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EVALUATION OF ANTIMICROBIAL ACTIVITY OF SILVER NANOPARTICLES FROM SILVER RESISTANT ISOLATE *ENTEROBACTER CLOACAE* (MK163462) AGAINST CERTAIN CLINICAL PATHOGENS

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ABSTRACT

A silver resistant bacterial isolate JWS-1 ioslated from jewellery waste contaminated soil was identified as Enterobacter cloacae (MK163462) based on 16S rRNA sequencing studies. The culture filtrate from Enterobacter cloacae was used for silver nanoparticle synthesis, the resultant silver nanoparticles were characterized by UV-visible spectrophotometer, FT-IR, SEM and XRD analysis. The synthesized silver nanoparticles were spherical and in the size range of 12-30nm. The silver nanoparticles demonstrated antimicrobial activity against certain gram positive and gram-negative pathogenic bacteria and fungi. The bacterium Enterobacter cloacae (MK163462) JWS-1 could use for the efficient synthesis of antimicrobial silver nanoparticles in an economical way.

KEY WORDS

Silver resistant bacteria, Silver nanoparticles, Enterobacter cloacae, FT-IR, SEM and XRD analysis.

INTRODUCTION

Nanotechnology is an interdisciplinary science that deals with several research areas such as medicine, biology, physics and chemistry (Narayanan and Sakthivel, 2010). Silver has been recognized as having inhibitory activity on microbes present in medical and industrial process.

Silver-ions have been reported to possess strong biocidal effects (Singh *et al.,* 2008). The silver-compounds are used as disinfection agents from the ancient time. The silver nanoparticles (AgNP) are effective against multidrug resistant bacteria, as well as against fungi and viruses. The antibacterial mechanisms silver nanoparticles are partially understood. Silver ions can bind with the DNA bases, and hinder the DNA replication (Feng *et al.,* 2000).

Silver nanoparticles are having wide range of applications in biomedical area such as antibacterial activity against bacterial pathogens and food borne pathogens (Rajeshkumar and Malarkodi, 2014), antifungal activity against *Aspergillus niger, Aspergillus fumigatus*, *Candida albicans, Aspergillus flavus* and *Fusarium sp* (Rajeshkumar *et al.,* 2014). In recent times, there has been growing interest in the preparation and study of silver nanoparticles (AgNPs) due to their extensive applications in different fields including wound and burn healing, implants (bone and dental), and for their antimicrobial activities (Khalil *et al.,* 2014; Rai *et al.,* 2014). There are many advantages of AgNPs to be used as effective antimicrobial agents. They are highly effective against a broad range of microbes even at a with low systemic toxicity toward humans (Ouay and Stellacci, 2015).

In the present investigation, the culture filtrate of *Enterobacter cloacae* was utilized for the production of silver nanoparticles (AgNPs). The nanoparticles were characterized by using a UV-Visible spectrophotometer, FTIR, SEM and XRD analysis to confirm structure, morphology, crystalline nature of silver nanoparticles.

The synthesized AgNPs were further evaluated for their antimicrobial activities.

MATERIALS AND METHODS

Isolation of silver resistant bacteria

Soil samples were collected from jewellery processing waste contaminated areas in Chidambaram, Cuddalore (district), Tamil Nadu, India. They were serially diluted up to10^{-5} using sterile distilled water. Then, the dilution were poured into the Petri plates and added with sterilized modified Nutrient Agar added with 1 Mm $AgNO_3$. The plates were then incubated at 37º C for 24 hrs.

Biosynthesis of silver nanoparticles using bacterial isolates

For study, the bacterial culture was inoculated in to 100ml of nutrient broth. The flask was incubated in a shaker at 200 rpm for 48 hrs at room temperature. The bacterial culture was centrifuged at 12000 rpm for 10 minutes. The supernatant was used mixed it with sterilized $AgNO_3$ solution at 1 mM final concentration. Then they were incubated on shaker (200 rpm) at room temperature for a period of 72 hrs in bright condition (Das *et al.,* 2014).

Identification of the silver nanoparticles producing bacterial isolate

The morphological, physiological and biochemical characterization of silver nanoparticles producing bacterium was studied according to the methods mentioned in Bergey's manual of determinative bacteriology (Holt *et al.,*1994). Further species level identification was done by 16S rRNA sequencing method. The bacterial culture was submitted to Yaazh Xenomics, Coimbatore for sequencing studies.

Characterization of silver nanoparticles

The formation of silver nanoparticles were characterized by visible color change, UV-visible spectroscopy and Fourier transform infrared spectroscopy (FTIR), Scanning Electron Microscopy (SEM) and X-ray diffraction methods.

Antimicrobial activity of silver nanoparticles

The antimicrobial activities of AgNPs synthesized by the isolate JWS-1 were demonstrated by disc diffusion method. Briefly, various pathogenic gram-positive bacteria such as *Staphylococcus aureus*, *Streptococcus pyogenes, Bacillus cereus* and *Streptococcus mutans*, gram negative bacteria like *Klebsiella pneumoniae, Pseudomonas aeruginosa, E. coli, Salmonella typhi* and fungi such as *Candida albicans, Cryptococcus neoformans, A. niger, Candida tropicalis* and *Candida parasilopsis* were swabbed on the solidified muller hinton agar plates (for bacteria) or PDA (for fungi) plates. The sterile disc was dipped in AgNPs solution at different concentrations (5µg, 10µg, 15µg and 20µg/ml) and placed on the agar plate and incubated at 37°C for 24h along with standard antibiotics Tetracycline(bacteria) and Fluconazole (fungi)). The zone of inhibition formed after incubation was measured in mm (Sudha *et al.,* 2013).

Minimum Inhibitory Concentration (MIC)

The broth dilution methods were followed to determine the minimum concentration of silver nanoparticles required for the inhibition of bacterial strain growth. Nutrient broth was supplemented with different concentration of nanoparticles and inoculated with bacterial suspension to (10^{-6} CFU/ml). The MIC was determined after 24 hours of incubation at 37°C by measuring the optical density at 600 nm (Kora and Arunachalam, 2011).

Minimum Bactericidal Concentration (MBC)

About 50 µl of sample MIC from all tubes in which no visible growth was transferred in to MHA plates and was incubated for 24 hours at 37 ̊C. The lowest concentration of antimicrobial agent that kills 100% of the bacterial population is called MBC (Ansari *et al.,* 2011).

Minimum Fungicidal concentration (MFC)

The MFC of the AgNPs was determined by plating loopful of culture from MIC tubes onto SDA plates and incubated under aseptic condition for 72 hrs at 28°C.

The lowest concentration of the silver nanoparticles that showed no visible growth on solid media was recorded as the MFC.

RESULTS AND DISCUSSION

The silver resistant bacterial isolate obtained from jewellery waste contaminated soil were used in the present study. The silver nanoparticle producing isolate was identified as *Enterobacter cloacae* based on the results of 16Sr RNA studies. BLAST search of the nucleotide sequence with the nucleotide sequences available in the data bases revealed that the isolate was similarity to as *Enterobacter cloacae*.

Figure-1: The phylogram showing the position of *Enterobacter cloacae* with other *Enterobacter* species based on 16S rRNA gene sequence and phylogenetic tree analysis.

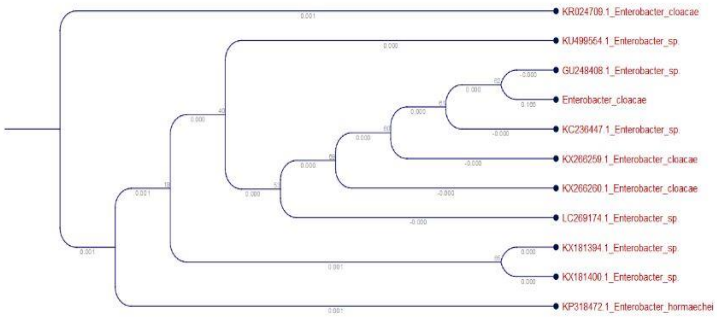

Fig-2. Raw nucleotide sequences of *Enterobacter cloacae*

ACACTGCTAGTCGAGCGGATCACGATGAAGCAGCTTTGCT
TCGCTTCGCTGACGAGTGGCGGAGGGGAGAGTCATGTGT
GGGACCCTGCCTGATGACTGGGGATAACTACGGAAAACG
GAACTTAAGACCGAAAAACGTCGCAAGACCAAGGAGGGG
GACCTTCGGACCTCTTGGCTTCGGATGTGCCCTGATCCCAT
TACCTAGTAGGTGGGGTAACGGGTCATGTATGCCACGAG
CCCTAATTGGTCTGAGAGGATGACCGGCCACACTGGACCT
GGGACCTGGTCCACACTCCTAACTCAGGCAGGAGTGGGG
AATATTGCATCTGGGCGCTGGCCTGATGCCTGATGCCGCG
CGTATGAAGAAGGCCTTCGGGTTGTAAAATACTTTCACTC
GGGGATGAAGGAAAAAAGGTTGATAACCTTGCTGATTGA
CGTTACCCGCAGACTAAGCACCGGCTAACTCCGTGCCACC
AGCCGCGGTAATACGGAGGGCGCAAGCGTTAATCGGAAT
TACTGAATTATTGAGCGCACGCCGCGCGTCTGTCAAGTTC
GGATGTGAAGATCCCCGGGCTCAACCTGGGACCTGCATTC
GAAACTGGAAACTAGAGTCTTTGTAGAGGGGGGTAGAAT
TCCAGGTGTCCATGTGTAAATGCGTAAAGATCTGGAGGA
ATACCGGAACACCAAGTGCGGCCCCCTGACACTTCTGACT
GACGCTCTGAGTGCTGAAAGCGTGAGAGAGCAAACGAGA
TTAGATGACCCTGATACCCCCACGCCGCCACACCATGTCA
CACATGAGAGGTTGTGCGCTTTGAGGCGTGGCTTCCCGTT
AGCTGCTGCGTTAAGTCGATCGCCTGGTCCAGCACGGGG
CGCACGATCAAAACTCTGATGAATCTGACGGGAGGTCAC
GCGACGACCGGCACGAGCATGTGGATTTTATGTGCGATG
CATCGCAAACAACGCTTTACCATACTCTACGACATC

Das *et al*. (2014) isolated silver resistant Bacillus strain C11 from industrialized area of Kerala and used it for the extracellular synthesis of silver nanoparticles. *Enterobacter cloacae* is ubiquitous in nature, they present in terrestrial and aquatic environments.

The biosynthesis of silver nanoparticles by the culture filtrate of *Enterobacter cloacae* initially confirmed through the appearance of a pale yellow to brown color filtrate in the reaction vessels after incubation at room temperature. In negative control (silver nitrate alone and culture filtrate without $AgNO_{3)}$, no color change was observed (Figure 2A & 2B). In flask containing bacterial supernatant with silver nitrate solution a color change from pale yellow to brown color was observed (Figure-2C). The silver nanoparticles were concentrated and separated after centrifugation.

Figure-2. Cell filtrate (72 h) of *Enterobacter cloacae* silver ion (1 mM): (A) silver nitrate solution, (B) culture filtrate without $AgNO_3$ (C) culture filtrate + $AgNO_3$ (after 6 hrs).

El-Baghdady *et al*. (2018) demonstrated the synthesis of silver nanoparticles using the silver resistant bacterial isolate *Enterobacter cloacae* Ism 26 recovered from soil contaminated with industrial waste. The silver nanoparticle synthesizing isolate was identified based on 16S rRNA gene sequencing method. *Enterobacter cloacae* Ism 26 synthesized silver nanoparticles were spherical with an average size of 15nm. Herein, the characterization studies like UV-Visible spectrophotometer, FTIR, SEM and XRD analysis revealed that the isolate JWS-1 *Enterobacter cloacae* (MK163462) synthesized spherical, 12-30 nm sized silver nanoparticles.

In the present investigation, the results clearly indicated that culture filtrate could be sufficient to produce silver nanoparticles. Numerous investigations demonstrated siver nanoparticles synthesis using cell free supernatants of bacteria (Natarajan *et al.,* 2011,Shivaji *et al.,*2011, Das *et al.,* 2013) (Karthick and Radha 2012; Kushwaha *et al.,* 2015).On contrary, the studies of El-Baghdady *et al.* (2018) demonstrated that silver nanoparticles were produced only by sonicated cell lysate supernatant obtained from *Enterobacter cloacae* Ims 26 both culture filtrate and bacterial pellets did not produce silver nanoparticles. The components of bacterial culture involved in the synthesis of silver nanoparticles vary according to bacterial species, which was confirmed in our study (El-baghdady *et al.,* 2018).

Karthick and Radha (2012) utilized *Enterobacter aerogenes* (MTCC 111) for the biosynthesis of silver nanoparticles. The silver nanoparticles synthesis using *Enterobacter aerogenes* was found to be faster and reliable. The very first report on extracellular biosynthesis of silver nanoparticles by *Klebsiella pneumoniae*, *E.coli* and *Enterobacter cloacae* has been reported by Minaeian *et al.,* (2008).

The antimicrobial activity of AgNPs against different human pathogenic microorganisms (Table-1), the highest antibacterial activity was observed against *Staphylococcus aureus* (15.5±0.6mm) and *E.coli* (15.3±0.5 mm) which was indicated by the wider zone of inhibition. The lowest activity was observed against *Pseudomonas aeruginosa* (11.7±0.4mm) at high concentration of AgNPs used in the study. The AgNPs from *Enterobacter cloacae* possess antifungal activity which was evident from the results of the present study. The synthesized AgNPs had greater inhibition against the growth of yeast then filamentous fungi. The maximum activity was seen with *Candida parasilopsis* (16.3 ±0.5mm). The lowest antifungal activity was observed against filamentous fungi *Aspergillus niger* (11.9 ±0.5mm) at 20µg/ml concentration.

Table-1: Antimicrobial activity of silver nanoparticles synthesized by the isolate JWS-1 (*Enterobacter cloacae*) against clinical pathogens.

Bacterial Pathogens	Zone of inhibition (mm) Concentrations used (µg/ml)				Standard Tetracycline (5 µg/disc)
	5	10	15	20	
Gram Positive bacteria					
Staphylococcus aureus	7.3± 0.4	9.0± 0.5	10.6± 0.4	15.5± 0.6	20.3± 0.5
Streptococcus pyogenes	8.3± 0.5	10.6± 0.5	13.7± 0.3	12.4± 0.5	20.4± 0.5
Bacillus cereus	7.2± 0.5	8.5± 0.5	10.6± 0.4	12.5± 0.6	19.3± 0.5
Streptococcus mutans	8.3± 0.4	9.3± 0.5	11.7± 0.5	13.5± 0.4	20.3± 0.1
Gram Negative bacteria					
Klebsiella pneumoniae	7.3± 0.5	8.7± 0.5	11.1± 0.4	14.1± 0.4	24.5± 0.5
Pseudomonas aeruginosa	7.5± 0.3	9.0± 0.5	10.6± 0.4	11.7± 0.4	18.4± 0.5
Salmonella typhi	8.3± 0.4	9.8± 0.2	11.7± 0.4	13.9± 0.8	23.3± 0.5
E.coli	8.5± 0.5	10.6± 0.4	11.5± 0.5	15.3± 0.5	25.3± 0.5
Fungal Pathogens					**Standard Fluconazole (5 µg/disc)**
Candida albicans	7.3± 0.5	9.7± 0.4	11.4± 0.5	13.8± 0.5	21.4± 0.3
Cryptococcus neoformans	8.2± 0.3	9.7± 0.4	11.9± 0.5	14.6± 0.7	20.3± 0.5
Aspergillus niger	7.1± 0.4	8.8± 0.5	10.6± 0.3	11.9± 0.5	20.1± 0.4
Candida tropicalis	9.3± 0.5	11.1±0.4	13.3± 0.5	15.7± 0.5	22.5± 0.4
Candida parasilopsis	9.6± 0.2	10.9±0.4	12.4± 0.4	16.3± 0.5	23.5± 0.4

The results for MIC, MBC and MFC of synthesized AgNPs are presented in the Table 2. The MIC values ranged between 3.1 to 12.5µg/ml for bacteria, 3.1 to 25µg/ml for fungal pathogens. The lowest MIC value 3.1 µg/ml

was recorded against *Streptococcus pyogenes* and *Streptococcus mutans*. This was followed by *Staphylococcus aureus* and *E.coli* (6.3µg/ml). The highest MIC value (25µg/ml) was recorded against *Pseudomonas aeruginosa.*

The MBC and MFCs values were two-fold greater than MIC. The lowest MBC recorded against *Streptococcus mutans* and *Streptococcus pyogenes* (6.3µg/ml). The lowest MFC (6.3µg/ml) recorded against *Aspergillus niger* whereas the highest MFC valued recorded against *Cryptococcus neoformans* (50µg/ml) (Table-8).

Table-2. Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) of AgNPs synthesized by JWS-1 (*Enterobacter cloacae*)

Bacterial Pathogens	**MIC (µg/ml)**	**MBC (µg/ml)**
Staphylococcus aureus	6.3	12.5
Streptococcus pyogenes	3.1	6.3
Bacillus cereus	12.5	25
Streptococcus mutans	3.1	6.3
Klebsiella pneumoniae	12.5	25
Pseudomonas aeruginosa	25	50
Salmonella typhi	12.5	25
E.coli	6.3	12.5
Fungal Pathogens	**MIC (µg/ml)**	**MFC (µg/ml)**
Candida albicans	12.5	25
Cryptopcoccus neoformans	25	50
Aspergillus niger	3.1	6.3
Candida tropicalis	6.3	12.5
Candida parasilopsis	12.5	25

Prakash *et al.* (2011) have also reported a similar result for antibacterial effects of the AgNPs on Gram negative (*E. coli*) and Gram positive (*Streptococous pyogenes*) bacteria. Whereas, Dipak and Sankar *et al.* (2014) and Priyadarshini *et al*. (2013) have reported the highest and the lowest zone of inhibition formation against *E. coli* and *Staphylococcus* respectively.

Our results are similarity with the studies of Prakash *et al*.,2011. They showed antibacterial effects of silver nanoparticles on gram positive (*E.coli*) and gram positive (*Streptococcus pyogenes*) bacteria.

Said *et al.* (2016) reported extracellular synthesis of silver nanoparticles using Enterobacteriace. The shape of silver nanoparticles produced by *Enterobacter cloacae* were spherical with an average size of 92nm. The antibacterial activity of biosynthesized silver nanoparticles from *Enterobacter cloacae* was tested against *Acinetobacter baumanni*, *E.coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*. The highest antibacterial activity was observed against *Staphylococcus aureus* (23mm). Chudasama *et al.* and Ramgopal *et al.* (2011) have reported that *S. aureus* (Gram positive) greatly inhibited compared to that of *E. coli* (Gram negative). In the present study, AgNPs from *Enterobacter cloacae* exhibited higher bactericidal activity among both the Gram positive and Gram-negative bacterial species indicating its broad-spectrum antibacterial action.

The antimicrobial activity of *Enterobacter cloacae* synthesized silver nanoparticles was tested against some gram negative and gram-positive pathogenic bacteria (El-Baghdady *et al.,*2018). The synthesized silver naoparticles displayed antibacterial activity against both gram positive and gram-negative bacteria to different extents. Silver nanoparticles concentration of 75 µg/ml inhibited growth of all tested bacteria. In our study, silver nanoparticles concentration of 20 µg/ml displayed wider zone of inhibition the other concentration used. According to Pal *et al.* (2007) spherical nanoparticles were found to have greater inhibiton activity than other nanoparticles, which was confirmed in our study.

In conclusion, silver nanoparticles are synthesized extracellular by using the culture supernatant of *Enterobacter cloacae*. The biosynthesized silver nanoparticles displayed greater microbiocidal activity

even at low concentrations. Extracellular synthesis of silver nanoparticles could be advantageous in terms of simple downstream process and it is more economical and ecofriendly method. Therefore, biosynthesized silver nanoparticles can be used as strong microbicidal agent to combat various infections caused by clinically important pathogens.

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