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Research Article

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Extracelluler biosynthesis of silver nanoparticles (Ag-NPs) using *Fusarium oxysporium* (MTCC-2480) and its antibacterial efficacy against gram negative human pathogens

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ABSTRACT

Nanotechnology has developed as an emerging field and modern research with potential biomedical sciences. Emerging of new infectious diseases and the occurrence of drug resistance bacteria is challenging to the researchers. This would initiate the search for unique constituent to be predictable new antimicrobials. Extracellular biosynthesis of silver nanoparticles (Ag-NPs) was carried out by Fusarium oxysporum (MTCC-2480). The colour change of culture supernatant from pale to brown colour indicates the reduction of silver metals into silver ions. Silver nanoparticles were further characterized and confirmed by performing thin layer chromatography (TLC). A single separate band of silver nanoparticle was observed by TLC with R_f value of 0.75. Silver nanoparticles have been evaluated for antibacterial activities against selected human pathogens by disc and well method. Silver nanoparticle exhibited highest antibacterial activity against Salmonella typhi followed by Pseudomonas aeroginosa. Silver nanoparticles were subjected to study the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) against the selected human pathogens. MIC revealed that 20µl/ml of Ag-NPs exhibited highest inhibition activity against Klebsiella pneumoniae followed by Pseudomonas aeruginosa and MBC revealed that 100% inhibition was observed against Pseudomonas aeruginosa. The nanobiotechnological fugal based biosynthesis of silver nanoparticles has high potential as antibacterial bio-resource.

Key words: silver nanoparticles, antibacterial activity, human pathogens

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INTRODUCTION

Nanotechnology is a research for the design, synthesis and manipulation of structure of particles with the dimension smaller than 100nm. Now-a-days nanotechnology has dynamically developed as an important field of modern research with potential effects in electronic and medicine [1&2]. A new and emerging branch of nanotechnology is nanobiotechnology. Nanobiotechnology combines biological principles with physical and chemical procedures to generate nano-sized particles with specific biological functions. Nanobiotechnology represents an economic and eco-friendly alternative for chemical and physical methods of nanopaticles synthesis. In the last two decades, a number of nanoparticle based therapeutic and diagnostic agents have been developed for the treatment of life threatening diseases such as cancer, diabetes, asthma, allergy, infectious diseases, and so on. Current scenario, the

clinical application of nanoparticles is attaining popularity with an increasing number of nanoparticle based therapeutics in clinical development. The most important anticipated application in medicine includes drug delivery, both in vitro and in vivo diagnostics. One of the greatest values of nanotechnology will be in the development of new and cost effective nanomedicine to treat the human diseases [3].

Nanoparticles can be made from vast range of materials such as gold, silver, metal oxides, inorganic material, polymetic materials and lipids [4]. Both unicellular and multicellular organisms are known to produce inorganic materials either intra or extra cellular [5].Various microbes are known to reduce metal ions to the metals. Although it is known that bacteria, yeast, cyanobacteria and actinomycetes can reduce toxic metals through reduction of metal ions. Among these fungi are extremely good candidate group of organisms in the synthesis of metal nanoparticles. Nanobiotechnologist understood that fungi play an important role in remediation of toxic metals through reduction of the metal ions this was considered interesting as nanofactories very recently [6]. Using these exceptional properties of fungi, the biosynthesis of inorganic nanomaterials by eukaryotic organisms such as fungi may be used to grow nanoparticles of gold and silver [7].

The extracellular synthesis of silver and gold–silver nanoparticles by fungus *Fusarium* sp biomass had a contribution on the formation of nanoparticles [8]. The reduction of silver ions by *Fusarium* sp has been attributed to a nitrate dependent reductase by extracellular process. In this context the present research aims to synthesis the silver nanoparticles from *Fusarium oxysporum* (MTTC-2480) strain in order to understand the process of silver metals through reduction of silver ions. The main objective of present work is green synthesis of Ag-NPs using selected fungal strain and to evaluate the antibacterial efficacy against gram negative human pathogens. The antibacterial activity of the Ag-NPs was assessed by determining the well method, disc diffusion method, minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). Silver nanoparticles were characterized by visual analysis and thin layer chromatography (TLC) to determine the dynamic stability of nanoparticles.

EXPERIMENTAL SECTION

Fungal strain

Fusarium oxysporium (MTCC-2480) fungus was procured from Institute for Microbial Technology (IMTECH) culture collection Chandigarh, India for the extracellular biosynthesis of silver nanoparticles and characterization studies.

Culture maintenance

Fungal stain was rescued by inoculating in Saubaurd Dextrose Broth (SDB) at 24±2^{o}C for 3 days. Then it was sub-cultured in Saubaurd Dextrose Agar (SDA) slants and stored at 4^{o}C for further studies.

Human pathogens

Most common gram negative bacterial pathogens such as *Eschertia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Vibrio cholerae* were collected from Christian Medical College (CMC) Vellore, India for the present antibacterial susceptibility study.

Fungal biomass preparation

Fungal biomass was prepared by growing the fungus in Potato Dextrose Broth (PDB) aerobically. A loop full of inoculum was inoculated into the flask containing 100 ml of sterile PDB and incubated on Orbital shaker (NEOLAB) with 120 rpm agitation at 24±2^{o}C for 5 days. The biomass was harvested by filtering the culture through Whatman No.1 filter paper. The biomass was washed thrice with sterile distilled water to remove the traces of chemical components.

Biosynthesis of Silver nanoparticles (Ag-NPs)

Ten gram (wet weight) of biomass was aseptically weighed and transferred into 250 ml Erlenmeyer flask containing 100 ml of Milli-Q water and kept on Orbital shaker **(**NEOLAB**)** with 120 rpm at 24± 2^{o}C for 24 hours. The culture was centrifuged at 12000 rpm for 5 minutes. The clear supernatant was used for the extracellular biosynthesis of silver nanoparticles. The supernatant was transferred to the reaction vessel containing silver nitrate ($AgNO_3$) at the concentration of 3mM. The biochemical reaction between the culture supernatant and the silver ions was performed under dark conditions for 30 minutes. The biochemical reduction of the silver ions in the reaction mixture was

monitored by changing the colour of the solution. The synthesized silver nanoparticle (Ag-NPs) was further subjected to perform antibacterial studies.

Antibacterial determination by well diffusion method

Silver nanoparticles were assayed for antibacterial activity by well diffusion method against selected human pathogens. The overnight broth cultures of each test pathogens were prepared in Muller Hinton Broth (MHB) medium at 37^{o}C. Each pathogen (10^8 cells) were mixed with sterile Muller Hinton Agar (MHA) and poured on to the sterile Petri disc separately. The plates were left for few minutes for solidification. After solidification in each plate 5 wells of 6 mm diameter size with equal center to center distance were made using gel puncture aseptically. Using variable micropipette 10µl, 15µl, 20µl & 25µlof silver nanoparticle solutions were poured onto each well in triplicates for each test pathogens. Control samples of culture supernatant (25µl) were poured onto the center well on all plates as negative control to compare the antibacterial efficacy. The inoculated plates were incubated at 37^{o}C for 18 - 24 hours. Triplicates were maintained for each test pathogens to obtain mean value of antibiogram. After incubation the different levels of zone of inhibition was measured using Vernier caliber and recorded.

Antibacterial determination by disc diffusion method

Antibacterial activity of silver nanoparticle was evaluated by disc method followed by Bauer et al [9]. Twenty five microlitre of silver nanoparticle was impregnated with commercially available sterile empty disc (Hi-media Laboratory Private Limited, Mumbai, India) with the size of 6mm diameter for antibacterial assay. Sterile MHA plates was prepared and swabbed with overnight broth cultures of each test pathogens (10^8 cells). Silver nanoparticle impregnated disc was placed at the center of the plate aseptically. Triplicates were maintained for each test pathogens to obtain mean value. The disc impregnated with culture supernatant (25µl/disc) was used as a negative control to compare the antibacterial efficacy. All the inoculated plates were incubated at 37^{o}C for 18 - 24 hours. After incubation the different levels of zone of inhibition around the discs were measured and recorded in mm diameter for each test pathogen.

Determination of minimal inhibitory concentration (MIC)

Silver nanoparticles were determined for minimal inhibitory concentration (MIC) by standard micro dilution method against selected human pathogens. The test pathogens (10^5cells/ml) were inoculated in MHB. Different concentration of silver nanoparticles *viz.*, 5µl, 8µl, 11µl, 14µl, 17µl & 20µl were tested forMIC leading to the inhibition of bacterial growth [10]. The MIC was examined in spectrophotometer at 600nm after 24 hours of incubation at 37^{o}C as the tested nanoparticle inhibit the growth of test pathogens. Negative control tubes are also maintained for each pathogen without adding Ag-NPs. The MIC is the lowest concentration of silver nanoperticle that visually inhibits 99% growth of inoculated bacteria.

Determination of minimal bactericidal concentration (MBC)

The viability of test bacteria was determined in the culture tube containing no visible turbidity in MICs to determine the MBCs of silver nanoparticles. One milliliter of MIC dilutions was drawn from each test tube for serial dilution. Hundred microliters of aliquots were spread evenly on sterile nutrient agar (NA) plates separately. The inoculated plates were incubated at 37^{o}C for 24 hour to determine the MBC. The number of bacterial colonies were counted using colony counter and determined as maximum growth, minimum growth and no growth were recorded [11]. The MBC end point is defined as the lowest concentration of silver nanoparticle that kills 100 per cent of the inoculated bacterial population.

Thin Layer Chromatography (TLC) of silver nanoparticles

Silver nanoparticles were characterized by thin layer chromatography (TLC) using pre-coated silica gel plates of 0.25 mm thickness (Merck, India) to identify the silver ions which have bioactivity. To develop a chromatogram, chloroform and acetic acid (1:1 v/v) was used as a mobile phase. The activated TLC plate was observed to elucidate the presence of Ag-NPs. The eluted spot in the TLC plate was visualized in the iodine chamber and the retention factors (R_f) value of silver nanoparticles was calculated by the following formula.

$$R_f = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

Chemicals and culture media
All chemicals and culture media components were procured from Hi-media Laboratories Private Limited, (Mumbai, India) for the execution of the present investigation.

Statistical analysis
The antibacterial efficacy results were calculated as mean diameter of zone of inhibition in mm ± standard deviation (mean ± SD).

RESULTS AND DISCUSSION

Since roman period silver has been used for its antimicrobial properties however the advances in the biosynthesis of Ag-NPs have made possible restoration of the use of silver as a most powerful bactericidal agent [12]. Extracellular biosynthesis of silver nanoparticles is an important area of nanoscience research. Recently many researchers have been conducted research on extracellular synthesis of Ag-NPs using microorganisms to explore the nanomedicine as a potential biosources [13 &14]. Among the different microbial sources fungi are known to secrete much higher amounts of proteins and have significantly higher productivity of nanoparticles [15]. In the present study extracellular biosynthesis of silver nanoparticles (Ag-NPs) was carried out by the culture supernatant of *Fusarium oxysporum* (MTCC-2480). It was observed that the culture supernatant has a pale colour before reaction. When the supernatant contact with silver ions the reaction starts with in few minutes and the colour of the reaction mixture became brown after 30 minutes on completion of the reaction. The colour change of reaction mixture was caused by the surface Plasmon resonance of Ag nanocrystals in the visible spectral region [16].This observation indicates the release of extracellular enzymes into culture supernatant by *Fusarium oxysporum* (MTCC-2480) and suggested that the possible mechanism for the reduction of metal ions present in the reaction vessel. The actual mechanistic aspects of nanoparticles reduction are still debate an open question, however this process occur in the fungal case probably either by reductase action or by electron shuttle quinones or both. It is considered that metal ions are reduced by extracellular reductase enzymes and proteins produced by the strain leading to the nanoparticle formation [17].

Table 1: Antibacterial activity of Ag-NPs synthesized from *F.oxysporium* (MTCC-2480) against selected human pathogens by well method

Human pathogens	Zone of inhibition in [mm] in diameter				
	control	Concentration of Ag-NPs			
		10µl	15µl	20µl	25µl
Eschertia coli	-	8.8 ± 0.83	12.0± 0.70	16.4 ± 1.14	17.2 ± 1.10
Salmonella typhi	-	10.2± 0.83	14.2± 1.22	18.6± 1.34	20.6± 1.24
Klebsiella pneumoniae	-	8.2 ± 0.83	10.4 ± 1.14	14.2±1.22	16.4±1.24
Pseudomonas aeruginosa	-	8.8 ± 0.83	12.0± 0.70	16.4 ± 1.14	18.2 ± 1.44
Vibrio cholerae	-	8.2 ± 0.83	10.4 ± 1.14	12.0± 0.70	14.0± 0.10

Reference; - No activity; each value is the mean ± SD of three individual estimates

Table 2: Antibacterial activity of Ag-NPs synthesized from *F.oxysporium* (MTCC-2480) against selected human pathogens by disc method

Human pathogens	Zone of inhibition in [mm] in diameter	
	control	Concentration of Ag-NPs 25µl/disc
Eschertia coli	-	16.4 ± 1.14
Salmonella typhi	-	18.6± 1.34
Klebsiella pneumoniae	-	14.2±1.22
Pseudomonas aeruginosa	-	16.4 ± 1.14
Vibrio cholerae	-	12.0± 0.70

Reference; - No activity; each value is the mean ± SD of three individual estimates

Silver nanoparticles were further characterized and confirmed by performing thin layer chromatography (TLC) and retention factors (R_f) value was calculated. The TLC was done on silica gel plate with 0.25mm thickness (Merck, India) using the mixture of chloroform and acetic acid (1:1v/v) as a mobile phase. The running sample was visualized in the iodine elution chamber for the conformation and stability of Ag-NPs in the reaction mixture. A single separate band of silver nanoparticle was observed by TLC with R_f value of 0.75. This was corroborated by TLC analysis on silica gel plate using chloroform-methanol-acetic acid (95:5:1) showed a spot with R_f value of 0.65 [18].

Table 3: Minimal inhibitory concentration (MIC) of Ag-NPs synthesized from *F.oxysporium* (MTCC-2480) against selected human pathogens

Human pathogens	(Blank)	Concentration of Ag-NPs in µl/ml					
		5µl	8µl	11µl	14µl	17µl	20µl
Escherchia coli	0.00	1.05	0.94	0.86	0.78	0.70	0.62
Salmonella typhi	0.00	0.91	0.76	0.61	0.41	0.33	0.18
Klebsiella pneumoniae	0.00	0.98	0.79	0.60	0.41	0.23	0.03
Pseudomonas aeruginosa	0.00	1.00	0.83	0.66	0.49	0.32	0.15
Vibrio cholerae	0.00	1.06	0.81	0.72	0.59	0.45	0.33

Values are optical density (OD) at 600nm

Table 4: Minimal bactericidal concentration (MBC) of Ag-NPs synthesized from *F.oxysporium* (MTCC-2480) against selected human pathogens

Human pathogens	Serial dilution of MIC tube				
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Escherchia coli	+++	+++	++	+	-
Salmonella typhi	+++	++	+	-	-
Klebsiella pneumoniae	++	+	-	-	-
Pseudomonas aeruginosa	+	-	-	-	-
Vibrio cholerae	++	+	-	-	-

+++ denotes maximum growth; ++ denotes moderate growth; + denotes minimum growth; - denotes no growth

Silver nanoparticles have been evaluated for their antimicrobial activities against a wide range of pathogenic microorganisms [19]. The synthesized silver nanoparticles has been evaluated for antibacterial activities by well and disc method against selected human pathogens. The results of antibacterial efficacy are depicted in table 1&2 respectively. Biosynthesized silver nanopatrticles had antibacterial activity against selected gram negative bacterial pathogens. Silver nanoparticle (25µl/disc) impregnated disc exhibited highest antibacterial activity against *Salmonella typhi* (18.6± 1.34mm) followed by *Pseudomonas aeroginosa* (16.4 ± 1.14mm) by disc diffusion method. In addition, maximum antibacterial efficacy was noticed against *Salmonella typhi* (20.6± 1.24mm) followed by *Pseudomonas aeroginosa* (18.2 ± 1.44mm) by well method. However minimum activity was observed against *Vibrio cholerae* (14.0± 0.10mm). In the present study silver nanoparticle exhibit recommended level of antibacterial activity against all the tested gram negative pathogens. Feng et al [20] reported that inhibitory activity of silver nanoparticle is higher in case of gram negative bacteria than gram positive bacteria. This might be due to the thin peptidoglycan (PG) layer in gram negative bacteria cell wall which may induce to some extent by the action of the silver ions. It also revealed that silver nanoparticles synthesized from novel *Streptomyces* sp had highest antibacterial activity against *Salmonella typhi* followed by *Pseudomonas aeroginosa*, *Proteus vulgaris*, *E. coli* and the least was noticed against *Klebsiella pneumoniae* [21].

Silver nanoparticles were subjected at different concentration to study the minimal inhibitory concentration (MIC) against the selected pathogens were portrayed in table 3. MIC revealed that 20µl/ml of Ag-NPs exhibited highest inhibition activity against *Klebsiella pneumonia*e followed by *Pseudomonas aeruginosa* and *Salmonella typhi*. The MIC culture tube containing no visible turbidity was selected to determine for further MBCs to find out the break point of silver nanoparticles. The finding of MBCs is displayed in table 4. It revealed that 100% inhibition was observed at the concentration of 20µl/ml of Ag-NPs for the bacteria *Pseudomonas aeruginosa*. These results clearly indicate that biosynthetic silver nanoparticles could provide an eco-friendly and safer alternative for the conventional chemotherapeutic agents to treat infectious disease caused by gram negative bacteria. Zarei et al [22] observed that antibacterial effect of silver nanoparticle against gram negative pathogens showed MIC and MBC value of 3.12µg/ml and 6.25µg /ml respectively. Several studies have been investigated for the interaction of nanosilver with bacteria. The actual bactericidal mechanism of silver nanoparticles is not clear and still confuse. It revealed that the majority of the nanosilvers were localized on the membrane of treated microbes [23]. It is assumed that silver ion has high affinity towards sulfur and phosphorus molecules containing amino acids inside or outside of bacterial cell membrane protein are the key element of the antimicrobial effect. This in turn affects the osmotic stability leads to bactericidal activity. It was also suggested that silver ions which is released from silver nanoparticles can interact with phosphorus moieties in nucleic acid. As a result inactivation of DNA replication or can react with sulfur containing proteins leading to the inhibition of enzymes required for bacterial metabolisms [24]. This may causes bacteriostatic or bactericidal effects of treated organisms [25].

CONCLUSION

In conclusion, we have been described biosynthesis silver nanoparticle by *Fusarium oxysporium* (MTCC 2480) using cost effective method. The colour change of culture supernatant indicates the reduction of silver metals into silver ions. The bactericidal activity results showed that nanoparticle is active against selected gram negative human pathogens. The results proved that silver nanoparticles showed exceptional antibacterial activity which revealed silver nanoparticle as novel antimicrobial material that can be used in topical nanomedicine and reconstructive surgery applications.

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