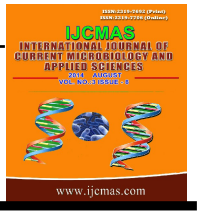
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 8 (2014) pp. 384-396**
http://www.ijcmas.com



Original Research Article

Silver nanoparticles as antimicrobial agent from *Kluyveromyces marxianus and Candida utilis*

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Keywords

Extracellular biosynthesis, Silver nanoparticles, *Kluyveromyces marxianus*, *Candida utilis 22*, Antimicrobial effect, Human pathogens

A B S T R A C T

A green approach for synthesizing the nanoparticles using the yeast have been suggested as promising ecofriendly alternative to chemical methods. The present study involves the extracellular biosynthesis of silver nanoparticles (Ag-NPs) using *Kluyveromyces marxianus, Candida utilis 22* and evaluating the antibacterial and antifungal efficacy against *Staphylococcuc aureus* ATCC 6538, *Escherichia coli* ATCC 10536, *Pseudomonas flourescence* ATCC 50090, *Candida albicans*, *Candida glabrata*, *Candida krusei* as multi-drug resistant human pathogens. Morphological observation and characterization of biosynthesized silver nanoparticles were performed by UV-visible spectroscopy, Transmission electron microscopy and Fourier transform infrared spectroscopy. The biosynthesized silver nanoparticles from each yeast strains showed a maximum absorption in the visible region at 430-450 nm and at 400-430 nm respectively and the size was ranged from 3-12 nm and 6-20 nm respectively. The interaction between protein and Ag-NPs was analyzed and the stabilization of Ag-NPs by protein is a clear possibility. Further more, Ag-NPs have the highest antibacterial and antifungal efficacy against all the tested microorganisms. Conclusion, silver nanoparticles from each strains have great potential to be an effective to antibacterial and/or antifungal agents for future therapies in multi-drug resistant human pathogens of bacteria and *Candida* infections.

Introduction

An important area of research on silver and metal nanoparticles has focused on their multifunctional role in diverse fields of science and technology. Silver nanoparticles are considered attractive building blocks for nonomaterial architectures and have been high specific surface area and high fraction of surface atoms. These nanoparticles serve as a platform for drug, gene delivery (Dobson, 2006) and cancer treatments (Cai *et al.*, 2008) for biomedicine applications. The characteristics and functionality of nanoparticles are closely related on their size, shape and controlled on monodispersity. Typical top-down synthesis methodologies involve complex chemical processes that use high temperature, high pressure and finally the release of toxic

pollutant to environment. Consequently, bottom-up biological nanoparticles synthesis has been explored as an alternative in developing environmentally friendly nanoparticles synthesis processes (Ahmad *et al.*, 2003).

Recently, many studies were conducted to explore the synthesis of Ag-NPs using microorganisms as a potential bio-sources, when the fungi *Saccharomyces cerevisiae* was exposed to silver ions, Ag-NPs were formed. The extracellular biosynthesis of Ag-NPs would make the process easier for downstream processing (Saravanan *et al.*, 2013).

Among these nanoparticles, Ag-NPs have been shown to have strong inhibitory and bactericidal effects as well as a broad spectrum of antimicrobial activities. With the emergence and increase of infectious diseases caused by various pathogenic microbial organisms which resist multiple antibiotics (Conlon *et al.*, 2004), the interest of synthesizing Ag-NPs is acknowledged (Ilić *et al.*, 2009). For instance, Ag-NPs have been studied to control bacterial growth in a variety of applications including catheters (Samuel and Guggenbichler, 2004) and wound healing (Ülkür *et al.*, 2005). It was also reported that Ag-NPs have shown to be powerful biocides against bacteria (Kim *et al.*, 2007), and fungi (Zhang *et al.*, 2008).

Although antimicrobial effects of silver are renowned, bactericidal and fungicidal mechanism are still undetermined. It has been proposed that bactericidal mechanism happens due to the release of silver ions which generates reactive oxygen species (ROS) (Martinez-Gutierrez *et al.*, 2010) and causes the deposition of silver sulphur granules on the microbial cell wall, whereas others reported that the interaction of silver ions (Ag+) interact with vital enzymes such as NADH dehydrogenases resulting in the uncoupling of respiration from ATP synthesis hence causing their inactivation. It was also reported that exposure of Ag+ to bacterial cells would suffer morphological changes such as cytoplasm shrinkage and detachment of cell wall membrane, DNA condensation and cell membrane degradation (San and Don, 2013). Although antifungal activity (Jung *et al.*, 2008) has been reported, the mechanistic action of silver on fungi is still unverified.

The present work has been focused on the development of an extracellular biosynthesis of Ag-NPs using culture supernatant of *Kluyveromyces marxianus* and *Candida utilis 22* and the identification of antibacterial and antifungal properties of Ag-NPs against human pathogens of Gram Positive and Gram negative bacteria including *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas flourescence* and *Candida* infections including *Candida albicans*, *Candida glabrata* and *Candida krusei.*

Materials and Methods

Culture and Media

The pure culture of *Kluyveromyces marxianus* and *Candida utilis 22* used in this work were obtained from Microbiological Resources Centre (MIRCEN), Ain Shams University, Cairo, Egypt. The cultures were grown on universal medium (UM) slants at 30°C for 24 h and maintained at 4°C in a refrigerator.

Synthesis of silver nanoparticles

Kluyveromyces marxianus and *Candida utilis 22* were inoculated separately on UM broth (1% glucose, 0.5% peptone, 0.3%

yeast extract and 0.3% malt extract, pH 6). The cultured flasks were incubated at 30°C for 48 h, then the cultures were centrifuged at 10,000 rpm for 10 min and the supernatants were used for the synthesis of Ag-NPs. The supernatants with 0.6 of tri-sodium citrate were added to the reaction vessels containing silver nitrate at a concentration of 0.085 g dissolved in 5 ml deionised water. The reaction between the supernatant and silver ions was carried out at 140°C on an orbital shaker in bright condition. Control containing cell free filtrate without silver nitrate solution was run simultaneously as standard with the experimental flask. The bioreduction of silver nitrate into Ag-NPs was identified by the change of the colour of the cell free filtrate from yellow to brownish or brown over a period of time.

Characterisation of silver nanoparticles Absorbance measurement

Change in colour from yellow to brownish or brown was observed in the silver nitrate solution. The UV-visible spectra, which represent surface resonance absorption band of Ag-NPs was recorded using UV-visible spectrophotometer (Spectronic 21, Bauch and Lomb, USA). The surface Plasmon resonance spectra of Ag-NPs in samples were measured at a resolution of 1 nm between 200-800 nm wavelengths.

Transmission electron microscopy

High resolution transmission electron microscopy (TEM) has been performed using the JEOL JEM 1200 EXII instrument operated at an accelerating voltage of 80 kv to examine the morphology and size of Ag-NPs. The samples suspension was sonicated for 15 min to separate the agglomerated particles and make the solution homogeneous. Immediately after sonication, a drop of the suspension was sampled and the drop is placed on a film on a support grid. The samples were ready for examined under TEM after the solvent has completely evaporated.

FTIR spectroscopy analysis

Fourier transform infrared spectroscopy is used for determination of the bonds starching vibration present in the Ag-NPs. The FTIR spectrum of the dried samples was recorded on Perklin Elmer instrument in the range of 450 to 4000 cm^{-1} at a resolution of 4 cm^{-1}.

Analysis of antibacterial and antifungal activity of Ag-NPs

The silver nanoparticles synthesized from *K. marxianus* and *C. utilis 22* were tested for antibacterial and antifungal activity by well-diffusion method against multi-drug resistant human pathogens, three bacterial strains (*Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 10536, *Pseudomonase* fluorescence ATCC 50090) obtained from TCS bioscience LTD, Botolph Claydon. Buckingham, MK 1821 R. Stock cultures were maintained on nutrient agar at 4°C and three yeast strains (*Candida albicans*, *Candida glabrata*, *Candida krusei*) isolated from patients who have *Candida* urinary tract infections and stock cultures were maintained on UM. Muller Hinton agar (MHA) was prepared and poured on sterile Petri plated. These MHA plates were inoculated by swabbing the 24 h old test human pathogens individually and then wells of 6 mm diameter were made using a gel puncture. 20 µl of Ag-NPs was loaded into each well. After incubation at 35°C for 24 h, the susceptibility of the test microorganisms was determined by measuring in mm the diameter of inhibition zone around the well. Cefotaxime (30 µg),

Novobiocin (30 µg) and Itraconazol (10 µg) as a standard antimicrobial agents were used against Gram positive bacteria (*S. aureus*), Gram negative bacteria (*E. coli*, *P. flourescence*) and all the tested yeast strains respectively. The interpretive criteria published by the National Committee for Clinical and Laboratory Standards (NCCLS) (Wayne, 1997) and the Clinical and Laboratory Standards Institute (CLSI) (Wayne, 2004) were followed.

Results and Discussion

Today, the development of environmental friendly nanoparticles has recently been of increasing interest to both academic and industrial sectors. Silver and its compound, which have been used as an antimicrobial agent in the past have recently received renewed interest. This is because some of bacterial strains have demonstrated resistance towards antibiotics. Hence,, synthesizing Ag-NPs using biological method, which has higher antimicrobial activity are of particular concerned. It was reported that extracts from bio-organisms such as enzymes/proteins, amino acids, polysaccharides and vitamins may act as reducing and capping agent in the Ag-NPs synthesis, making the synthesis is more environmentally friendly than chemical synthesis (Jagadeesh *et al*., 2004 and Xie *et al*., 2007). A comprehensive study of extracellular biosynthesis of Ag-NPs from *K. marxianus* and *C. utilis 22* was carried out in this research work

Characterization of synthesized silver nanoparticles

Aqueous silver nitrate ions were reduce during exposure to the cell filtrate of *K. marxianus* and *C. utilis 22*, visual observations showed a change of colour in silver nitrate solution from yellow to brownish in case of *K. marxianus* and dark brown in case of *C. utilis 22* (Fig. 1), whereas no colour change was observed in the control (the cell free filtrat without silver nitrate). The appearance of a brownish and brown colour in silver nitrate treated culture supernatant suggested the formation of silver nanoparticles (Sastry *et al*., 2003 and Jeevan *et al*., 2012). A similar observation was made by Duran *et al*., (2003); Jeevan *et al*., (2012) and Saravanan *et al*., (2013) in the biosynthesis of Ag-NPs by *Fusarium oxysporum*, *P. aeruginosa* and baker's yeast respectively by extracellular process. The brown colour of the medium could be due to the excitation of surface Plasmon vibration of Ag-NPs (Ahmad *et al*., 2005)

The exact mechanism of biosynthesis of Ag-NPs is not known. However, it has been hypothesized that silver ions required the NADPH-dependent nitrate reductase enzyme for their reduction, which was secreted by the microorganisms in its extracellular environment (Kalishwaralal *et al*., 2008 and Jevean *et al*., 2012). The use of this enzyme has previously been demonstrated in the *in vitro* synthesis of silver nanoparticles under anaerobic conditions. Nitrate reductase is known to shuttle electron from nitrate to the metal group. Thus, these results substantiate the role of nitrate reductase enzyme in the biosynthesis of silver nanoparticles (Gajbhiye *et al*., 2009 and Jeevan *et al*., 2012).

The synthesized Ag-NPs and its stability were characterized by UV-visible spectroscopy, this technique has proved to be very useful for the analysis of nanoparticles (Fig. 2). In the UV-vis absorption spectrum, a strong, broad peak located between 430-450 nm and 400-430 nm were observed for the silver nanoparticles prepared from *K. marxianus*

and *C. utilis 22* respectively. This is very specific for silver nanoparticles (Kathiresan *et al*., 2009 and Saravanan *et al*., 2011 & 2013). Observation of these peaks, assigned to a surface Plasmon, is well documented for various metal nanoparticles with sizes ranging from 2-100 nm (Tillmann, 2004).

TEM provide further insight into the morphology and particle size distribution profile of the Ag-NPs and revealed pattern similar to the biosynthesized Ag-NPs characterized using TEM. The present data obtained from transmission electron-micrograph showed distinct shape and size of nanoparticles. The particles from *K. marxianus* and *C. utilis 22* were spherical in shape in the range of 3~12 nm (Fig. 3 A & B) and of 6~20 nm (Fig. 4 A & B) respectively and uniformly distributed without significant agglomeration in case of *C. utilis 22* and with agglomeration of Ag-NPs presence during the synthesis in case of *K. marxianus*. FTIR measurements of the freeze-dried samples of nanoparticles synthesized from *K. marxianus* and *C. utilis 22* were carried out to identify the possible interactions between silver salts and protein molecules, which could account for the reduction of silver ions and stabilization of silver nanoparticles. (Fig. 5 A & B). The amide linkages between amino acid residues in proteins give rise to well known signatures in the infrared region of the electromagnetic spectrum in both *K. marxianus* and *C. utilis 22*. FTIR spectrum reveals the bands at 3398.6, 3402.2 cm^{-1} and 2976.7, 2977.9 cm^{-1} were assigned to the stretching vibrations of primary and secondary amines respectively. The bands seen at 1388.7, 1385.6 cm^{-1} and 1036.0, 1035.5 cm^{-1} respectively corresponds to -C-N stretching vibrations, while the band at 1589.7, 1592.0 cm^{-1} respectively is characteristic of –C=O carbonyl groups and –C=C- stretching, which may be present between amino acid residues. The overall observation confirms the presence of protein in samples of silver nanoparticles from *K. marxianus* and *C. utilis 22*. It has also been reported earlier that protein can bind to silver nanoparticles through their free amine groups or cysteine residues (Gole *et al*., 2001 and Jeevan *et al*., 2012), or through free amide groups (Bansal *et al*., 2004; Shiv Shankar *et al*., 2004 and Saravanan *et al*., 2013). so that the protein could most possibly to form a coat covering around Ag-NPs and it stabilize the aqueous synthetic medium. This evidence suggests that the biomolecules could possibly perform the function for the formation of stable Ag-NPs in aqueous medium (Fig. 5 A & B).

Analysis of antibacterial and antifungal activity of Ag-NPs

The present work of antimicrobial study of Ag-NPs mostly emphasised on multi-drug resistant bacteria including *S. aureus* ATCC 6538, *E. coli* ATCC 10536, *P. flourescence* ATCC 50090 and *Candida* infections in patients which have been contributing to the increasing morbidity and mortality of these patients, especially associated to yeast resistance to antifungal therapy including *C. albicans*, *C. glabrata* and *C. krusei* isolated from patients with urinary tract infections (Ashour *et al*. under publish). The increase in antibiotic resistant microorganisms has prompted interest in the use of Ag-NPs as an antimicrobial agent (Monteiro *et al*., 2013). The results of inhibition study showed in Table (1) that Ag-NPs produced from *K. marxianus* and *C. utilis 22* have great potential effect than Cefotaxime and Novobiocin as standard antibacterial agents for Gram positive and Gram negative, also Itraconazol as standard

antifungal agent against all the tested pathogens whereas *S. aureus* was resistant to Cefotaxime while, *E. coli* and *P. flourescence* were resistant to Novobiocin. Also, *C. albicans*, *C. glabrata* and *C. krusei* showed a resistance for Itraconazole. The Ag-NPs from *C. utilis 22* has the largest inhibition against *S. aureus* than Ag-NPs from *K. marxianus* which further support the work done by Shahverdi *et al*., (2007) and San and Don (2013) that concluded Ag-NPs have an antimicrobial effect on *S. aureus*. However, Ag-NPs from *K. marxianus* and/or *C. utilis 22* have highest antibacterial efficacy observed against multi-drug resistant *E. coli* and moderate activity noticed against *P. flourescence*, these findings are in agreement with previous studies that examined the antimicrobial activity of Ag-NPs against *E. coli* (Sondi and Salopak-Sondi 2004) and on contrary with Saravanan *et al*., (2013) who reported that Ag-NPs from *S. cerevisiae* more efficacy against *P. aeruginosa*.

On the other hand, Ag-NPs from *K. marxianus* have great potential effect against *C. albicans* as well as Ag-NPs from *C. utilis 22* against *C. krusei* and moderate activity notices against *C. glabrata*, these findings are in agreement with Studies of Panácek *et al*., (2009) that examined the antifungal activity of Ag-NPs against *C. albicans* and *C. glabrata*. Regarding, the present results of antimicrobial action of nanoparticles may differ according to the size of nanoparticles produced, and different species of bacteria or fungal produces varying size of nanoparticles (Raimondi *et al*., 2005 and San and Don, 2013).

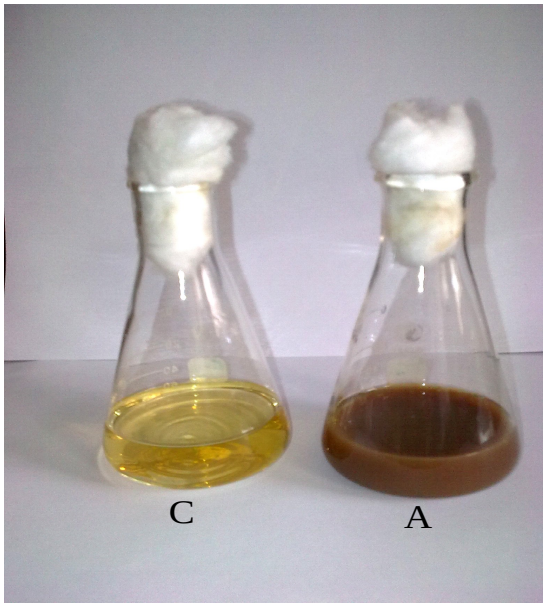
Generally, the results revealed that Ag-NPs synthesized were the key element that acting as antibacterial and antifungal agents. Silver has been used for its well known antimicrobial properties since roman time however the advances in generating Ag-NPs have made possible revival of the use of silver as a powerful bactericide and fungicide against various multi-drug resistant clinical isolates (Kim *et al*., 2008 and Saravanan, *et al*., 2011). Nanomateriales are the leading requirement in the field of nanobiotechnology and nanomedicine. Although no sufficient information is available on the adverse effects of Ag-NPs on human health and environment, many Ag-NPs are small enough to penetrate into human skin into organs as well as deposition of metal on biological species such as fish, microorganisms etc. (Oberdörster *et al*., 2004). Hence, assessment of environmental risks and human health associated with Ag-NPs is required before they are utilised in various applications.

The present study demonstrated the extracellular biosynthesis of Ag-NPs from *K. marxianus and Candida utilis 22*. The synthesized Ag-NPs from each strains have great potential effect against the mult-drug resistant human pathogens and to be an alternative to antibacterial and/or antifungal agents. Moreover, extracellular biological synthesis approach would make the process simpler, easier for downstream processing and cost effective alternative to conventional chemical and physical methods. The present research work explored that the synthesized nanoparticles from *K. marxianus* and/or *C. utilis 22* was ready for the application in the field of nanomedicine against multi-drug resistant human clinical pathogens of bacteria and *Candida* infections. Further studies are required on understanding the cellular and molecular mechanism of Ag-NPs and the effect of microbes are of the essence to biomedical applications.

Table.1 Representation of antimicrobial activity of silver nanoparticles synthesized from *Kluyveromyces marxianus*, *Candida utilis 22* and standard antibacterial and antifungal agents against multi-drug resistant human pathogens

Clinical pathogens	Mean zone of inhibition (mm)				
	Ag-NPs by *K. marxianus* (20 µl)	Ag-NPs by *C. utilis* (20 µl)	Cefotaxime (30 µg)	Novobiocin (30 µg)	Itraconazole (10 µg)
Gram positive					
S. aureus	37.8	45.8	14.0	----	-----
Gram negative					
E. coli	43.8	41.5	-----	17.0	-----
P. flourescence	30.2	32.0	-----	15.0	-----
Yeast infection					
C. albicans	60.0	40.0	----	----	13.0
C. glabrata	42.0	25.0	----	----	00.0
C. krusei	42.2	46.6	----	----	00.0

Fig.1 Biosynthesis of silver nanoparticles from A: *Kluyveromyces marxianus* and B: *Candida utilis 22* and C: Control (cell free filtrate without silver nitrate)



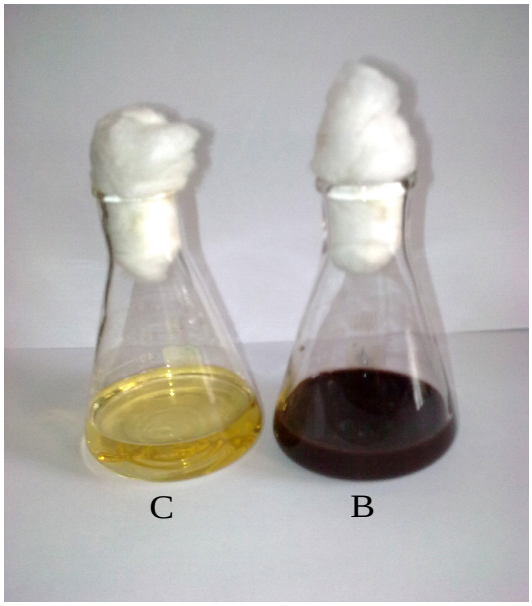


Fig.2 UV-visible absorption spectra of silver nanoparticles synthesized by the culture supernatant of A: *Kluyveromyces marxianus* and B: *Candida utilis 22*

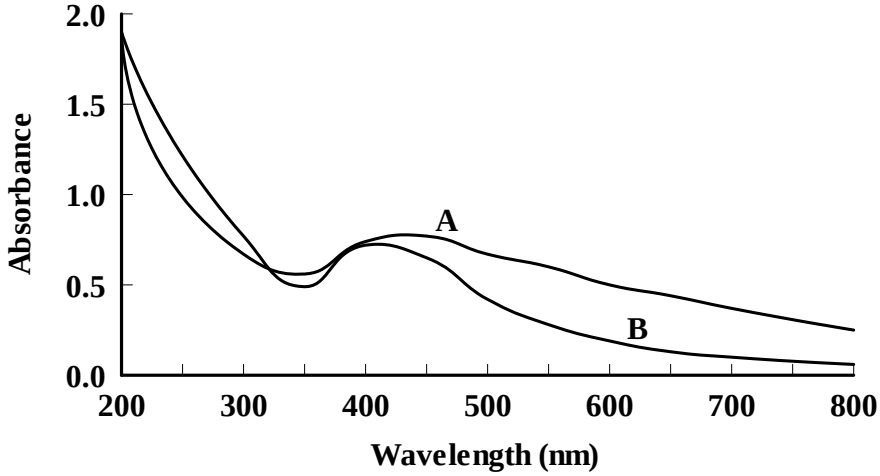
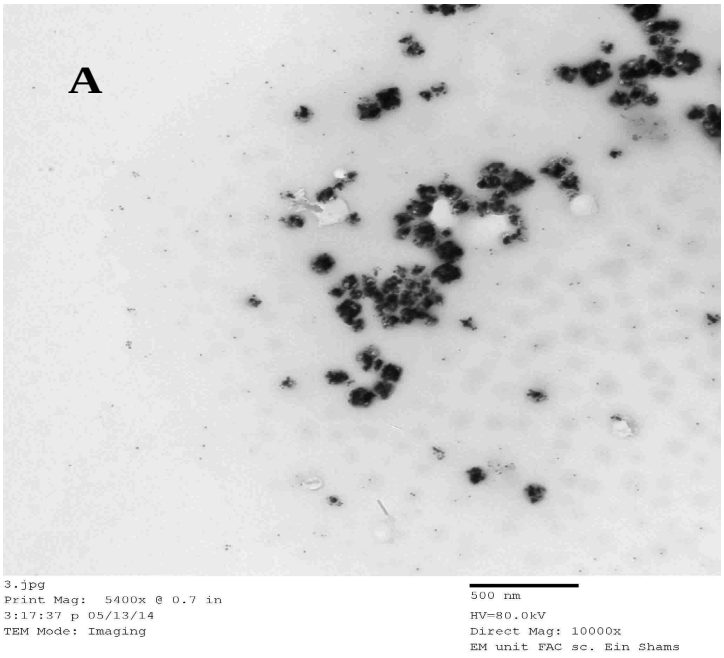


Fig.3 A & B. Transmission electron micrograph of the silver nanoparticles synthesized by *Kluyveromyces marxianus*



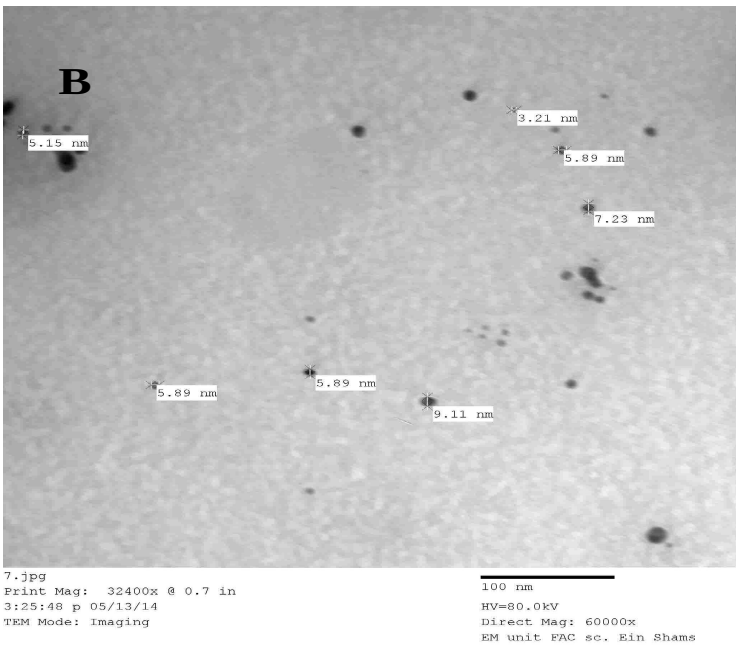
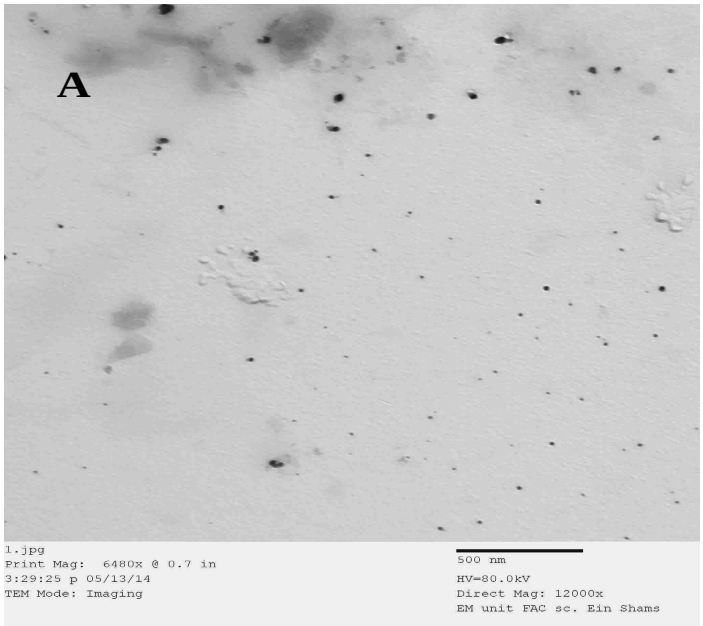


Fig.4 A & B. Transmission electron micrograph of the silver nanoparticles synthesized by *Candid utilis 22*



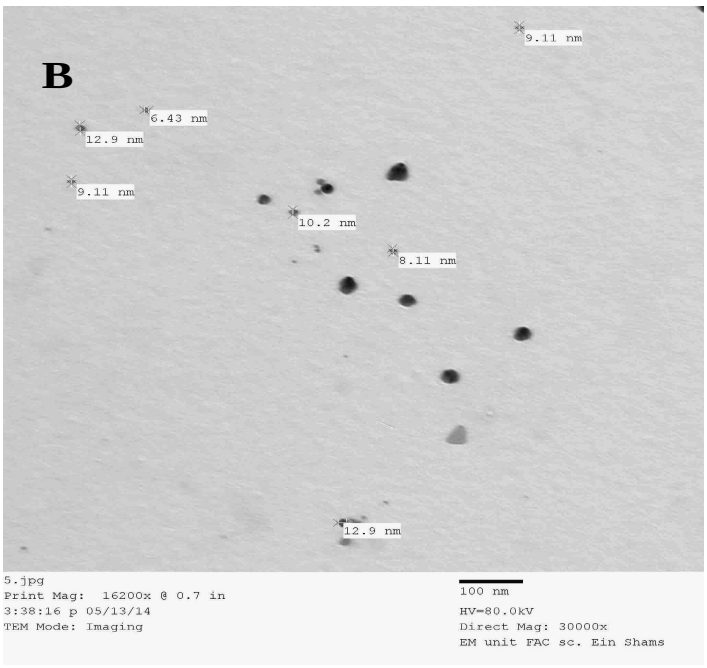
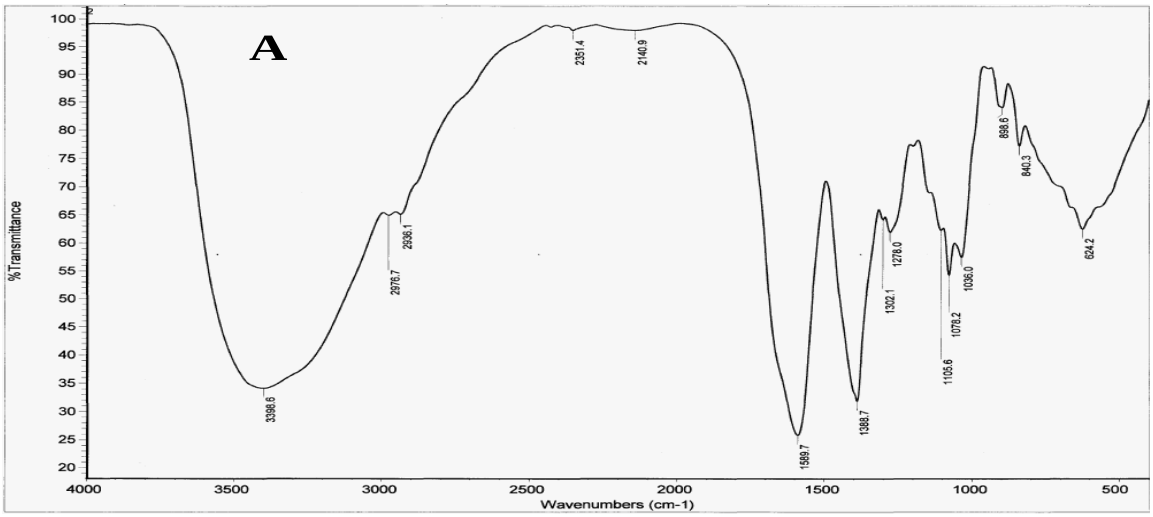
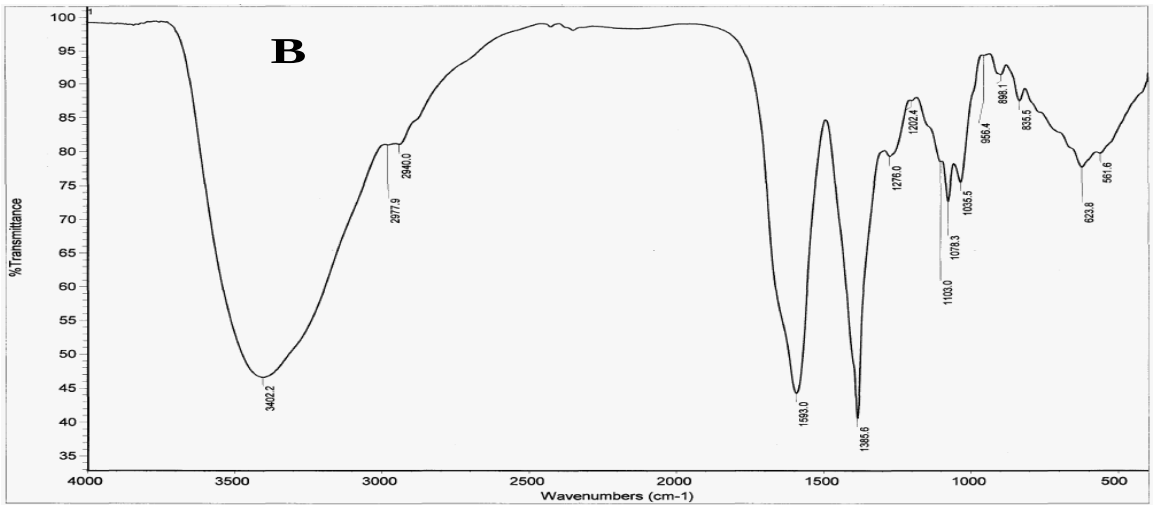


Fig.5 FTIR spectral analysis of silver nanoparticles synthesized from A: *Kluyveromyces marxianus* and B: *Candida utilis 22*





Acknowledgment

The author thanks the management of the Petroleum Research Institute and the Central Lab in Faculty of Science, Ain Shams University, Cairo, Egypt. The author also thanks Dr. Zeinab Hassan Kheiralla, Prof. of microbiology in Women's College for Arts, Science and Education, Ain Shams University, Cairo, Egypt.

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