

**Controllable Biogenic Synthesis of Intracellular Silver
Nanoparticle by Endophytic Ascomycetes Fungi from
Calotropis gigantea (L.) R. Br Seeds**

PROJECT PHASE - II (SBTAPROJ1)

Submitted in Partial Fulfilment of the requirement for the award of
Master of Technology degree in Biotechnology

by

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DEPARTMENT OF BIOTECHNOLOGY

SCHOOL OF BIO & CHEMICAL ENGINEERING

SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of Kavi Praba (41810008), who carried out the Review entitled "**Controllable Biogenic Synthesis of Intracellular Silver Nanoparticle by Endophytic Ascomycetes Fungi from *Calotropis gigantea* (L.) R. Br Seeds**" under our supervision from January 2023 to May 2023

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DECLARATION

I, KAVI PRABA A hereby declare that the Project Report entitled “**Controllable Biogenic Synthesis of Intracellular Silver Nanoparticle by Endophytic Ascomycetes Fungi from *Calotropis gigantea* (L. Br) seeds**” done by me under the guidance of Dr. V. Ramesh Kumar, Head of the Department of Biotechnology, Sathyabama Institute of Science and Technology, Chennai is submitted in partial fulfillment of the requirements for the award of Master of Technology degree in Biotechnology.

DATE: 5/5/2023

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ABSTRACT

The biosynthesis of monodispersed nanoparticles, along with the determination of potentially responsible biomolecules, is the major bottleneck in the area of bio-nanotechnology research. The current investigation focused to synthesize the Silver Nanoparticles (AgNPs) using an endophytic fungus isolated from *Calotropis gigantea* (L. Br) seed, located in Chennai, India. The fungus was identified for its morphological and molecular characteristics. The fungus identified as *Pichia kudriavzevii* strain based on 16SrRNA sequencing. The produced particles were ----- shaped in nature and shows the surface plasma resonances---. FTIR spectra of nanoparticle showed the C=C bonding, N-compound, C-O stretching vibration denotes the presences of alkene, protein and aromatic compound on the surface of silver nanoparticle produced by *Pichia kudriavzevii*. Synthesised NanoParticle revealed thee mean negative zeta potential and electrophoretic mobility of -29.8 mV and -0.000255 cm²/Vs respectively. The nanoparticles produced were proteinaceous compounds as capping agents with -29.8mV zeta potential and revealed the antibacterial activity against gram positive bacterial strain.

Keywords: Biosynthesis, Endophytic fungus, *Calotropis gigantea* seed, copper nanoparticles, SEM, XRD.

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LIST OF ABBREVIATION

AFM	-	Atomic Force Microscope
Ag NP	-	Silver Nano Particle
CIA	-	Collagen Induced Arthritis
Cu NP	-	Copper Nano Particle
DLS	-	Dynamic Light Scattering
EDX	-	Energy Dispersive X-Ray
EFNP	-	Endophytic Fungi Nano Particle
FESM	-	Field Emission Scanning Electron Microscope
FTIR	-	Fourier Transform Infrared Spectroscopy
ICAM 1	-	Intracellular Adhesion Molecule
IL	-	Interleukins
NP	-	NanoParticle
SC	-	[S] Curvularin
SEM	-	Scanning Electron Microscope
TNF	-	Tumor Necrosis Factor
UV	-	UV Visible Spectroscopy
XRD	-	X-Ray Diffraction

CHAPTER 1 - INTRODUCTION

The creation of a variety of nanomaterials is valued at the moment for the advancement of environmentally friendly technology in metal synthesis (Bhattacharya D *et al.*,2005). Despite their tiny assembly, the extent of reactivity is caused by their crystallographic nature, which increases surface area (Osaka T *et al.*,2006) (Singh M *et al.*,2008). The creation of ultrafine particles has been demonstrated using various physical and chemical techniques. Natural synthesis is important since chemical processes are expensive to utilize, dangerous, and have low productivity (Kowshik M 2003). A lot of debate is currently being had on the synthesis of NPs (Bansal V *et al.*,2005) (Kumar SA *et al.*,2007) due to probable, physiological mechanisms from high angiosperms species. The creation of inorganic materials can take place either extracellularly or intracellularly, and these environmental biological systems offer excellent examples of nanophasic materials operating from evolution over a long period with substantially optimal properties (Dickson DPE *et al.*,2000) (Senapati S *et al.*,2004). The rate of NPs fabrication can be influenced by exposure to temperature, pH, and substrate concentration (Gericke M.,2006). Fungi have been verified for the huge gage generation of NPs among various biological systems, including bacteria, actinomyces, fungi, plants, protozoa, and mammals. Bacteria typically produce NPs intracellularly since it is difficult and expensive to purify NPs on a large-scale production. Fungi produce NPs extracellularly, in contrast to bacteria, and are simple to employ and purify for huge gage manufacturing (Rai M *et al.*,2021).

Endophytes are a collection of incredibly diverse microorganisms that were associated with plants. The term endophytes were created by Anton de Bary in the 19th century to refer to the fungi that live in the plant's tissues. (Martin Muthee Gakuubi *et al.*,2018).Different criteria are used to classify the endophytes. They are classified into clavicipitaceous and non- clavicipitaceous endophytes based on ecology and sexual and asexual endophytes based on transmission.

The natural qualities of the metal NP like silver have quorum quenching, antioxidant, antibacterial, anti-viral, anti-cancer, and insect repellent, have been discovered (Dinda G *et al.*,2015) (Fouda A.H *et al.*,2015). The researchers created the AgNPs by synthesizing the metal because of its significant features (Hassan S

et al.,2011). Because of their widespread optical features, AgNPs were promoted as effective free radical scavengers (Gharib M et al., 2018) (Fathima J.B et al., 2018).

Calotropis gigantea, a participant of the small genus *Calotropis* family Apocynaceae, is the species that appear most frequently because it contains polar phytochemicals like terpenoids, lignans, and flavonoids, the Apocynaceae plant *Calotropis gigantea* (L.) R. Br has been tested and shown to have a variety of pharmacological activities. Its seeds have historically been used to treat hypertension, fever, and catarrh. Utilizing *C. gigantea* to make nanomaterials is risk-free and sustainable. *Calotropis gigantea* seed can grow a variety of seasonal microbes, particularly fungi.

Calotropis gigantea is a shrub that is about 10 meters tall. It grows in a dry coastal area along the sheltered shores of lagoons and has many branches at the bottom banks. The plant has a source of fiber. *Calotropis gigantea* shrub has vital medicinal values and is rich in fiber. The powdered flowers are used to treat colds and coughs. Intestine worms, rheumatics, and epileptic attacks can be treated with an infused medicine. In the tropics, it is cultivated as an ornamental and sometimes grown for fiber from its stem.

There are potential sources of biologically active natural products in the form of endophytes, which are symbiotically associated microorganisms of living plants. They are found in every plant species studied and are estimated to be around a million or more. There is a chance to find a novel and interesting microorganism with significant therapeutic efficacy. A better understanding of the mechanism of endophytes' existence and their interaction with the surroundings can be achieved by the selection of suitable higher plants. A new natural product might be revealed through this procedure. The number of useful microorganisms is larger than the amount of utilization for food and health industries.

A chemical synthesis process is the easiest and most convenient way to synthesize AgNPs. Several toxic reagents are used to synthesize AgNPs. Many metal particles are used in creams and cosmetics. The use of plants and microorganisms has been proposed as a way to synthesize metal nanoparticles. It requires less effort to synthesize nanoparticles from plants than to maintain and

culture them. The use of plant biomolecules in the synthesis of AgNPs is beneficial because of their low toxicity to living cells.

The purpose of the current study was to explore the use of *Calotropis gigantea* for the synthesis of AgNPs using secondary metabolites from the seed extract. The synthesis process of AgNPs compounds was characterized using different qualitative and quantitative analyses to evaluate the formation of silver nanoparticles.



CHAPTER 2- LITERATURE SURVEY

2.1) ENDOPHYTIC FUNGI

Endophytic fungi have gained a significant amount of attention in the last two decades as producers of bioactive products. (Schulz B *et al.*, 2002) (Aly AH *et al.*,2011). Flavonoids, phenolic acid, amides, chlorinated metabolites terpenoids, sesquiterpenes, lignans, phenyl prostanoids, isocoumarin derivatives, diterpenes, phenol, alkaloids, aliphatic compounds, and so on are just some of the functional secondary metabolites (Zhao J *et al.*,2009).

(Stierle A *et al.*,1993) studied that endophytes can produce compounds that are related to those found in their higher plants. Some endophytic fungi, like Diosgenin, paclitaxel, hypericin, vinblastine, camptothecin, and podophyllotoxin, can generate identical bioactive substances as their habitats (Kusari S *et al.*,2009).

2.2) STUDIES ON ENDOPHYTIC FUNGI

2.2.1) Anti-inflammatory activity of endophytic fungi

(Deshmukh S.K *et al.*,2009) reviewed, Endophytic fungi were isolated from leaves of the medicinal plant *Mimosops elengi*(bakul) which has anticancer properties. (Moharram AM *et al.*,2017) The extracts of *Emericella nidulans*, *Pleospora tarda*, and *Penicillium funiculus* were effective in reducing inflammation. The greatest inhibition of bovine albumin denaturation was shown by *Emericella nidulans* and *Pleospora tarda*. (Pretsche A *et al.*,2014) studied the impact of the organisms on *Propionibacterium acnes* and its calming properties.

2.2.2) Anti-rheumatoid activity of endophytic fungi

(Schmidt N *et al.*,2010) reviewed that, To distinguish between the signal transducer and Janus kinase inhibitors and record pathway activators, a screening framework was used. *Penicillium*'s parasitic macrocyclic lactone (S)- curvularin (SC) is used as a calming specialist. In an in vivo model of chronic autoimmune disease, they investigated the possibility that SC might lower the expression of proinflammatory genes. Therefore, the side effect of SC and dexamethasone were

contrasted in the mouse model of collagen-induced arthritis (CIA). (Abdel-Azeem AM *et al.*, 2016) studied that the endophytic *Chaetomium globosum*-produced secondary metabolites of *Adiantum capillus-veneris* from the dry Sinai have anti-rheumatic activity.

2.3) ENDOPHYTIC FUNGI WITH OTHER MICROBIOMES

Endophytic fungi are forced to have complex and dynamic communication with other plant microbiota, such as endosphere-associated bacterial and fungal strains. This holds whether or not these microorganisms are symbiotic or pathogenic in their natural environments. Endophytes from bacteria and fungi both have Endophytes that exhibit antagonistic activity against phytopathogens that cause disease and suffer less damage from those pathogens (Strobel G., 2018)

In addition to producing various antioxidants that suppress pathogens, they produce numerous bioactive metabolites that are antiviral and antimicrobial (Gouda S *et al.*, 2016). Further studies identified, that a wide variety of fungal species, particularly endophytic fungi, can protect the insect population (Litwin A *et al.*, 2020). Nitrogen fixation, soluble minerals, and metabolic production involve a variety of bacteria, including *Bacillus*, *Pseudomonas*, *Pedobacter*, and *Acidobacterium*. A variety of fungal strains, including *Acremonium spp.*, *Chaetomium globosum*, *M. robertsii*, *B. metarhizium*, and *Beauveria bassiana*, are successful at safeguarding plants (Grabka R *et al.*, 2022). Due to its wide range of hosts, endophytic fungus outperforms other biocontrol agents.

2.4) FUNCTION OF BIOGENIC SYNTHESIS IN NANOPARTICLES

Production and use of diverse ultrafine particles are the subjects of the science field of nanotechnology. The term "nanoparticle" refers to anything between 100 and 1000 nm in size that, because of its size, may differ from the bulk material. Silver, alginate, titanium, and zinc, are recently being used to make a variety of metallic nanomaterials (Mukherjee P *et al.*, 2001). Nanoparticles are being used for a wide range of applications, including medical treatments, energy storage in batteries, and widespread inclusion in the cosmetics and apparel sector (Tripp SL

et al., 2002). A wide range of microorganisms has been used in the biological method, which has benefits over other chemical reactions because it is more eco-friendly and energy-efficient (Hakim L.F *et al.*, 2005). In contrast to Nanoparticles made by chemical processes, NPs are biocompatible because they have biological molecules coated on their surface (Huang J *et al.*, 2015). The applications of bio-inspired NP's biocompatibility in biomedicine and related fields are very intriguing. NPs of diverse sizes and interesting morphologies can be created using biogenic methods; For instance, silver nanoparticles ranging from it sizes of 25 to 12 nm have been produced by exposing *Verticillium spp.*, adding microbial biomass to silver ion solution in water (Riddin T *et al.*, 2010) (Schroff A *et al.*, 2014). Because the fungal cells (biomass) continue to grow, the NPs are not toxic. Negatively charged enzymes with a carboxyl group interact electrostatically with the fungus's cell wall and the silver ions allow the nanoparticles to grow on the surface of mycelia (Mukherjee P *et al.*, 2001). The cell-soluble sulfate-reducing bacteria extract protein and were used to make NPs of a specific morphology (Riddin T *et al.*, 2010). Because the use of exclusive chemicals was limited and these NPs had a higher catalytic activity, they highlighted those who used chemical methods for synthesizing. *Penicillium rugulosum*, a fungus with industrial significance, was used to produce uniform-sized gold nanoparticles, that are easy to handle than those produced by other bacteria or yeast (Mishra A *et al.*, 2012).

2.4.1) Fungi's intracellular nanoparticle synthesis

This method uses enzymes to transport subatomic particles into microbial cells to form ultrafine particles. The organism itself produces nanoparticles that are smaller than those that are reduced outside of the cell. The nucleation of particles within the organisms is probably related to the size limit (Narayanan K.B and Sakthivel N.,2010).

2.4.2) Fungi's extracellular synthesis of nanoparticles

Extracellular synthesis of ultrafine particles has a wider range of applications than intracellular synthesis because it removes unnecessary cell-to-cell components. Most of the time, fungi are capable to fabricate nanoparticles outside of the cell because they have a lot of secretory components that help break down and cover nanoparticles (Narayanan K.B. and Sakthivel N.,2010).

2.4.3) Microbes for the production of nanoparticles

Either intracellular & extracellular inorganic materials are produced by unicellular and multicellular organisms (Shiv Shankar *et al.*,2004). Metal ultrafine particle synthesis is a new upcoming research on microbes. Due to their resistance and capability to bioaccumulate metals, fungi are the most dominant studies on the biological production of metallic nanoparticles (Sastry M *et al.*, 2003).

2.5) DIFFERENT ENDOPHYTIC FUNGAL SPECIES FOR THE PRODUCTION OF METAL NANOPARTICLE

2.5.1) *Cladosporium perangustum*

(Govindappa M *et al.*.,2022) Reported that the ability to produce several phytochemicals that are required for biological functions and serve as caps was also demonstrated by endophytic fungal species. These phytochemicals facilitate the bioreduction and occurrences of silver ultrafine particles (Li *et al.*,2016). Silver nanoparticles from *Cladosporium* types of *Loranthus micranthus*, an endophytic growth of the stem *Aegiceras corniculatum*, have been integrated, and endophytic parasitic interceded silver nanoparticles have serious areas of strength for exhibited, against diabetic, and hostile to Alzheimer's sickness movement (Popli *et al.*,2018). Silver nanoparticles made from *Dendrophthoe falcata*'s endophytic *Cladosporium perangustum*. The procedure is simple, cost-effective, suitable, and ecologically safe. They synthesized Au NPs nanoparticles from *C. perangustum* and characterized them using UV-visible spectra, X-ray diffraction, Fourier-transformed infrared, and a Scanning electron microscope. Their research on nanotoxicology, cancer prevention, and antioxidants was published.

2.5.2) *Lasiodiplodia theobromae*

(Ranjani S *et al.*,2020) reported that, Isolating and identifying the endophytic fungi that were found on the petiole of the *Cinnamomum zeylanicum* tree. Based on its sequence, the endophytic fungus was identified as *Lasiodiplodia theobromae*. Green silver nanoparticles were mixed with *Lasiodiplodia theobromae*'s watery concentrate. One more physiochemical portrayal of the endophytic fungi nanoparticles was completed by FTIR and FESEM (field outflow filtering electron microscopy) examination. The transition from a whitish to a dark brown color is the first confirmation of the synthesis of silver nanoparticles. They were tested for rate of growth, bacteriostatic and bactericidal activity, and pyocyanin assays after treatment.

2.5.3) *Penicillium oxalicum*

(Parikshana *et al.*,2020) Investigated, for the mycosynthesis of iron nanoparticles, an endophytic fungus from *Tecomella undulata*, *Penicillium oxalicum*, was utilized. The synthesized Iron ultrafine particles were delineation by different approaches. The Ultraviolet-visible spectroscopy absorption spectrum revealed an iron nanoparticle-specific 300 nm absorption band. The Fourier-transformed infrared spectrum of the iron ultrafine particles obtains retention peaks that ranged from 3430.70 cm to 466.83 cm. SEM pictures uncovered around 140 nm particles. In AFM images, the particle's height and width were found to be 3.9 nm and 100 nm, respectively. With the assistance of the formed iron nanoparticles, the destaining of methylene blue stain in the presence of water was investigated. The stain was effectively decolored to 99.17 percent in just six hours. The destaining procedure was arbitrated by the mycosynthesis of ultrafine particles opening the door to a long-term strategy for getting rid of toxic textile effluents.

2.6) SILVER NANOPARTICLE

silver is an evolution metal with a specific Gray-white coloration and copper brilliance. It has a nuclear number of 29 and a mass of 63.546. These characteristics make silver an essential component in the functioning of society. Additionally, silver

is an essential component due to its reasonable tensile strength (Egziabher G., 2012). Silver ultrafine particle has a high-volume ratio, that tends to behave as an extremely reactive particle. Silver is considered an important metal for human health as it contains enzymes that make it function in all body organs (Goyer RA.,1997). They are more effective against bacteria because they can easily interconnect with other molecules (Smith AM *et al.*,2006) (Theivasanthi T and Alagar M.,2011). When compared to other metals copper is enormously responsive to air and has more stable oxidation stages. (Jeong S *et al.* ,2008)

2.6.1) Methods used for the synthesis of Silver nanoparticles

(Khanna *et al.*,2007) reported, he was able to produce silver nanoparticles by reducing silver nitrate salt with a surfactant. silver particles are formed by oxidizing the silver nitrate ultrafine particle. Ag NPs are typically protected from oxidation by being encased in organic materials with a stabilizing and capping agent, or inorganic materials (Esteban-Cubillo A *et al.*,2006). Various methods for the synthesizing of Silver nanoparticles include Heat reduction (Habibi MH *et al.*, 2010), radiation method (Ponce AA and Klabunde KJ 2005), microemulsion technique (Joshi S *et al.*,1998), laser ablation (Qi L *et al.*,1997), mechanical attrition (Tilaki R and Mahdavi S., 2007), chemical reduction (DeCastro CL and Mitchell BS., 2002) and biological synthesis (Shantkriti S and Rani P., 2014) (Salvadori MR *et al.* ,2014) and green approaches (Singh S., 2017).

2.6.2) Biosynthesis of AgNPs using different plant extracts

(Valodkar M *et al.*, 2011) Reported that Silver nanoparticles are made with peptides attached by using the pedicel of Euphorbia nivulia, also known as "common milk hedge," as a capping agent and reducing agent. (Valodkar M *et al.*,2011) Observed, the biological changes of prepared AgNPs on tumor cells and their applications as antimicrobials. (Lee H.J *et al.*,2011) conducted research to extract Silver nanoparticles from the leaves of Magnolia kobus. Copper nanoparticles were produced using aqueous Calotropis procera seed extract (Harne S *et al.*, 2012), and the cytotoxicity of these compounds toward cancer cells has been studied.

(Subhankari and Nayak.,2013) Observed, the antimicrobial properties of shaped NPs in ginger (*Zingiber ofcinale*) and *Syzygium aromaticum* (Clove) concentrates. (Saranyaadevi K *et al.*,2014), reported that, *Nerium oleander* leaf (Angrasan J and Subbaiya R., 2014), and *Vitis vinifera*. Recently, the *Tilia* sample was used for the synthesis of Silver ultrafine particles. (Padma PN *et al.*,2018) investigated the possibility of green synthesizing AgNPs by extracting the fruit rind of *Punica granatum*. The *Syzygium aromaticum* bud was taken out and ultrafine particles are examined by using properties such antimicrobial and physical properties.

2.7) OPTICAL PROPERTIES OF AgNPs

The typical absorption peak of Silver nanoparticles in UV-visible light ranges from 400 to 500 nm. It was agreed that the difference in biodynamic particles that are responsible for the decreasing cycle in silver nitrate precursors is the cause of the variety of retention tops. Consequently, the particle size is influenced by both the plant's nature and the method of preparation. This was shown in various green blend strategies (Lee H.J *et al.*,2011). The Silver nanoparticle retention peak was at 560 nm, with a 45-110 nm molecule size range (Harne S *et al.*,2012). (Subhankari I and Nayak P.,2013) identified a similar characteristic retention peak at 470 nm. The X-ray diffraction and Transmission electron microscope results indicate the size of the particle is 15nm. (Saranyaadevi K *et al.*, 2014) They observed the 531 nm peak of the Ag NPs absorption. According to TEM analysis, the average particle size was 5 nm, while XRD analysis indicated a range of 50 to 100 nm. (Caroling *et al.*, 2015) Observed the retention peak at 576 nm, with a minimum size of 30-50 nm in SEM results; (Nasrollahzadeh M and Mohammad Sajadi S., 2015) reported the peak at 460 to 480 nm, with TEM results ranging from 15 to 20 nanometers in size. One more study (Jayandran M *et al.*, 2015) reported that Silver particles with the highest absorption peak at 585 nm had sizes varying from 60 to 100 nm by TEM and a standard size of 45 nm by XRD. Karimi and Mohsenzadeh discovered that the Silver absorption peak was 478 nm and that the standard particle size was 40 nm using FESEM. (Kaur P *et al.*, 2016) reported that in TEM, the peak is 485 nm, and the particle size is 15–20 nm. (Rajesh k *et al.*, 2018) studied, the surface plasmon peak of Ag NPs biosynthesized with *Tilia* extract could be seen at 563 nm. The AgNPs typical molecule size was determined to be 27.6 nm in XRD, while TEM shows 4.7-

17.4 nm (Hassanien R *et al.*.,2018). A silver peak with an ultrafine particle size of 12 nm was observed in the bud of *Syzygium aromaticum* using the Scherrer formula at 480nm (Nagar N and Devra V., 2018). The maximum retention peak was observed at 460 nm and the characteristic of silver colloids of absorption peak was observed at 488 nm with an XRD moderate crystalline size of 76 nanometers (Ismail M *et al.*, 2019).

2.8) FACTORS AFFECTING BIOSYNTHESIS OF NANOPARTICLE

2.8.1) pH

pH is the first and foremost significant factor affecting nanoparticle size, texture, and (Armendariz V *et al.*, 2004) (Bawaskar M *et al.* 2010). The various biological system extracts have varying pH. Different plant extracts have varying pH. (Sathishkumar M *et al.* 2009). Variation of pH solution in media, ultrafine particle morphology can be detected (Suchomel N *et al.* 2018). The morphology of biosynthesized ultrafine particles, especially AgNPs from *Chrysosporium tropicum* and *Fusarium oxysporum* (Soni N and Prakash S., 2011), *Zingiber officinale* (Aziz WJ and Jassim HA., 2018), and AuNPs from *Avena sativa* (Armendariz V *et al.*, 2004). [Dubey *et al.*,2010] ensured that nanoparticles incorporated at a lower pH were larger than those incorporated at a higher pH.

2.8.2) Temperature

Temperature is one of the ideal factors that disturb ultrafine particle biosynthesis (Bawaskar M *et al.*, 2010). Using conventional physical and chemical methods, ultrafine particle synthesis necessitates extremely high temperatures of 350 degrees Celsius. (Patra J.K and Baek K.H.,2004) state that although biosynthesis can take place at room temperature (less than 100 degrees Celsius), temperature variations still play a significant role in determining nanoparticle morphology. (Liu H *et al.*,2017) The size of the AgNPs made from *Cinnamomum camphora* leaf extract slightly raises the reaction temperature rises from 70 to 80 degrees Celsius but reduces further temperature rises from 80 to 90 degrees Celsius. They concluded that the high temperature was to blame for the shrinking

as a result. (Dwivedi A.D and Gopal K.,2010) Demonstrated that higher temperatures are responsible for forming AuNPs with platelet- and nano-rod-shaped shapes and a faster rate of AuNP synthesis. Spherical AuNPs, on the other hand, formed at lower temperatures. The biosynthesis of each kind of nanoparticlerequires a specific temperature. The synthesis of AgNPs with an extract of *Trichoderma longibrachiatum* was attempted at temperatures of 23, 28, and 33 degrees Celsius; (Elamawi R.M et al.,2018) Reported that, While the other two temperatures did not result in any synthesis at all, the optimal temperature for AgNPs synthesis was 28-degree Celsius.

CHAPTER 3- AIM AND SCOPE

- To Extract Endophytic fungi from *Calotropis gigantea* seed
- To Identify Molecular characteristics of Endophytic Fungi
- To Purify AgNPs From Endophytic Fungi
- To Evaluate the synthesized AgNPs
- To Determine the effect of pH and Temperature, to analyze its biogenic nanoparticle

CHAPTER 4 - MATERIAL AND METHODOLOGY

4.1) ISOLATION, PURIFICATION OF FUNGAL STRAINS

Fungi are collected from *Calotropis gigantea* seed. The seeds were placed in PDA (Potato Dextrose Agar) and incubated at room temperature for 6-7 days. After incubation days, spores are obtained from the seed, placed in another PDA Petri plate by using sterilized wire loop, and incubated for 6-7 days at 37 degrees Celsius. For purification purposes, subculturing was performed to obtain a pure fungiculture. The purified fungi culture was stored at 4 degrees Celsius for further purposes.

4.2) IDENTIFICATION OF FUNGAL STRAIN

Fungi sample are characterized based on morphology, and molecular characteristics. The Color, Shape, and nature of fungi colonies were observed on Potato Dextrose Agar plates for 7 days. A Cotton Blue Lactophenol test was performed to identify the Morphology structure of isolated fungi.

4.3) MOLECULAR CHARACTERIZATION

4.3.1) DNA extraction

- 1) The genomic DNA of fungal isolates was extracted. Transfer 500ul of broth culture into 1.5ml new Eppendorf tube.
- 2) Centrifuge the sample at 4000rpm for 5 minutes.
- 3) Decant the supernatant and wash the pellet with 3 ml of saline.
- 4) Vortex the sample for 30 seconds.
5. Centrifuge the sample at 4000rpm for 5 minutes.
6. Decant the supernatant.
7. Add 100 µl of PBS, 400µl Cell Lysis Buffer, and 5µl proteinase into the pellet tube. Keep at water bath at 55°C for 1-2 hours.
8. Add saturated phenol of 500µl to the sample. Mix for 5 minutes by inverting.
9. Centrifuge at 12000 rpm for 10 minutes. Transfer the supernatant to a 1.5 ml tube.

10. Add 25:24:1 of phenol: chloroform: Isoamyl alcohol to the tube. Mix for 5 minutes by inverting.
11. Centrifuge at 12000 rpm for 10 minutes.
12. Transfer the supernatant and add 500µl of chloroform. Mix for 5 minutes by inverting.
13. Centrifuge at 12000rpm for 10 minutes.
14. Transfer the aqueous layer carefully into the new tube and add an equal volume of 100% Ethanol or Isopropanol. Keep at -20C for 1 -2 hours.
15. Centrifuge at 14000rpm for 20 minutes. Decant the Supernatant.
16. Add 1 ml of 70% Ethanol. Centrifuge at 14000 rpm for 10 minutes.
17. Decant the supernatant. Air dry the pellet at Room Temperature to remove the droplets.
18. Add 50 - 60ul of Elution buffer.

4.3.2) Gel Electrophoresis and Quality check:

The quality and quantity of DNA was measured using Nanodrop spectrophotometer and agarose gel electrophoresis. A solo band of high-molecular-weight DNA has been observed.

4.3.3) PCR Amplification

The microbial 16SrRNA gene, region was determined using universal primers by PCR from the above-isolated DNA. A single discrete PCR amplicon band of 1523bp was observed when resolved on Agarose Gel. The PCR product was purified to remove contaminants.

4.3.4) Purification by Column:

1. Cut the DNA fragment from the agarose gel slice using a scalpel or razor and weigh the gel. Add 3-fold of binding buffer (i.e. For 100mg gel slice and 300ul of binding buffer) and keep in the dry bath at 50°C for 10 minutes.
2. Allow the gel slice to dissolve completely by vortexing the tube in between incubation.
3. After the gel has dissolved completely, add 10ul of 3M sodium acetate (pH. 5.00).
4. Add 1-fold of isopropanol (i.e. for 100 mg gel slice add 100ul of isopropanol) to the dissolved gel, vortex, and load onto the SMS column.
5. Centrifuge at 8000rpm for 30 seconds and discard the flow through.
6. Repeat the centrifugation step for large volumes greater than 700ul until you process the entire volume.
7. Centrifuge one more time at 10,000g for 15 seconds and discard the flow through.
8. Place the SMS column into a new collection tube and centrifuge for 90 seconds at the rate of 13,000 rpm to remove the residual ethanol.
9. Transfer the column to a new 1.5 ml tube, add 20ul of buffer SE onto the membrane, and wait 1 minute. It is optional to incubate the columns after adding the buffer SE for 5 minutes to improve the elution efficiency.
10. Centrifuge the tubes at 13,000 rpm for 1 minute for PCR product elution and discard the columns. Store the product at -20°C.

4.3.5) Sequencing

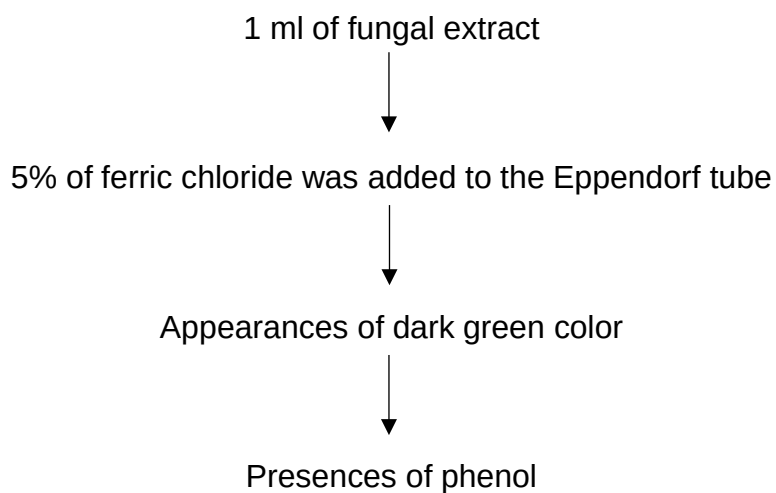
The purified sample was sequenced by using universal forward and universal reverse primers. Sequencing is performed with the help of ABI 3500 genetic analyzer by BDT v3.1 cycle sequencing kit.

4.4) PHYTOCHEMICAL ANALYSIS

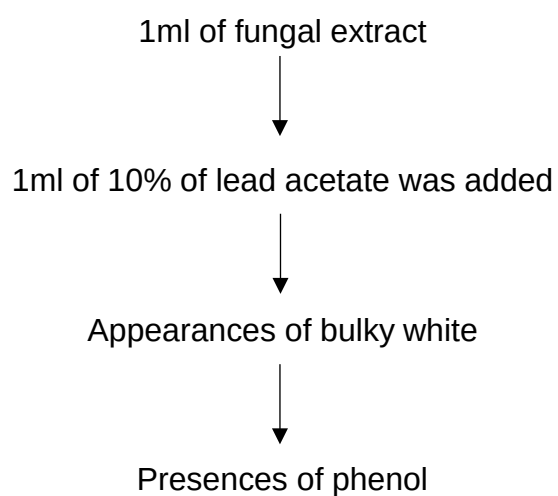
4.4.1) *Qualitative analysis*

A) Phenol:

i) Ferric chloride test:

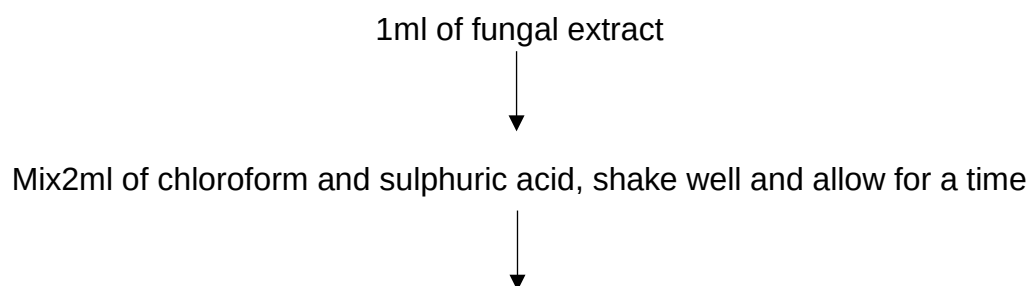


ii) Lead Acetate Test:



B) steroids:

I) Salkowski test



Appearances of reddish brown color



Presence of steroids

C) Terpenoids

i) Liebermann- Burchard test

1ml of fungal extract



drop of acetic acid followed by 1ml of sulphuric acid was added



Appearances of deep red colour junction of 2 layers



Presences of terpenoids

D)Tannin

i) Ferric chloride test

1ml of fungal extract



few drops of 10% ferric chloride was added



Bluish black color appearances



Disappearances by adding drop of sulphuric acid

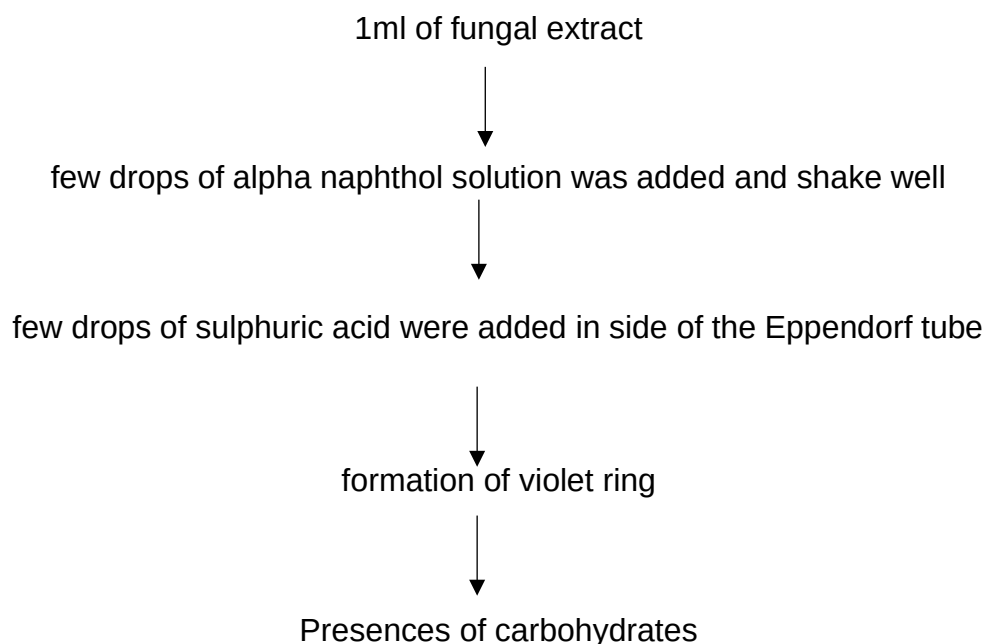


Formation of yellow brown precipitate

Presences of tannin

E) Carbohydrates

i) Molisch test



4.5) Fungal biomass preparation for AgNPs biosynthesis

Loop full of inoculum was suspended in PDB (potato dextrose broth) and incubated at 27degree Celsius in shaker incubator. After 7- 10 days of incubation the pale white color fungal mycelium is observed in the PDB. Then the biomass was separated by filtration process. The filtered biomass was resuspended in 100 ml of denoised water and kept in shaker incubator was 2 days. This cell filtrate sample is used for further analysis.

4.6) Biosynthesis of silver nanoparticles

The experiment was conducted on the cell filtrate. Initially the sample was sonicated for 20-25 mins. After sonication, the sample was centrifuge at 6000 rpm for 20 mins. The supernant was collected and it is filtered by using Whatman filter paper grade no 3. The filtered sample was used for further analysis. By using

different concentration of AgNO₃ (2mM,4mM,6mM,8mM, and 10mM) the experiment was carried out.10 ml of fungal extract was mixed with 20 ml of AgNO₃ as per different concentration and kept on dark condition for 72 hrs. and observe the color change from pale white to brownish color. The synthesis of silver nanoparticle was monitored by UV-Visible Spectral in the wavelength ranges of 200- 800nm.

4.7) Characterization of AgNPs

4.7.1) UV Visible spectroscopy

The optical properties of Surface Plasmon resonance of the AgNPs and control were monitored by UV–Visible absorption. The liquid sample was diluted with distilled water and absorbance was taken in the range of 200–800 nm Jasco- UV-VIS NIR.

4.7.2) ATR-FTIR

ATR-FTIR analysis was carried out to find the functional groups in the fungal cell responsible for the reduction of silver ions and the formation of biogenic intracellular AgNPs. A pinch of powder sample was mixed with water and subjected to FTIR analysis in the Happ- Genzel FTIR spectroscopy device. 32 scans were taken at the range of 4000- 650 cm⁻¹ and the resolution was kept at 4cm⁻¹ for the attenuation of noise ratio.

4.7.3) Particle size Analyser and Zeta Potential

Particle size analyser and Zeta Potential analysis was carried out at scattered angel of 25°C by scattering laser intensity, using HORIBA AZ100 zeta potential analyser based on the laser doppler electrophoresis. Measurements were obtained in the range of -400 to +400mV.

4.8) Effect of Temperature

The effect of incubation temperature (30 °C, 40 °C and 60 °C) on the biogenic AgNPs was carried out by cell filtrate with 8 mM of AgNO₃ and kept in water bath for different temperature as mentioned above. Finally colour changes observed from pale white to brownish color and AgNPs were determined by UV-Vis spectral analysis.

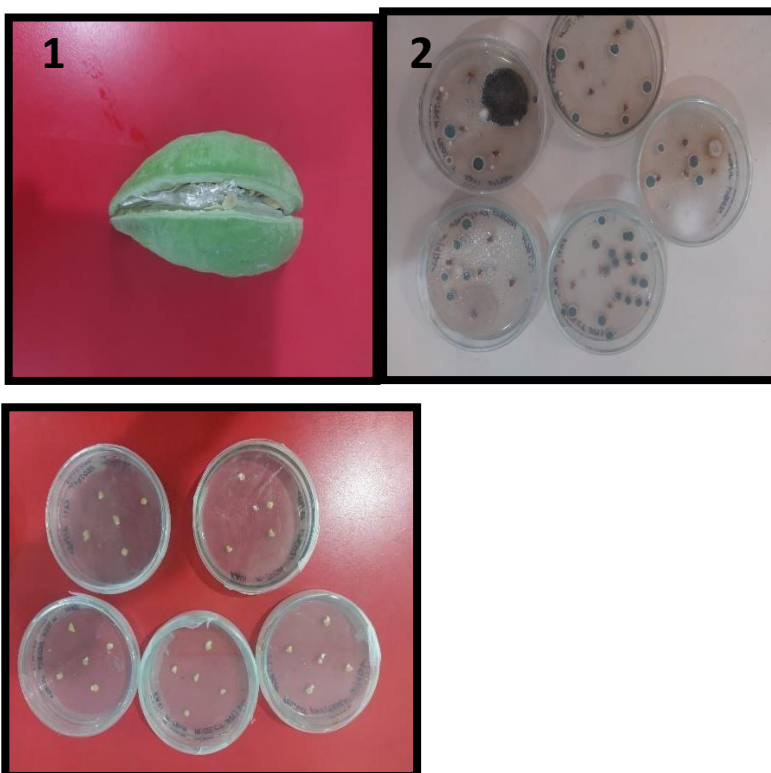
4.9) Effect of pH

The effect of incubation pH was carried out in acidic, neutral and alkaline condition (3, 7, 10) on the biogenic AgNPs was carried out by cell filtrate with 8mM of AgNO₃ and the pH is adjusted with 1N of NaOH and 0.1 N OF HCl for the above-mentioned pH. After adjustment of pH colour changes were observed from pale white to brown color and AgNPs were determined by UV-Vis spectral analysis.

CHAPTER - 5 RESULT AND DISSCUSION

5.1) Isolation and Identification of fungal strain

The *Calotropis gigantea* seed were placed in potato dextrose agar and kept incubation for 7days at room temperature (figure 5.1). Further subculturing was performed to obtain a pure culture. The Macroscopic and microscopic characteristics were identified in pure culture. After 7 days of incubation on Potato Dextrose Agar, they reveal the appearance of white color (Figure 5.2). Using the Cotton Blue lactophenol strain, the fungal spores were identified using an Electron Microscope with a 40X lens. (Figure5.3) They showed that fungal strains have smooth, unbranched, long conidiophores, Metula, Phialides, Ramuli, and Sterigmata.



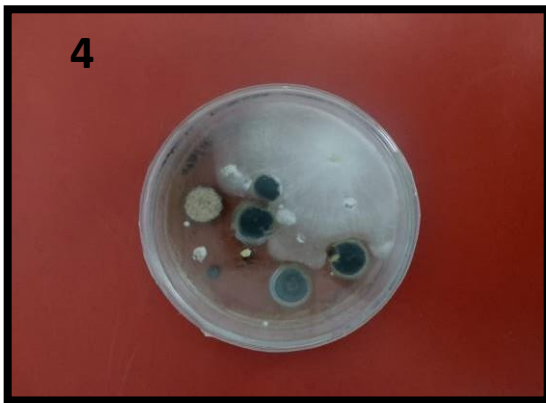


Figure 5.1) growth of an Endophytic fungus in Potato dextrose agar for 7 days of incubation period.



Figure 5.2: *Pichia kudriavzeii* strain F2-T427-10 Macroscopic characteristic of colonies of fungal strain

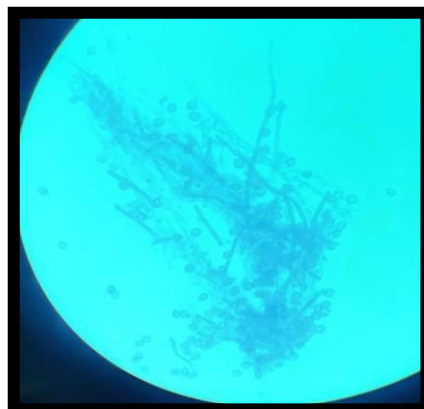


Figure 5.3: Microscopic analysis of *Pichia kudriavzeii* strains F2-T427-10 was determined by bright field microscopy at 40 X magnification

5.2) MOLECULAR CHARACTERIZATION

16sRNA forward sequence

>AGTTGAGCGCTGAAGGTTGTGTACTTGTACCAACTGGATGAGCAGCGAACG
GGTGAGTAA
CGCGTGGGGAATCTGCCTTTGAGCGGGGGACAACATTTGGAAACGAATGCT
AATACCGCA
TAAAAACTTTAAACACAAGTTTTAAGTTTGAAAGATGCAATTGCATCACTCAAA
GATGAT
CCCGCGTTGTATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGATGATA
CATAGCCG
ACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAACCTCCT
ACGGGAGGC
AGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGC
GTGAGTGAA
GAAGGTTTTTCGGATCGTAAACTCTGTTGGTAGAGAAGAACGTTGGTGAGAG
TGGAAGC
TCATCAAGTGACGGTAACTACCCAGAAAGGGACGGCTAACTACGTGCCAGCA
GCCGCGGT
AATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCA
GGTGGTTT
ATTAAGTCTGGTGTAAGGCAGTGGCTCAACCATTGTATGCATTGGAAACTG
GTAGACT
TGAGTGCAGGAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAG
ATATATGGA
GGAACACCGGTGGCGAAAGCGGCTCTCTGGCCTCTAACTGACACTGAGGCT
CGAAAGCGT
GGGGGGAGCAAACAGGGATTAGATACCCTGGGTAGTCCCCCCCCCTGTAAAC
GATGAGTGC
TAGGATGTAGGGAGGTAATAAGTTCTCCTGTATCGCAGCAACGCAATAAGCA
CTCTGCCT GGGGAAGTGAGGGCCGCAACCGTTGATCTTTTCG

16sRNA reverse sequence

>TCGGAGCGGGTGACTCTATAAGGAGCGGCGTCCTTGCATGTTAGGCAACCT
ACTTCGGGA

TATCCCAACTCCCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGT
ATTCACCGC
GGCGTGCTGATCCGCGATTACTAGCGATTCCGACTTCATGTAGGCGAGTTGC
AGCCTACA
ATCCGAAGTGAAGAATGGTTTTAAGAGATTAGCTAAACATCACTGTCTCGCGAC
TCGTTGT
ACCATCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCGATGATGATTT
GACGTCAT
CCCCACCTTCCTCCGGTTTATCACCGGCAGTCTCGTTAGAGTGCCCAACTTA
ATGATGGC
AACTAACAATAGGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGA
CACGAGCT
GACGACAACCATGCACCACCTGTATCCCGTGTCCCGAAGGAACTTCCTATCT
CTAGGAAT
AGCACGAGTATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAAC
CACATGC
TCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGT
CGTACTCC
CCAGGCGGAGTGCTTATTGCGTTAGCTGCGATACAGAGAACTTATAGCTCCC
TACATCTA
GCACTCATCGTTTACGGCGTGGACGACCAGGGTATCTAATCCTGTTTGCTCC
CCACGCTT
CTAGCCTCAGTGTCAGTTACTGGCCAGAGAGCCGCTTTCGCCACCGGTGTTC
CTCCCTAT
ATCTTCTCAATTCATCGCTCACATGGAAGTCCCCTCTCCTCTCCCGCACTCAA
GTCTACC
AATTTCCAATGCAAACAGGGGTTGAGCCACTGCCTTTTACACCAAAACCTAAA
AAACCAC
CTGCGCTCGCTTTACGTCCCACCAATGCGGACAACGGTGGGGGACCTGCCT
ATTACCGCG
GGCGCCGGGCGCGGAGTTAGCCGTCCCCTTCCTGGTAGTGTGCTGTTCCCC
TGAAGGAGA
TGACGACTCTCACCAACATAACCGCTTCTCCACCCGAGTAGTACATAGTCGAG
AAAACGTT

CATACACTCTACCCGCAGTGCACCGTTTCTGGAGTTCAGTCCATTGCGGAAG
AATGCTCG
TACAAACTAACCCCCCCCCCGCCACTTAGTGAAGGATGGAGTGGGGTGGCG
GATGTGTTC TATA

5.3) The consensus sequence was aligned using BLAST tool

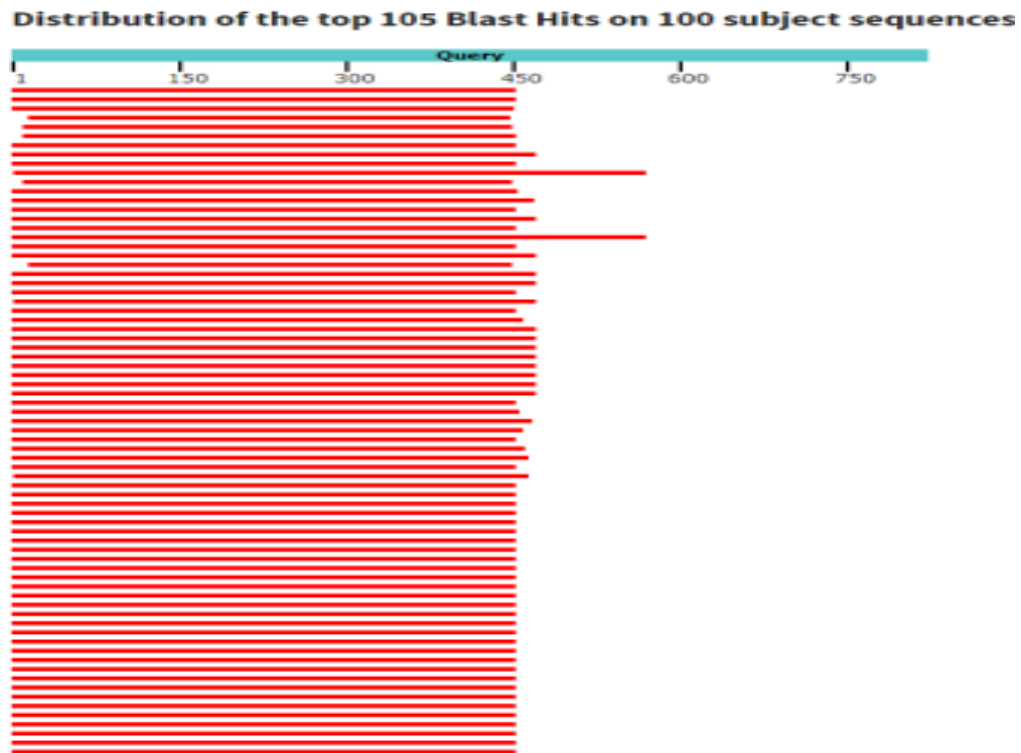


Figure 5.3: The consensus sequence was aligned using BLAST tool

5.4) sequencing

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☒ Pichia kudriavzevii strain F2-T427-10 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, complete sequence	Pichia kudriavzevii	540	540	54%	2e-148	88.33%	481	MK329988.1
☒ Pichia kudriavzevii isolate CFM-1400.47 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, complete sequence	Pichia kudriavzevii	540	540	54%	2e-148	88.30%	473	MZ677198.1
☒ Pichia kudriavzevii strain SLDY-022 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, complete sequence	Pichia kudriavzevii	527	527	54%	1e-144	87.86%	475	MH1748627.1
☒ Pichia kudriavzevii isolate NCL 45 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, complete sequence	Pichia kudriavzevii	523	523	52%	2e-143	88.48%	440	FJ231424.1
☒ Pichia kudriavzevii isolate NCL 19 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, complete sequence	Pichia kudriavzevii	523	523	53%	2e-143	88.18%	440	FJ231421.1
☒ Issatchenkia orientalis isolate G7 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, complete sequence	Pichia kudriavzevii	518	518	53%	7e-142	87.81%	516	EU315753.1
☒ Pichia kudriavzevii strain AUMC 13420 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, complete sequence	Pichia kudriavzevii	512	512	54%	3e-140	87.22%	466	MH534936.1
☒ Pichia kudriavzevii isolate H-237 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, complete sequence	Pichia kudriavzevii	508	508	57%	4e-139	86.29%	502	MG015957.1
☒ Pichia kudriavzevii 281 Ca kr genes for 18S ribosomal RNA, internal transcribed spacer 1, 5.8S ribosomal RNA, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, complete sequence	Pichia kudriavzevii	501	501	54%	8e-137	86.78%	664	LC567131.1
☒ Pichia kudriavzevii isolate LV17 ITS1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, complete sequence	Pichia kudriavzevii	490	490	60%	2e-133	82.41%	1002	ON262429.1
☒ Pichia kudriavzevii isolate NCL 11 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, complete sequence	Pichia kudriavzevii	490	490	53%	2e-133	86.85%	440	FJ231418.1
☒ Pichia kudriavzevii strain UMY534 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, complete sequence	Pichia kudriavzevii	486	486	55%	2e-132	86.03%	468	KP171557.1
☒ Pichia kudriavzevii 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, complete sequence	Pichia kudriavzevii	483	483	56%	3e-131	85.26%	497	JQ083432.1
☒ Pichia kudriavzevii isolate CC-5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, complete sequence	Pichia kudriavzevii	481	481	54%	1e-130	85.93%	501	MG015961.1
☒ Pichia kudriavzevii internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, complete sequence	Pichia kudriavzevii	481	740	57%	1e-130	85.20%	1370	KP998095.1
☒ Pichia kudriavzevii isolate N68 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, complete sequence	Pichia kudriavzevii	479	479	54%	4e-130	85.90%	515	MK215802.1
☒ Pichia sp. QAU003 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, complete sequence	Pichia sp. QAU003	479	479	69%	4e-130	82.02%	633	KU870986.1
☒ Issatchenkia orientalis isolate G18 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, complete sequence	Pichia kudriavzevii	479	479	54%	4e-130	85.90%	517	EU315756.1
☒ Pichia sp. strain PPS isolate Y117 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, complete sequence	Pichia sp.	477	477	57%	1e-129	85.05%	476	MG752902.1
☒ Pichia kudriavzevii isolate NCL 123 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	Pichia kudriavzevii	477	477	52%	1e-129	86.47%	437	FJ231419.1

Figure 5.4: Based on the maximum identity score first ten sequences were selected for further analysis.

5.5) Phylogenetic tree

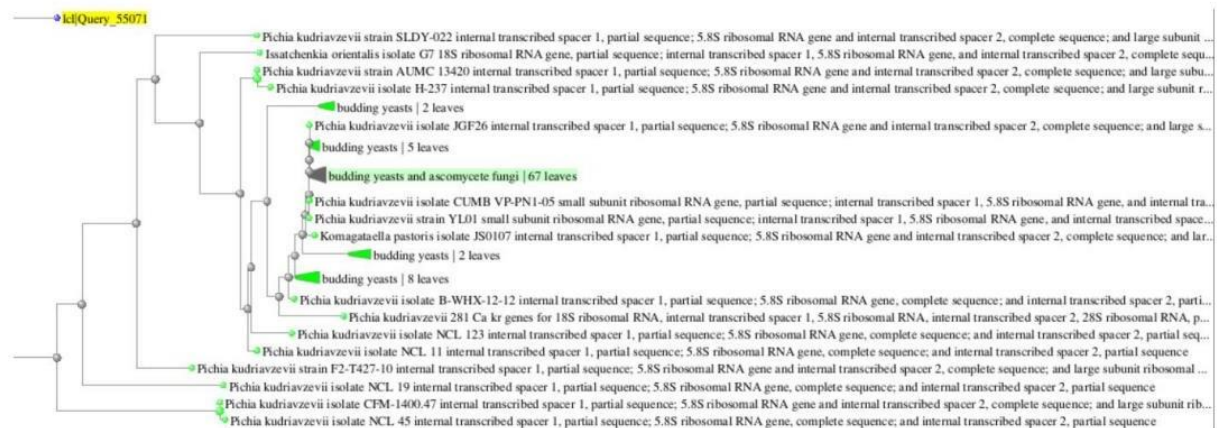


Figure 5.5: Using NCBI software, the sequence distance was calculated and the phylogenetic tree was constructed

Based on sequence homology and phylogenetic analysis, the Microbial culture was found to be *Pichia kudriavzevii* strain F2-T427-10, complete genome with GenBank Accession Number MK329988.1

5.6) Qualitative Analysis

The result of qualitative phytochemical analysis inferred that the fungal extract shows the positive result for presents of Carbohydrates, Phenol and Tannin. But Steroids and terpenoids shows the absent in the fungal extract (Table 5.6).

Table 5.6) Shows the quality result for *Pichia kudriavzevii*. ++ highly present; -- Absent; + present.

Test	Result
Phenol	++
Steroids	--
Terpenoids	--
Tannin	+
Carbohydrates	++

5.7) Biosynthesis and characterization of Silver nanoparticle

After 3 days of incubation in dark condition the diverse color change was observed in the aqueous silver nitrate solution from pale white to reddish brown, this indicated the rapid synthesis of silver nanoparticles in the reduction of AgNO₃ ions to Ag⁺ ions. (figure 5.7)



Figure 5.7: A) *Pichia kudriavzeii* extract and AgNo3. B) synthesized silver nanoparticles.

UV-Visible spectral is an instrument that determine the accurate characterization metal NPs. From the above-mentioned different concentration 6mM,8mM and 10Mm shows the highest color differences and these samples were given for uv vis spectrophotometry analysis. From the figure 5.7.1 8mM shows the UV-Vis absorption peaks at around 456 nm. The λ_{max} for the as-prepared biogenic synthesis of Ag nanoparticles exhibits the absorbance at 456 nm, also morphologically indicated by brownish colour formation of the solution from precursor state blackish colour state. This indicates the maximum surface plasmonic resonance at the particular wavelength. Further flattening of the peaks in the UV-Vis range (200-700 nm), indicating agglomeration of the precursor molecules in the solution. There is a gradual increase in the steepness and bumping at the 456 nm wavelength (More pronounced for 6mM and 10mM), indicating good formation of Ag nanoparticles after repeated dilution and reaction conditions of the bottom-up synthesis of Ag nanoparticles from biogenic sources.

It is also understood from the figure that, owing to the different reactants involving the biogenic precursors like some functional groups, keto groups present in the biogenic (fungus) source, it is contributing in the reductional process of the Ag^{2+} to Ag^0 to form Ag nanoparticles in the aq. solution.

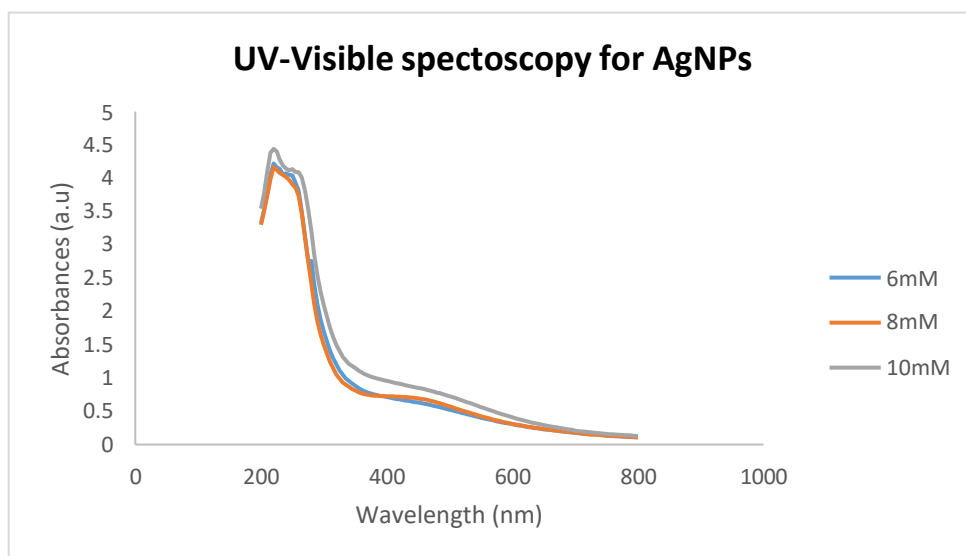


Figure 5.7.1: A) Ultraviolet-visible spectrophotometer for 6,8 and 10mM AgNPs.

By using the vibration and frequency the FTIR spectroscopy shows the atomic level of compound with specific chemical bonds that represent the molecular fingerprint for sample. (figure 5.7.2), shows the FTIR spectral bands of the fungal extract and AgNPs synthesized using *Pichia kudriavzeii* extract. Sharp transmittances peak was observed at 1149 and 993 cm^{-1} in freshly synthesized AgNPs. (Table 5.7.2) contain FTIR spectra of fungal extract obtained in wavelength range from 3500-650 cm^{-1} and primary functional group found in fungal extract.

A broad spectrum found between 3500-2500 cm^{-1} due to the OH, -CH stretching vibration of alkene and carboxylic acid functional group, which involves hydrogen bonding as the broad band of OH & -CH group in this region indicates presences of hydrogen bonding in the molecules (figure 5.7.2). A set of strong peaks observed in 1149 and 993 cm^{-1} , which generally represent CO stretching and C=C bending that associate the presences of aromatic amine and alkene. From the (figure 5.7.2) it clearly observed that functional group associate with fungal extract have imprinted their functional group on synthesized AgNPs. Thus, FTIR experiment, shows the bioactive compounds identified as alkene, nitro compound(protein) and carboxylic acid present in *Pichia kudriavzeii* play a vital role in covering agent and stabilizing AgNPs.

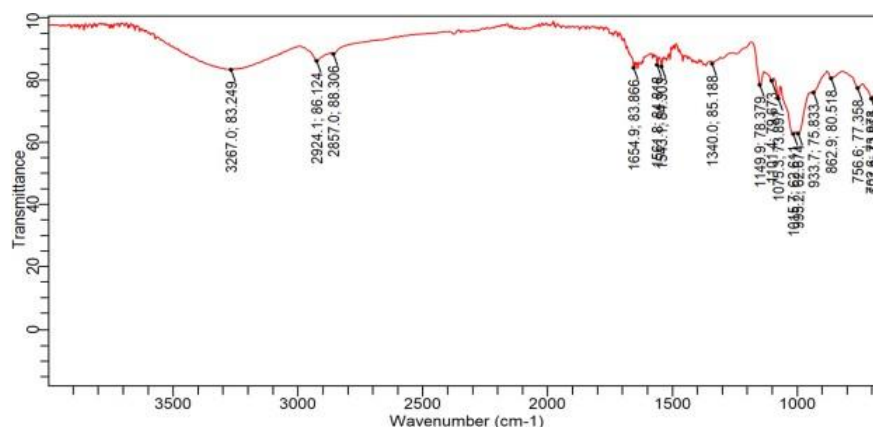


Figure 5.7.2) ATR-FTIR spectroscopy analysis if AgNPs from aqueous extract of *Pichia kudriavzeii*.

Table 5.7.2) FTIR Tabulation AgNPs from aqueous extract of *Pichia kudriavzeii*

Wavelength ranges	Transmittances	Functional group
3500-3000	3267	O-H stretching
3000-2500	2924	C-H stretching
	2857	C-H stretching
1670-1600	1654	C=C stretching
	1561	C=C stretching
1600-1300	1543	N-O stretching
1400-1000	1340	O-H bending
	1149	C-O stretching
	1101	C-O stretching
	1075	C-O stretching
	1015	-
1000-650	993	C=C bending
	862	C=C bending
	756	C=C bending
	702	C=C bending
	669	C-Br stretching

5.7.3) Particle Size Analyser

The accurate size and charges play a major role in the structural and functional properties of any particle used in biological application. In current investigation, the size and surface charge of AgNPs were calculated based on the laser diffraction using an Aqueous solution of AgNPs were Z- Average (nm): 33.84, Pdl: 0.487, Intercept: 0.871.(figure 5.7.3)

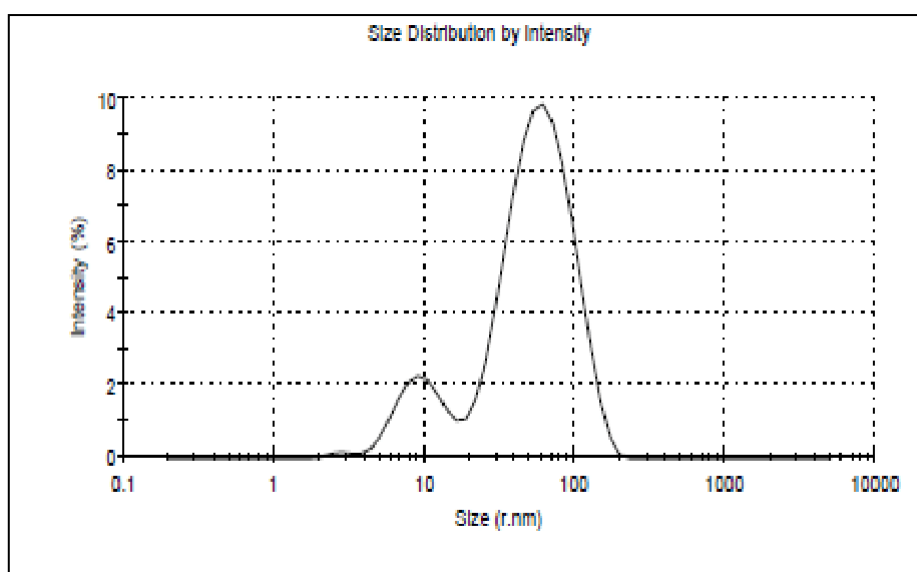


Figure 5.7.3) Graphical representation of Particle Size Analyser from aqueous extract of *Pichia kudriavzeii*

From this investigation, it is proven that particle size 0.487 shows to be good formation of AgNPs formation from our aqueous solution. (Table 5.7.3)

Table 5.7.3) Particle size analyser for AgNPs formation aqueous extract of *Pichia kudriavzevii*

Peak No.	Size(nm)	% Intensity	Width(nm)
1	63.71	85.3	30.75
2	9.698	14.2	3.189
3	2.987	0.5	0.5155

5.7.4) Zeta Potential

Zeta potential analysis is used to determine the surface charges and stability of the colloidal suspension of nanoparticle, is important in determining the interaction of cell membranes. The zeta potential of the biosynthesized AgNPs was found as sharp peak at -29.8mV (Figure5.7.4) (Table 5.7.4).

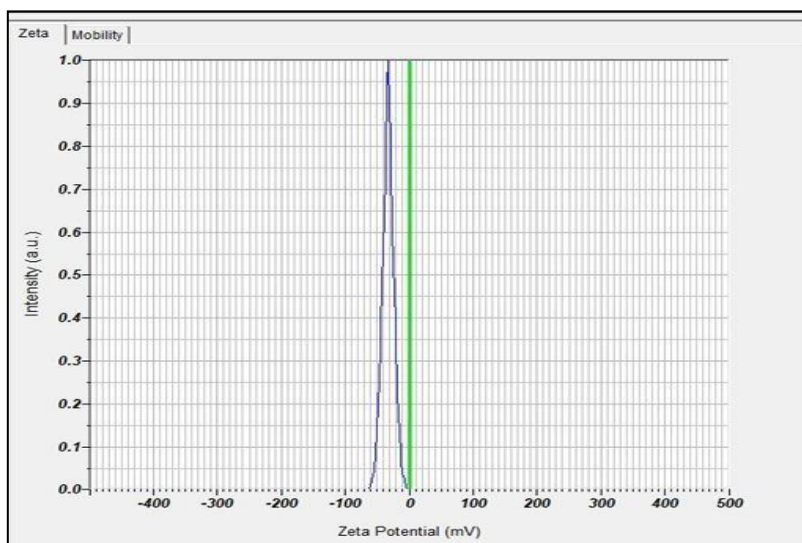


Figure 5.7.4) Graphical representation Of Zeta Potential analyser for AgNPs formation aqueous extract of *Pichia kudriavzevii*

Table 5.7.4) Tabulation of Zeta Potential analyser for AgNPs formation aqueous extract of *Pichia kudriavzevii*

Peak No.	Zeta Potential	Electrophoretic Mobility
1	-29.8 Mv	-0.000255 cm ² /Vs
2	--mV	--cm ² /Vs
3	--mV	--cm ² /Vs

Its suggested that there is strong interaction between the anionic and cationic in the dispersed medium. The negative value Confirms the repulsion among the particle and proves that they are more stable.

5.8) HRSTEM

5.9) Effect of Temperature on AgNPs formation

The diverse color change was observed in aqueous AgNO₃ solution was turns to Ag⁺ ions after heating process and confirms the synthesis of AgNPs (figure 5.9).

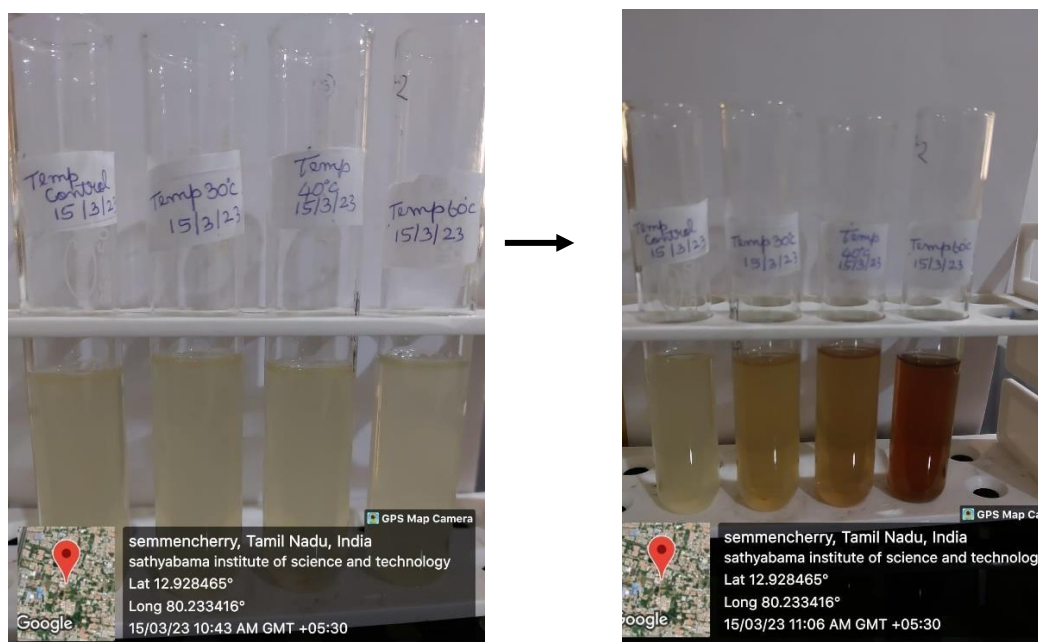
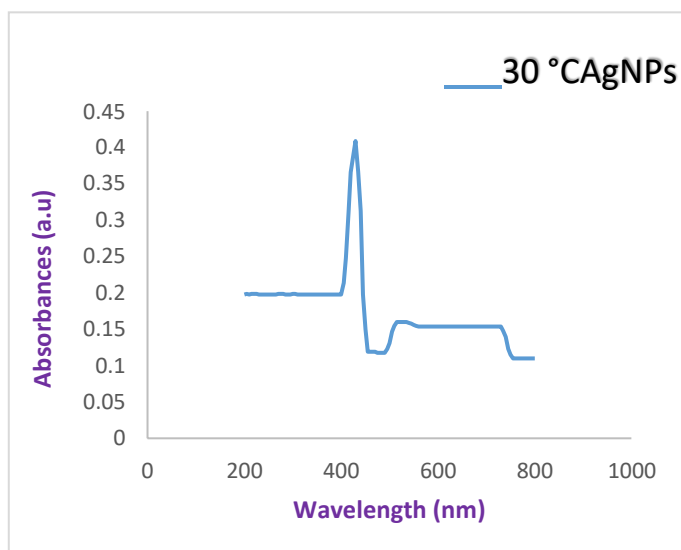


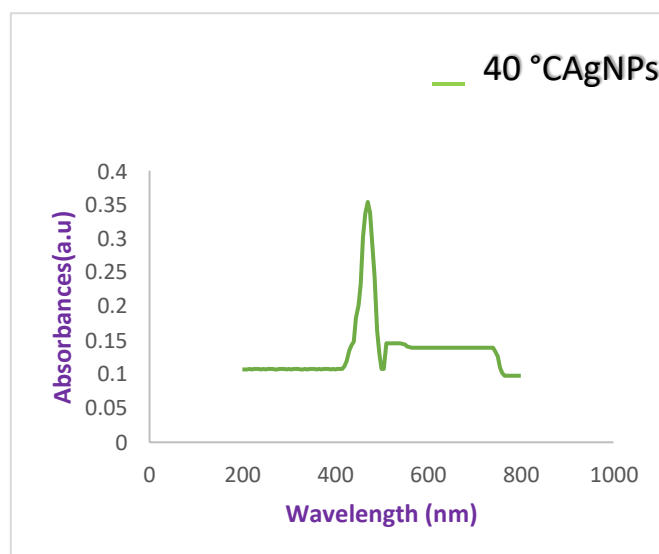
Figure 5.9: **Different temperature on AgNPs formation**

Figure 5.9.1 (A-C) Shows the effect of temperature on the AgNPs by pichia kudriavzeii. It is noticeable that the optimum temperature for the production of biogenic nanoparticles is between 30 °C - 40 °C. However, the thin peak is developed in higher wavelength region (475 nm at 60 °C), which indicates the formation of higher nanoparticles, whereas at lower temperature blunt peaks observed with smaller wavelength region of (420nm at 40 °C) and (415 nm at 30 °C) indicates the formation smaller NPs. These results show that if temperature

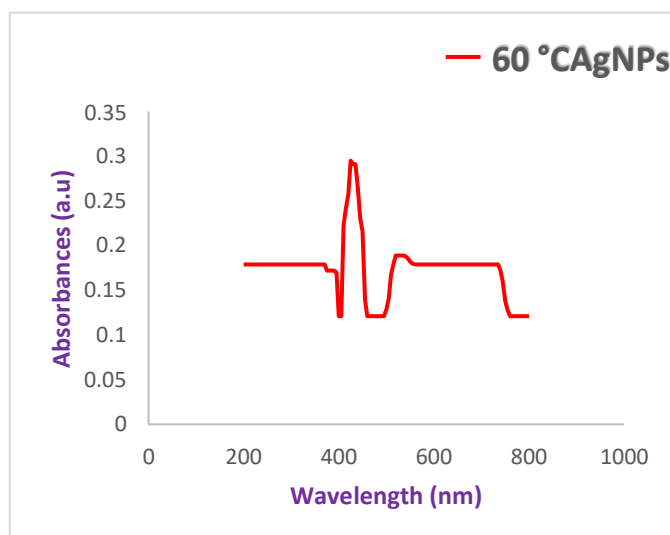
increases the reactant rate rapidly produce silver nuclei and prevent the reduction of secondary metabolites on surface of AgNPs.



A) 30 °C AgNPs



B) 40 °C AgNPs

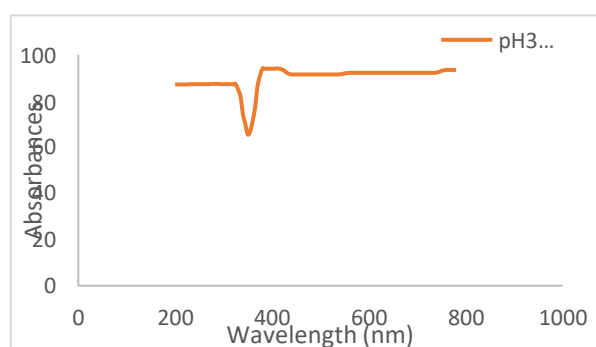


c)60 °C AgNPs

Figure 5.9.1 (A-C): shows the UV- Vis Spectral for the effect of temperature on AgNPs formation by *Pichia kudriavzeii*.

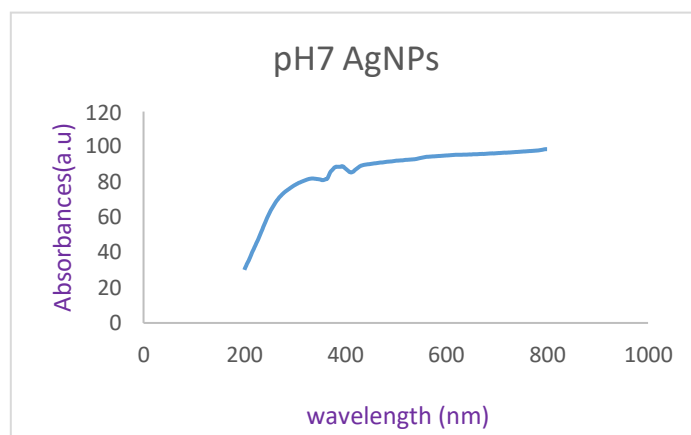
5.10) Effect of pH on AgNPs formation

The medium supplement with AgNO₃ solution changed from white to brown color based on different pH mixtures. UV-Vis spectral reading was recorded after the reaction at different pH in acidic, neutral and alkaline as pH 3, pH 7 and pH10(Figure 5.10). The maximum AgNPs bioproduction was at pH10, of which narrow peak developed, which indicates the formation of highly dispersed bio nanoparticles. At acidic condition, the polyssacrides and protein were reduced its function. Therefore, in present study, pH 10 was recorded for biogenic synthesis of AgNPs.

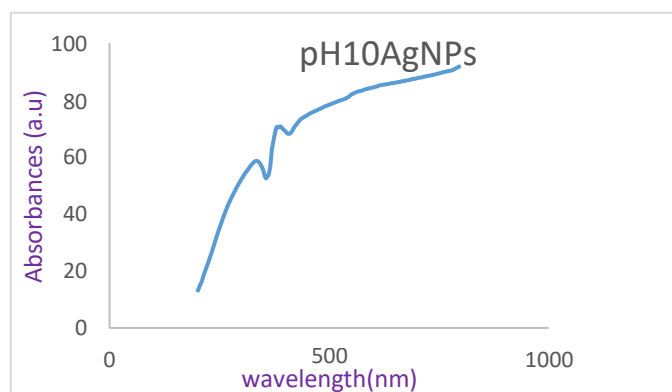


A)

pH3



B) pH7



c) pH10

Figure 5.10A-C) Shows the effect of pH on AgNPs formation by *Pichia kudravazeii* strain.

5.11) Antibacterial Activity for AgNPs Produced by *Pichia kudriavzevii*

Chapter 6-Conclusion

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