong signal for metallic nanoparticles. The appearance of signals was likely due to X-ray emission from carbohydrates/proteins/enzymes present in the cell wall of the biomass [22]. It has been demonstrated that the size, shape, surface area, solubility, chemical composition, dispersion factor of nanoparticles play exceptional roles in determining their biological responses [23]. Fig. 6(a) represents the silver nanoparticles of long elongated shape synthesized by *Candida diversa* strain JA1 with size predominantly of 92.8 nm and Fig. 6(b) confirms the presence of biologically synthesized silver through EDXA. Fig. 7 (a) demonstrates the zinc oxide nanoparticles of rectangular shape with the size of 73 nm and Fig. 7(b) confirms the presence of biosynthesized zinc oxide nanoparticles through EDXA.

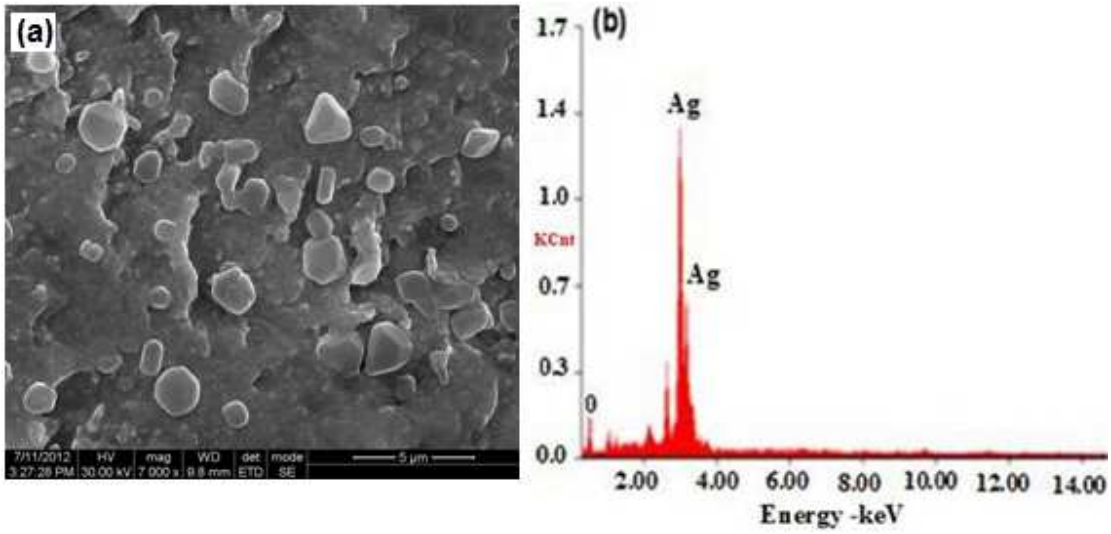

Fig. 6(a) Biosynthesized AgNPs by *Candida diversa* strain JA1 corresponds to 1 µm scale bar
6(b) EDXA analysis of extracellularly biosynthesized AgNPs by *Candida diversa* strain JA1

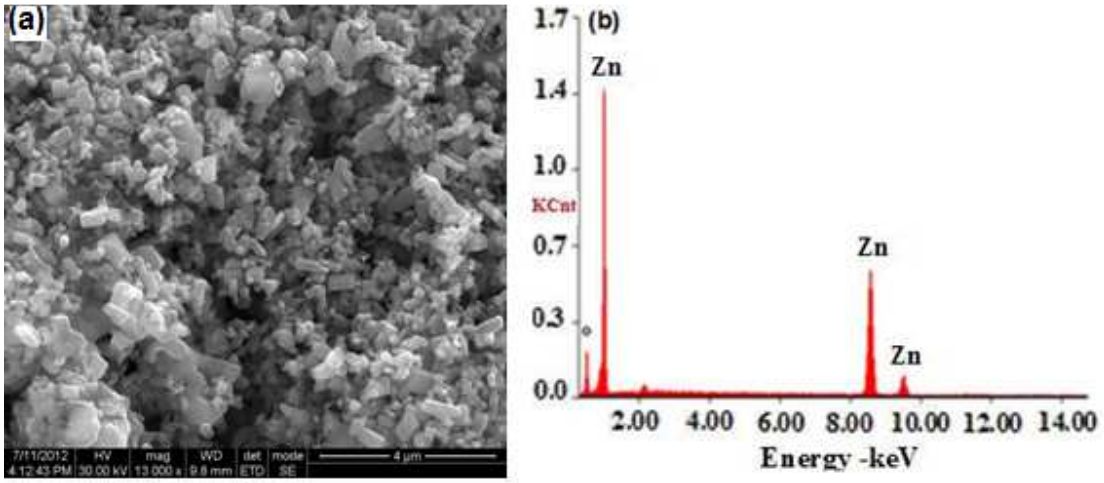

Fig. 7(a) Biosynthesized ZnO NPs by *Candida diversa* strain JA1 corresponds to 1 *μ*m scale bar
7(b) EDXA analysis of extracellularly biosynthesized ZnO NPs by *Candida diversa* strain JA1

The antimicrobial activity of extracellular biosynthesized AgNPs was evaluated against various pathogenic bacteria and fungi by Kirby-Bauer method. *Escherichia coli* (14.00±0.00), *Pseudomonas aeruginosa* (13.33±0.47), *Salmonella* sp. (14.33±0.47), *Staphylococcus aureus* (11.66±0.47), *Enterococcus* sp. (11.00±0.81), *Proteus mirabilis* (13.66±0.47). Biosynthesized AgNPs were also found to inhibit fungal plant pathogens with 22.33±0.47 diameter zone of inhibition against *Fusarium* sp. and 16.33±0.47 of zone of inhibition against *Aspergillus terreus* strain JAS1. Strong inhibitory activity of ZnO NPs was manifested against *Pseudomonas aeruginosa* (15.66±0.47) and *Fusarium* sp. (16.33±0.47). The antibiogram study of biosynthesized silver [3] and zinc oxide [24] nanoparticles has been previously reported by various researchers against pathogenic microorganisms. The antimicrobial effect manifested by metallic nanaoparticles synthesized using *Candida diversa* strain JA1 has shown pronounced inhibitory effect against various clinical pathogenic microorganisms. The destabilization of outer membrane, collapse of the plasma membrane and depletion of intracellular ATP by the metallic nanoparticles is responsible for the formation of zone of inhibition against clinical pathogens. The synergistic effect represents the combination of antibiotics with biosynthesized metallic nanoparticles against clinical pathogen. The antibacterial activity of tigecycline and ofloxacin increased in the presence of biosynthesized silver nanoparticles against test strains (Table 1). The increase in synergistic effect may be caused by the bonding reaction between antibiotic and nanomaterials. The antibiotic molecules contain many active groups such as hydroxyl and amide groups, which react easily with nanosilver by chelation. The combined effect of biosynthesized ZnO NPs with erythromycin, tigecycline and ofloxacin antibiotics was promising against various clinical pathogens and showed effective zone of inhibition (Table 2).

Table 1. Diameter of zone of inhibition of combined effect of extracellularly biosynthesized AgNPs with different antibiotics (with and without antibiotics) against Gram positive and Gram negative bacteria

Microorganisms	Antibiotics Diameter zone of inhibition (mm)											
	Tigecycline			Vancomycin			Erthromycin			Ofloxacin		
	Ab	Ab + NP	%	Ab	Ab + NP	%	Ab	Ab + NP	%	Ab	Ab + NP	%
E. coli	R	18	-	R	17	-	R	-	-	17	20	6.64
P. aeruginosa	12	15	25.00	R	20	-	30	35	16.66	13	15	15.38
Salmonella sp.	20	23	15.00	R	-	-	R	-	-	15	18	20.00
S. aureus	25	30	20.00	R	20	-	12	19	58.3	17	19	11.76
Shigella sp.	13	15	15.38	R	18	-	R	-	-	12	15	25.00
P. mirabilis	19	20	5.27	R	18	-	R	-	-	13	15	15.38
Enterococci sp.	R	10	-	R	14	-	18	22	22.2	16	19	18.75
K. pneumonia	12	15	25.00	R	17	-	17	19	11.76	19	20	5.56

*Note: Ab (a) – Antibiotic disc; Ab + Np (b) – Antibiotic disc dipped in nanoparticle; Over all percentile increase % = b-a/a*100*
R- Resistant

Table 2. Diameter of zone of inhibition of combined effect of extracellularly biosynthesized ZnO NPs with different antibiotics (with and without antibiotics) against Gram positive and Gram negative bacteria

Microorganisms	Antibiotics Diameter zone of inhibition (mm)											
	Tigecycline			Vancomycin			Erthromycin			Ofloxacin		
	Ab	Ab + NP	%	Ab	Ab + NP	%	Ab	Ab + NP	%	Ab	Ab + NP	%
E. coli	R	18	-	R	R	R	R	18	-	13	16	23.07
P. aeruginosa	25	30	20	R	21	-	20	23	15.00	15	33	120
Salmonella sp.	16	21	31.25	R	13	-	R	19	-	30	31	3.34
S. aureus	15	22	46.67	R	15	-	11	16	44.46	28	30	7.17
Shigella sp.	19	20	5.27	R	27	-	R	-	-	24	26	8.34
P. mirabilis	15	20	33.34	R	21	-	R	-	-	18	25	38.39
Enterococci sp.	R	22	-	R	21	-	20	23	15.00	16	27	68.75
K. pneumoniae	18	19	5.56	R	14	-	12	15	25	10	21	110

*Note: Ab (a) – Antibiotic disc; Ab + Np (b) – Antibiotic disc dipped in nanoparticle; Over all percentile increase % = b-a/a*100; R- Resistant*

The clinical pathogens exhibited multiple drug resistant to various commercially used antibiotics. All the bacterial clinical pathogens showed resistance towards vancomycin (30 μg/disc) followed by tetracycline (15 μg/disc) and erythromycin (15 μg/disc). Ofloxacin (5 μg/disc) was found to be sensitive against all the bacteria clinical pathogens (Table 3). The fungal strains were completely resistant to flucanazole (25 μg/disc) but the strains were sensitive to voriconazole (5 μg/disc) as shown in (Table 4).

Table 3. Antibiogram study of bacterial clinical pathogens

Clinical pathogens	Diameter zone of inhibition (mm) against antibiotic disc					
	Ofloxacin	Erythromycin	Tigecycline	Vancomycin	Ciprofloxacin	Tetracycline
E. coli	S (11)	R	S (19)	R	S (18)	R
Salmonella sp.	S (26)	R	S (16)	R	S (13)	S (14)
S. aureus	S (22)	S (18)	S (26)	R	S (19)	S (17)
P. mirabilis	S (28)	R	R	R	R	S (18)
Shigella sp.	S (31)	R	S (20)	R	R	S (22)
Enterococcus sp.	S (18)	S (23)	R	R	S (19)	R

Note: S- Sensitive, R- Resistant

Table 4. Antibiogram study of fungal clinical pathogens.

Microorganisms	Diameter of zone of inhibition antibiotic disc (mm)	
	Flucanazole (25 μg/disc)	Voriconazole (5 μg/disc)
Candida tropicalis	R	S (29)
Fusarium sp.	R	S (26)
Scedosporium sp. JAS1	R	S (27)
Ganoderma sp. JAS4	R	S (30)
Aspergillus terreus strain JAS1	R	S (33)

Note: S- Sensitive, R- Resistant

Biosynthesis of nanoparticles offers a valuable contribution to the field of medicine when compared to physical and chemical methods of nanopaticles synthesis. In the present investigation biosynthesized AgNPs and Zn ONPs showed good antimicrobial effect against clinical as well as plant pathogenic microorganisms.

CONCLUSION

In the present work, we were able to successfully synthesize Ag and ZnO nanapaticles using extracellular components of *Candida diversa* strain JA1. The biosynthetic methods followed in the present investigation in synthesis of nanoparticles employing *Candida diversa* strain JA1 are economical and nanoparticles have proven antimicrobial activity. The use of microbes such as yeast releases proteins, organic acids and polysaccharides which may have played an important role in stabilization of biosynthesized metallic nanoparticles. The biosynthesized nanoparticles showed pronounced antimicrobial and synergistic effect against clinical and plant pathogens. The biosynthesis have proven to be eco-friendly and non-toxic alternative over physical and chemical methods of synthesis. The distinct advantage of using microbes for nanoparticle synthesis over chemical reactions is the ease and handling of mass production of *Candida diversa* strain JA1 which in turn very convenient to mass produce metallic nanoparticles.

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