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Bacterial Growth Inhibition Potential of Nanoparticles Mycosynthesized from Soil *Penicillium Species*

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Abstract: ***Nanotechnology is one of the promising technologies for various science applications in 21st century. Nanoparticles synthesized through mycobiosynthesis are successfully used in wide area of science such as drug delivery agents in cancer biology, food processing, medicine, agriculture, biomedical, etc. The diverse applications of silver nanoparticles (AgNPs) instigated us to biosynthesis them from soil fungus, characterize and explore them for biological applications in the current investigation. Mycobiosynthesis of Penicillium species isolated from the soil was confirmed by filtrate colour from colourless to pink colour. Further production of AgNPs in fungal filtrate was witnessed by their distinct absorption peak in UV-visible spectrophotometer at 440nm and four distinct powder X-ray diffraction (XRD) peaks at 2θ value of 38.4°, 44.5°, 64.90°, and 77.4°. Fourier-transform infrared spectroscopy (FTIR) analysis of AgNPs revealed various functional groups possibly associated with biosynthesis of AgNPs. High resolution transmission electron microscope (HRTEM) analysis indicated presence of various sizes of AgNPs ranging from 10 to 90nm and 40-50nm being dominant sizes of nanoparticles. AgNPs evaluated for biological activity showed substantial antibacterial activity against E. coli and S. aureus. Nanoparticles indicated significant inhibition of Escherichia coli growth at exponential to death phase of bacterial growth curve. In addition to antibacterial potential, AgNPs also revealed significant anti-diabetic activity with IC_{50} of 182µg when screened from 12.5 µg to 400 µg concentration. It is inferred that bio-fabrication of AgNPs from Penicillium sp with potential biological applications was successfully achieved using an environmental friendly technique.***

Keywords: Soil fungi, Penicillium species, Mycobiosynthesis, Silver nanoparticles, Antibacterial activity, Bacterial growth curve, anti-diabetic activity.

I. INTRODUCTION

Nanotechnology revolutionized various fields of science finding its crucial applications in medicine, agriculture, food industry, pharmaceuticals, and other arenas. Today nanoparticles particularly silver nanoparticles (AgNPs) are extensively used in medicine for disease diagnosis, biomedical implants, cancer imaging, tissue engineering, drug delivery agents, etc [Abid Haleem *et al*., 2023]. Although various strategies of nanoparticle synthesis have been proposed after intensive research, based on possibility of nanostructure synthesis, cost, speed, and degree of quality they are broadly classified into two approaches: top-down and bottom-up approaches. In top-down approach a bulk material in converted into nano-sized particles (nanoparticles) using various recent technologies such as lithography and precision engineering. However, building of nano-sized molecules of 1-100nm by atom (or molecule) by atom (or molecule) from bottom using chemical and physical methods is called bottom-up approach [Iqbal *et al.*, 2012]. Nanoparticles are synthesized through chemical, physical and biological methods. Since chemical and physical methods possess several disadvantages biological methods have become more popular due to its low cost, eco-friendliness, non-toxic, and high stability. This eco-friendly synthesis of nanoparticles are mediated though fungi, plants, bacteria and algae [Karunakaran *et al*., 2023].

Currently nanotechnology is one of the dynamic fields of research whereby soil fungi found immense applications in mycobiosynthesis of AgNPs. Fungi are suitable reducing agents in biofrabracation of AgNPs due several reasons as they synthesis several proteins which might anticipate in nanoparticles production. Moreover, fungal species are easy to handle, they are ecofriendly as they produce less toxic substances, and easy to grow in laboratory. The biological activities of fungal-mediated AgNPs are more potent as bioactive compounds synthesized by fungi are coated onto nanoparticles [Guilger-Casagrande and de Lima, 2019].

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Recently biosynthesis of silver nanoparticles (AgNPs) getting more attention of scientists due to their immense applications in various arena of science. This is because of an excellent property of silver as a broad spectrum antimicrobial agent, silver has an immense history of being an excellent antiseptic and silver also used in daily life from our ancestor time, and hence it is safe. Today, many fungal species have been reported for biosynthesis of AgNPs which includes, *Trichoderma harzianum, Fusarium oxysporum, Colleotrichum sp. ALF2-6, Aspergillus oryzae, Rhizopus stolonifer, Aspergillus fumigatus, Isaria fumosorosea, Guignardia mangifera, Duddingtonia flagans, Penicilliumpurpurogenum, Epicoccum nigrum, Arthroderma fulvum, Sclerotinia sclerotiorum, Rhizoctonia solani, etc*. Fungi synthesize various extra cellular proteins in large quantities, hence they used as an excellent stabilising and reducing agents in mycobiosynthesis of AgNPs. Additionally these extra cellular proteins also act as coating agents for AgNPs to improve their stability and biological activity [Guilger-Casagrande *et al.,* 2019]. Although studied of antibacterial activity of AgNPs are reported widely however, studies associated with precise action on particular location of these the cells are rarely found on the literature.

II. MATERIALS

Sabouraud dextrose agar, Milli-Q water, Sodium chloride (NaCl), Lactophenol cotton blue stain, glass slides. Potassium dihydrogen phosphate (KH_2PO_4), Dipotassium hydrogen phosphate (K_2HPO_4), Magnesium sulphate ($MgSO_4.7H_2O$), Ammonium sulphate $(NH_4)_2SO_4$, Yeast extract, Glucose were purchased from Hi Media company, Bangalore. Alpha amylase from *Aspergillus oryzae*, NaH2PO4, Na2HPO4, Starch, DNS Reagent were procured from Sigma Aldrich, Bangalore.

III. METHODS

A. Isolation and identification of Penicillium species from soil.

Isolation of *Penicillium* species from soil was carried out by plating dilution technique in which 1g of soil sample was dissolved in 10ml of saline to get the dilution of 10^0. From 10^0 dilutions 1ml of soil suspension was transferred to 9ml of saline taken in next tube to get dilution 10^1 from which again 1ml is taken and transferred to next tube containing 9ml of saline to yield 10^2 dilution. Followed by the serial dilution, 1ml from each dilution *i.e.,* 10^0 to 10^2 was placed onto Petri dish to which 20 ml of sterile molten (SDA) containing 0.1% streptomycin antibiotic was added and plates were allowed to solidify. All SDA plates were incubated at 30°C for seven days. Post incubation, plates were observed for colony formation and results were recorded [Waksman, 1927]. Further fungi were stained using lactophenol cotton blue stain and observed under microscope. Based on the colony morphology, microscopic observation such as mycelia structure, type of spore fungus was identified at genus level with the help of standard manual [Gilman, 1966]. All fungal species isolated were sub-cultured in SDA slants and *Penicillium* sp was used for production of AgNPs.

B. Mycobiosynthesis of AgNPs from soil Penicillium species

The pure culture of *Penicillium* sp. isolated from soil was inoculated in 500ml of sterile AgNPs production medium with following composition (g/l): Glucose – 10.0 g, Yeast extract – 0.6 g, (NH4)2SO4 – 1.0 g, K2HPO4 – 2.0 g, KH2PO4 – 7.0 g, and MgSO4· 7H2O – 0.1 g.

The medium was incubated for 7 days at 30°C to achieve about 20 g fungal mats. After incubation period, the medium was decanted slowly and discarded. The mat of *Penicillium* sp. was washed twice with Milli-Q-water and filtrate was discorded. Resultant fungal mat was re-inoculated in 250ml of Milli-Q-water and incubated for 3 days at 30°C. After three days, the fungal mat was removed and filtrate was collected in separate conical flask which was used for bio-fabrication of AgNPs. In separate sterile container, 200ml of fungal filtrate was taken and 0.050 g of silver nitrate was added to get 1.5mM solution. Immediately filtrate was scanned from 250 to 500nm in UV-VIS spectrophotometer and results were recorded. The test conical flask (filtrate plus AgNPs), only fungal filtrate, and only 1.5mM $AgNO_3$ solutions were incubated in dark room. On every 2 hours AgNPs production was monitored by observing the change of colour in all three containers.

IV. ANALYTICAL CHARACTERIZATION OF AGNPS OF *PENICILLIUM* SPECIES

A. Spectrophotometric analysis

UV-VIS spectrophotometric analysis of AgNPs production was performed every 2hrs by scanning all three solutions from 250 to 500nm. The peak at which highest light absorption noticed was recorded and production of AgNPs was confirmed. The scanning of solutions was continued until maximum absorption peak noticed.

B. AgNPs analysis by X-ray Powder diffractometry (XRD)

After confirmation of AgNPs production in the container the fungal filtrate containing AgNPs was centrifuged at 3000rpm for 15 minutes and nanoparticle were recovered. AgNPs were analyzed by XRD generator (BRUKER, D8 Advance, Germany) by scanning at 20^o - 80^o range with 2θ angle at X-axis. After scanning, the XRD results were matched with published data of Joint Committee on Powder Diffraction Standard (JCPDS) *i.e.*, JCPDS File No. 03-09 for AgNPs. to confirm AgNPs production.

C. FTIR analysis of AgNPs

AgNPs of *Penicillium* sp. recovered from filtrate were mixed with pottassium bromide and made into round pellet by pressing the mixture in a presser. The round pellet was scanned in Fourier transform infrared (FTIR) Spectroscopy (Bruker, Germany) from 4000 cm^{-1} to 500 cm^{-1}. After the analysis the FTIR peaks were analysed for the detection of functional groups possibly anticipated in the mycobiosynthesis of AgNPs.

D. AgNPs analysis by High Resolution Scanning Electron Microscopy

The colloidal solution of AgNPs of *Penicillium* sp was centrifuged for 30 minutes at 2500 rpm and supernatant was discarded. Pellet thus obtained was washed twice with deionized water. The pellet containing AgNPs was made smear on the glass slide and air dried. The cover slip was directly observed through HRSEM. AgNPs were coated with coating material and images were taken using HRSEM.

V. BIOLOGICAL APPLICATIONS OF *PENICILLIUM* AGNPS

A. Antibacterial activity of AgNPs of Penicillium sp.

Antimicrobial potential of AgNPs against human pathogenic bacterial species were evaluated [Valgas *et al.*, 2007]. Before initiation of antimicrobial activity of AgNPs, *E. coli* and *S. aureus* were sub-cultured on nutrient agar and incubated at 37ºC for 24 hours. Both bacterial inocula were prepared by dissolving about 4-5 colonies of 24hours old culture in sterile saline and adjusting the suspension to 10^6cells/ml by measuring the absorbance at 600nm. On Mueller Hinton Agar plates an overnight bacterial culture of *E. coli* and *S. aureus* corresponded to 10^6 cells/ml was uniformly spread separately. Using sterile borer, about 6mm wells were made on MHA plates in which 100µl of AgNPs solution was loaded and plates were allowed to settle for some time. Plates were incubated at 37ºC for 24-48 hours in a bacterial incubator. Followed by the incubation time, zone of inhibition by AgNPs around the wells against *E. coli* and *S. aureus* were measured and results were recorded.

B. Study of effect of nanoparticles on the growth curve of bacteria

The antibacterial activity of AgNPs and their effect on growth curve of *E. coli* was investigated. A day prior to study, *E. coli* was sub-cultured on nutrient agar and plates were incubated at 37ºC for 24hours. Next day, in a sterile conical flask containing nutrient broth a loopful of *E. coli* inoculum was inoculated and 1000µg of AgNPs was added. Immediately, zero hour absorbance of the solution was measured at 600nm and flask was incubated at 37ºC and absorbance was recorded every 1hour till 24hours. Flask containing nutrient broth and fungal filtrate was inoculated with *E. coli* and maintained them as growth and filtrate control respectively.

C. Alpha-amylase activity of AgNPs

Anti-diabetic potential of AgNPs were measured using standard protocol [Manikandan *et al.*, 2016]. In a sterile vial AgNPs suspension was prepared at 8mg/ml concentration by dissolving 8mg of AgNPs in 1ml of 0.1% DMSO. From this stock, 100µl containing 800µg of AgNPs was taken in 96-well microtitire plate. Then it was serially diluted from 1^{st} to 6^{th} well by transferring 50µl from first well to next well containing 50µl of 0.02M sodium phosphate buffer of pH 6.9 to get the concentration of 400µg/ml, 200µg/ml, 100µg/ml, 50µg/ml, 25µg/ml and 12.5µg/ml. To all the diluted wells 50µl of 0.5mg/ml concentration of alpha-amylase prepared in 0.02M sodium phosphate buffer of pH 6.9 was added. Microtitre plate was incubated at room temperature for 30minutes. Followed by the incubation 50µl of freshly prepared 1% starch was added and incubated at room temperature for 10minutes. Blank was prepared similar to test except alpha-amylase enzyme and positive control was also prepared by replacing AgNPs with acarbose. After 10 minutes incubation, 100 µl of freshly prepared DNS solution was added to all wells and incubated in boiling water bath for 5 minutes. The colour changed from bright yellow to bright orange was read the absorbance at 540nm in microplate reader. Optical density (OD) of blank was subtracted in the OD of the test sample and the percentage of inhibition (I%) of alpha-amylase was calculated using below formula.

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I%=(Ac-As)/Ac) x 100

Where,

As - is the average absorbance of the Sample

Ac - is the average absorbance of the Control

The IC_{50} value was determined by using linear regression equation i.e. Y =*mx*+C. Here, Y = 50, m and C values were derived from the graph.

VI. RESULTS AND DISCUSSION

In present study, *Penicillium* sp. was isolated from soil and identified using cultural and microscopic techniques. Ecofriendly synthesis of AgNPs was carried out using *Penicillium* sp. and characterized through various analytical techniques. Finally, biological applications of these nanoparticles such as anti-bacterial and anti-diabetic activities of AgNPs were studied.

A. Isolation and identification of Penicillium species from soil sample

Serially diluted soil sample plated from various dilutions in SDA media shown different types of fungal colonies as shown in Figure 1. High populations of colonies were seen in 10^0 compared to remaining dilutions such as 10^1 and 10^2. Fungal colonies in each dilution were counted and expressed as colony forming unit per gram (cfu/g). The results of average number of colonies in one gram of soil are indicated in Table 1. Soil sample was dominated by *Aspergillus* species such as *A. niger*, *A. terreus*, and *Fisarium* sp. However, soil sample was also containing other fungal species namely *Penicillium*, *Rhizopus*, and *Helminthosporium* species.

Table 1. Average number of fungal colonies isolated from one gram of soil

Fungal species	Average cfu/g of soil
Aspergillus niger	45
Aspergillus terreus	16
Penicillium sp.	04
Rhizopus sp.	02
Helminthosperium sp.	05
Fusarium sp.	12

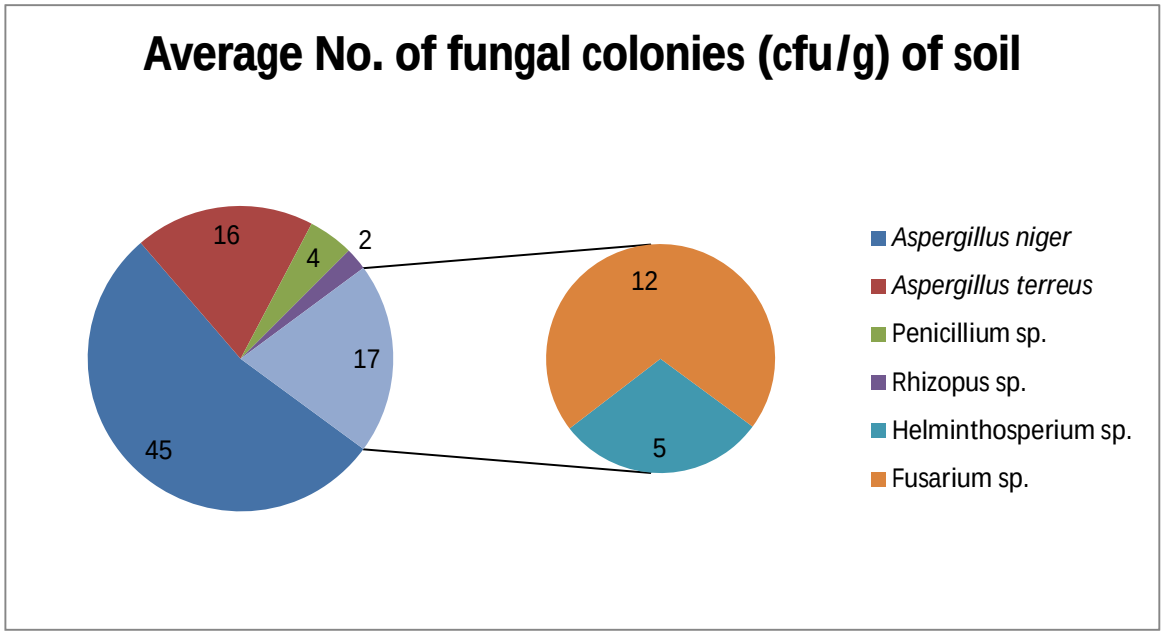

Figure 1. Isolation and identification of soil fungi in different dilutions of soil suspension.

Soil consists of huge population of fungal flora which is an immense resource of various biologically active molecules. Fungi play very role in biogeochemical cycling process and in contrast to plant they can be grown easily in the laboratory. Currently, several novel compounds with antibacterial, anticancer, anti-diabetic, anti-inflammatory, etc., properties have been isolated from fungi which includes quinones, alkaloids, phenols, steroids, flavonoids, terpenoids, etc,. [Zhang et al., 2022]. Aspergillus species versatile fungi commonly found in the soil, grains, decaying vegetation and seeds as saprophytes [Mousavi et al., 2016]. Penicillium species are found ubiquitously in wide range of environments. Currently, about 480 Penicillium species have been reported and approximately 1300 records have been available [Rodríguez-Andrade et al., 2021]. Since, the isolation of first antibiotic Penicillin, Penicillium species is great contributor of bioactive molecules. However, recently Penicillium species are successfully explored in nanotechnology for biosynthesis of different nanoparticles which showed promising medical applications [Sadhana and Ram Naraian, 2018].

B. Mycobiosynthesis of AgNPs by Penicillium species

In current study *Penicillium* species randomly chosen for biosynthesis of AgNPs indicated significant production of AgNPs. The filtrate of *Penicillium sp* turned slightly pink colour after addition of $AgNO_3$ and incubating it for 2hrs and this indicated the production of AgNPs. In contrast, no colour change was observed in the flask containing only filtrate and 1.5mM AgNO3. The results of AgNPs production are indicated in Figure 2. It has been reported that change of colour from colourless to pink is mainly due to reduction of Ag+ to Ag^0 by NADH-dependent nitrate reductase of fungus which act as capping agent [Mikhailova et al., 2020]. A characteristic surface plasmon band exhibited by AgNPs can be detected in UV-VIS spectroscopy that absorbs light at 420 to 490 nm [Gudikandula and Charya Maringanti, 2016]. Hence the production of AgNPs was further confirmed by scanning the AgNPs solution from 250nm to 500nm in which the highest absorption spectra was recorded at 490nm that corresponded to the AgNPs production. No absorption peak was noticed at 490nm either in only fungal filtrate or only $AgNO_3$ solution Figure 3.

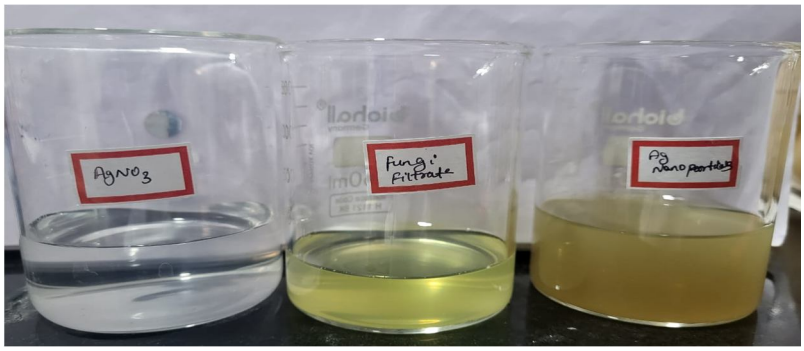

Figure 2. Mycobiosynthesis of AgNPs using soil fungus *Penicillium* sp.

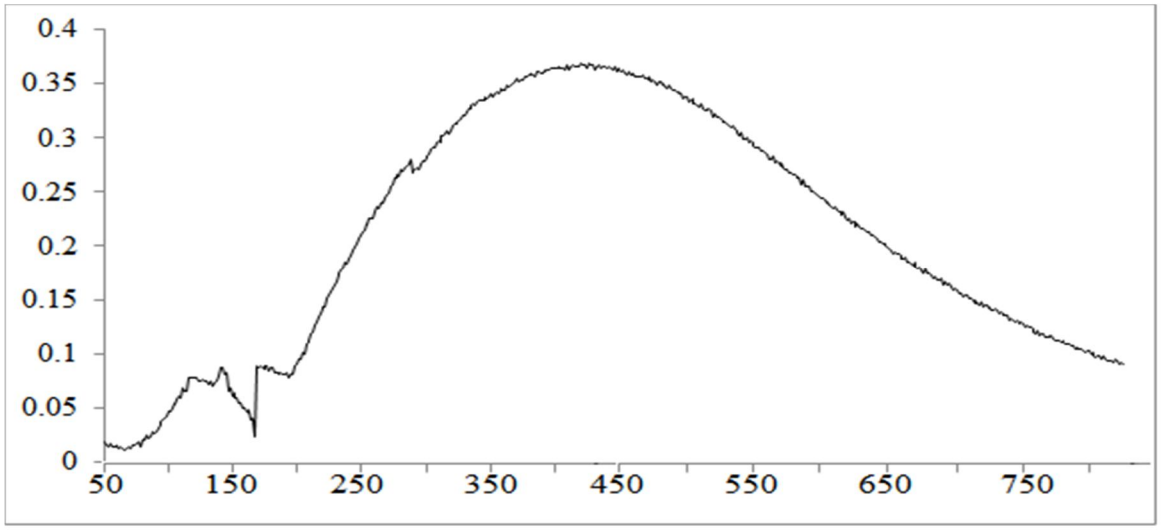

Figure 3. UV-Vis Spectrophotometric analysis of AgNPs of *Penicillium* sp.

The biosynthesis of AgNPs by *Penicillium* species in current study was assured by comparing the results with reported literature. The fungal filtrate showed formation of brown colour after addition of silver nitrate which is attributed to the surface plasmon resonance property of Ag metal confirming the production of AgNPs. The strong plasmon resonance property of AgNPs is responsible for excitation at 240nm results in high peak during scanning in UV spectroscopy. Further it is reported that, the capping and stabilization of AgNPs is brought about by proteins and enzymes mainly nitrate reductase secreted by microbes. In addition, the extracellular molecules synthesized by fungi particularly reducing sugars are anticipating in the biosynthesis of particular shape of AgNPs [Mohamed A. Yassin *et al.,* 2021].

C. XRD evidences of AgNPs production by Penicillium sp.

The information of an atomic structure of substance can be obtained through the XRD analysis. This tool is also useful in qualitative and quantitative detection of minerals in geological samples.

The technique is valuable in not only in detection of the structure of nanocrystals and also beneficial in determination of size and shape of the nanoparticles [Velavan S.Amargeetha A, 2018]. Therefore the technique is adapted in detect of AgNPs in the filtrate of *Penicillium* species. Biosynthesis of AgNPs by *Penicillium* sp was supported by the XRD data which indicated four different peaks of 38º, 46º, 64º, and 77.6º at 2θ angle at X-axis which corresponded to lattice planes of (111), (200), (220) and (311). The results of XRD indicated in Figure 4.0. The results are matched with JCPDS card no. 03-0921 of AgNPs which corresponded to face-centered–cubic nanocrystals of AgNPs.

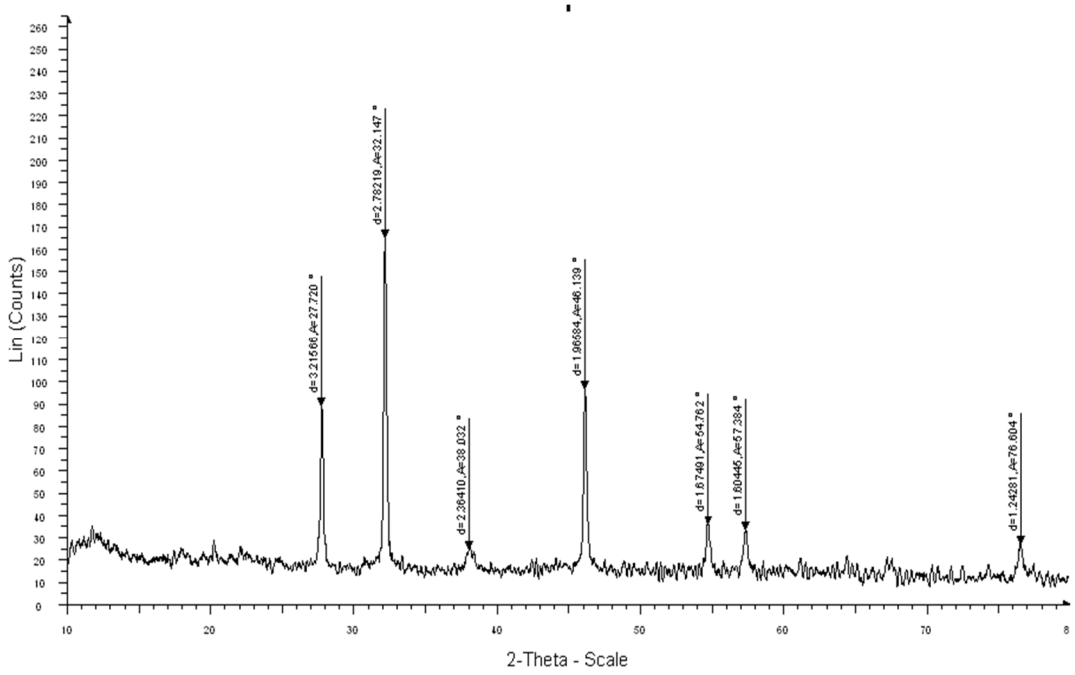

Figure 4. Powder-XRD analysis of AgNPs biosynthesized from *Penicillium* sp.

D. FTIR analysis of AgNPs of Penicillium sp

AgNPs of *Penicillium* sp analysed by FTIR to identify the possible functional groups associated with their biosynthesis. FTIR results of AgNPs of Penicillium sp. are shown in Figure 5. Analysis of AgNPs by FTIR indicated peaks 3915.85cm-1, 3411.74cm-1, corresponded to O-H stretch of hydroxyl group and O-H group of alcohol. The peak 2521.85cm-1, 2342.12cm-1, and 2073.61cm-1, were attribute to nitrile (C=N) group.

The chemical groups –C=C– stretch of alkenes is detected by peak 1639.88cm-1, 1370 cm-1 is attributed by C–H rock of alkanes, C–O stretch of carboxylic acids is detected by FTIR peak 1247.27cm-1, C–N stretch of aliphatic amines is corresponded to 1110cm-1. The chemical group =C–H bend of alkenes is attributed by 673.39cm-1. Detection of these functional groups by FTIR reveals there is a possible anticipation of proteins in mycosynthesis of AgNPs in which these chemical groups might be side chain molecules of enzymes involved in AgNPs biosynthesis.

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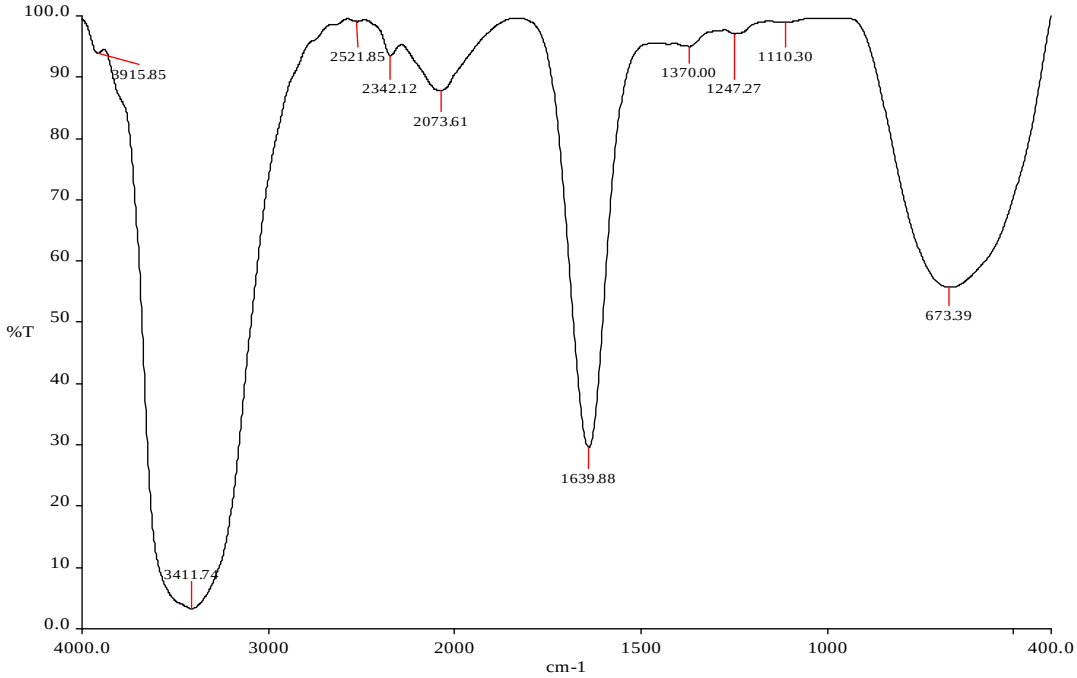

Figure 5. Detection of functional groups probably anticipated in AgNPs biosynthesis

E. HR-SEM analysis of Penicillium sp AgNPs

AgNPs analyzed using HR-SEM showed different size and shapes of nanoparticles. No agglomeration of AgNPs was observed in most of microscopic fields and AgNPs are found as separate entities. The sizes of the nanoparticles varied ranging from 5µm to 100nm. Microscopic image of AgNPs of HR-SEM are shown in Figure 6. The AgNPs with size ranging from 40-50nm were found to be highest *i.e.*, 56% followed by the 40% AgNPs of size of 30-40 nm. About 40% of nanoparticles were in the sizes ranging from 50-60nm and about 25% of AgNPs were in the smallest sizes of 10-20nm. The total ranges of different sizes of AgNPs and their respective percentage of distribution in the filtrate are shown in Figure 7.

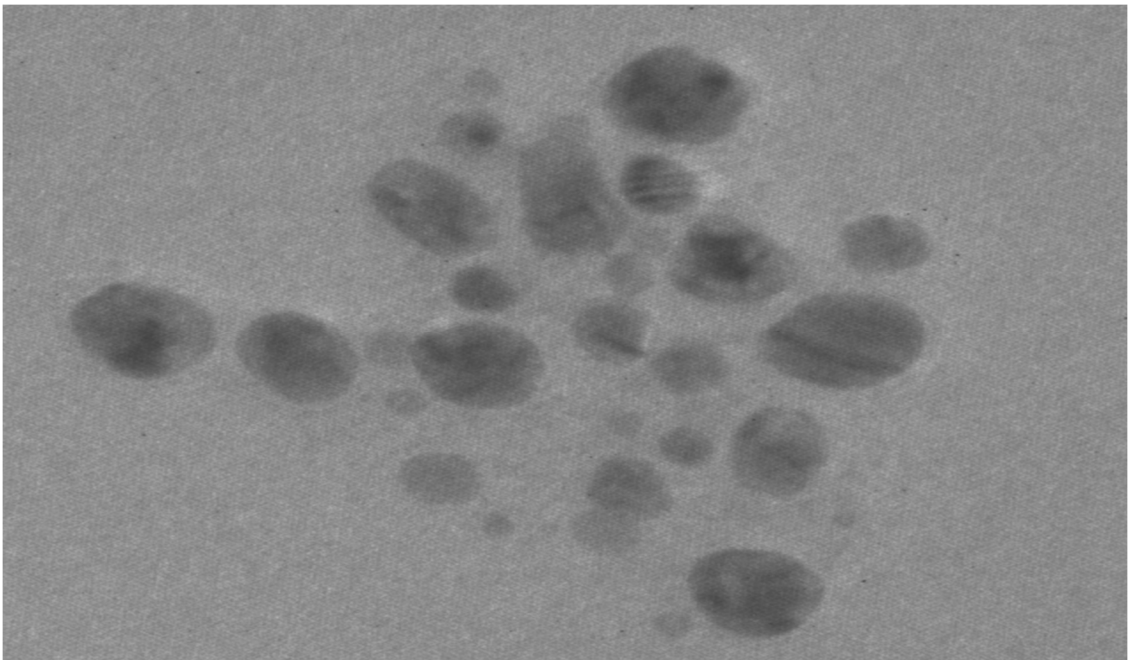

Figure 6. Microscopic images of AgNPs of *Penicillium* sp. observed under HRSEM

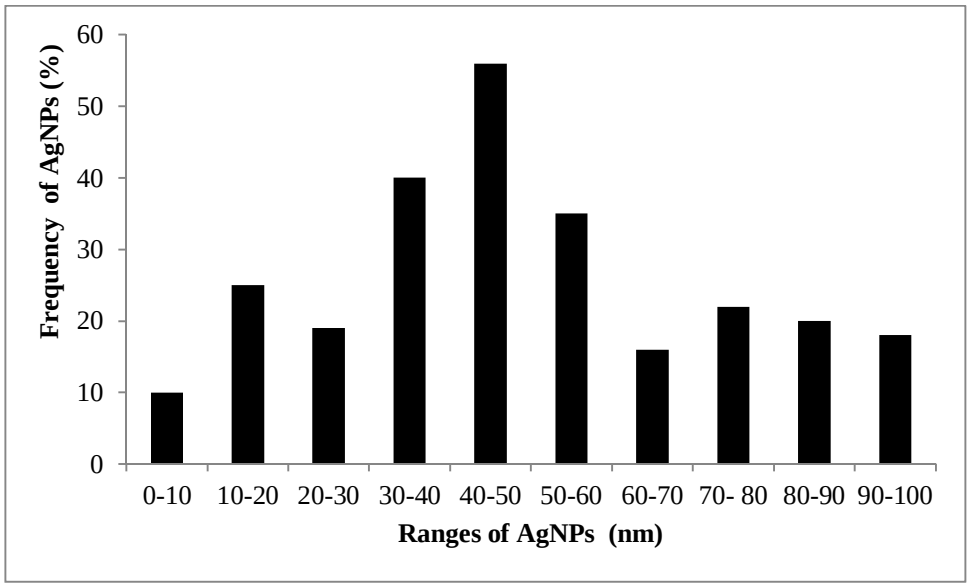

Figure 7. Size and percentage of AgNPs distribution in the fungal filtrate

F. Biological applications of AgNPs of Penicillium sp.

Antibacterial activity of AgNPs of *Penicillium* sp.

AgNPs mycosynthesized from *Penicillum* sp were evaluated for antibacterial activity against both Gram negative and Gram positive bacterial isolates. AgNPs revealed significant antibacterial activity against *E. coli* and *S. aureus* indicating the zone of inhibition of 2.6mm and 3.1 mm respectively. Antibacterial activity against Gram positive isolates was slightly higher than Gram negative bacteria. The results indicated that antibacterial activity against both bacteria was exclusively due to AgNPs as no antibacterial activity was observed in fungal filtrate and 1.5mM $AgNO_3$ solution. Fungi were referred to as bio-nano factories for production of AgNPs with potential biological properties such as antibacterial, antifungal, insecticidal, antiparasitic, and anticancer [Guilger-Casagrande *et al.*, 2019]. Soil Fungi isolated from stressful climate were reported for mycosynthesis bioactive AgNPs active against various human pathogenic bacteria namely *E. coli*, *S. aureus*, and *P. aeruginosa* [Ottoni *et al.*, 2017]. However in present study AgNPs of *Penicillium* sp have shown strong broad spectrum antibacterial activity.

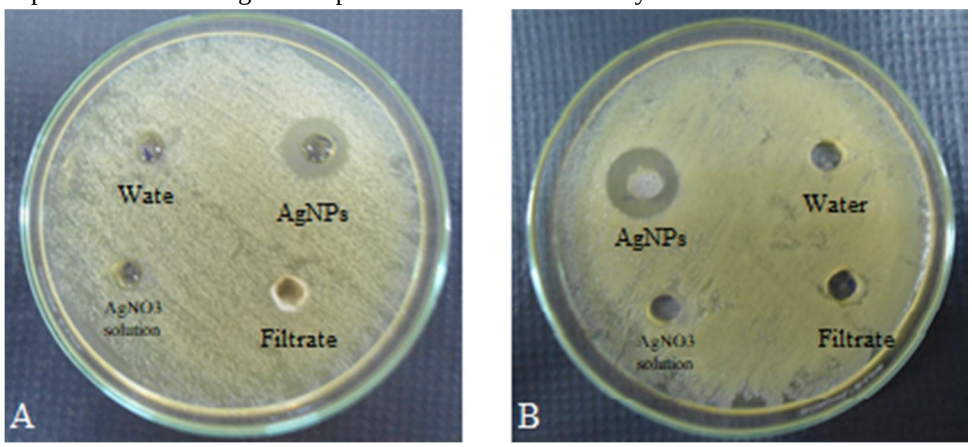

Figure 8 Antibacterial activity of AgNPs of *Penicillium* sp against *E. coli* (A) and *S. aureus* (B).

G. Study of effect of AgNPs on bacterial growth curve

The effect of AgNPs on the growth curve of *E. coli* bacteria was studied. The growth of the bacteria was inhibited when 1000µg of AgNPs were added and the absorbance was declined compared untreated control solutions and culture treated with fungal filtrate. Although AgNPs failed to inhibited the growth of initial inoculum at 0 to 4hours but subsequent hours i.e., 6 to 20hours AgNPs substantially inhibited the growth of *E. coli*. The bacterial growth in control broth followed usual bacterial growth curve pattern with declining the growth from 2hours to 24hours.

E. coli treated with filtrate also followed identical trend as that of control however the growth is slightly inhibited at initial growth phase *i.e.*, 0 to 4hours which further recovered from 6th to 20th hour and the results are shown in **Figure 8**. The AgNPs in current study successfully inhibited the growth of the bacteria by disturbing the growth curve at exponential, stationary and death phases.

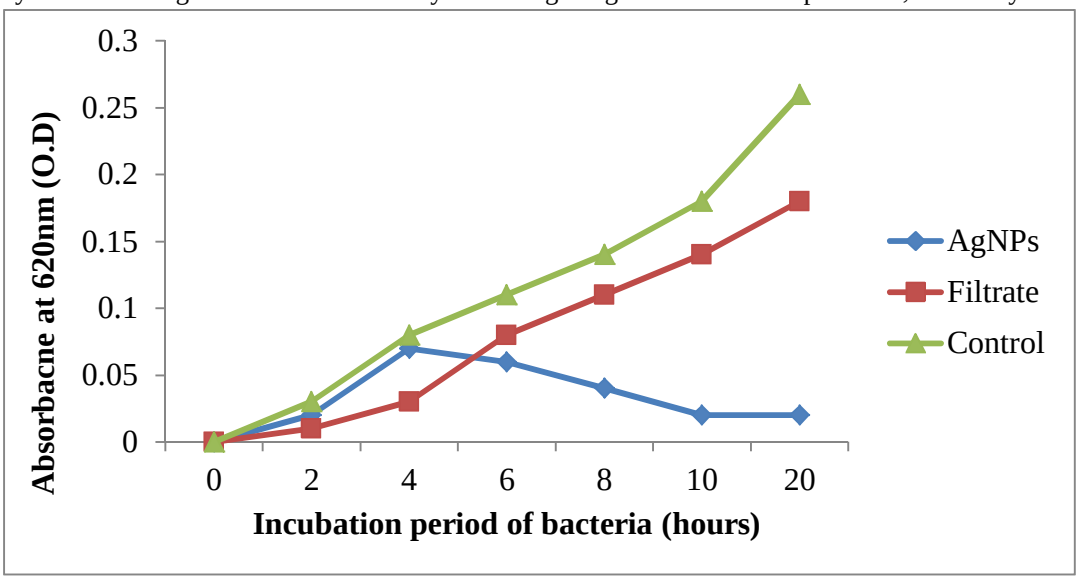

Figure 8. Study of effect of AgNPs on the growth curve of *E. coli* bacteria

H. Anti-diabetic activity of AgNPs

Alpha-amylase activity of different concentrations of AgNPs indicated dose-dependent anti-diabetic activity. AgNPs at 400µg concentration successfully inhibited about 65% of alpha-amylase inhibition compared to 99.14% inhibition of amylase activity by standard drug acarbose. The detailed anti-diabetic activity of AgNPs and acarbose and their IC_{50} values were in given in Table 2 and Figure 9. From the study it clear that AgNPs of fungal origin possess considerable anti-diabetic activity at lower concentration although activity is not par with standard anti-diabetic drug.

Table 2: Anti-diabetic activity of AgNPs of *Penicillium* species

Conc. of Acarbose/AgNPs (µg)	Alpha-amylase activity of Acarbose/AgNPs	
	Acarbose (% inhibition)	AgNPs (% inhibition)
Control	00.00	00.00
12.5	49.25	17.80
25	69.25	31.02
50	75.84	44.77
100	82.52	49.96
200	95.94	59.20
400	99.14	65.85
IC_{50} value (µg/ml)	15.47	182.00

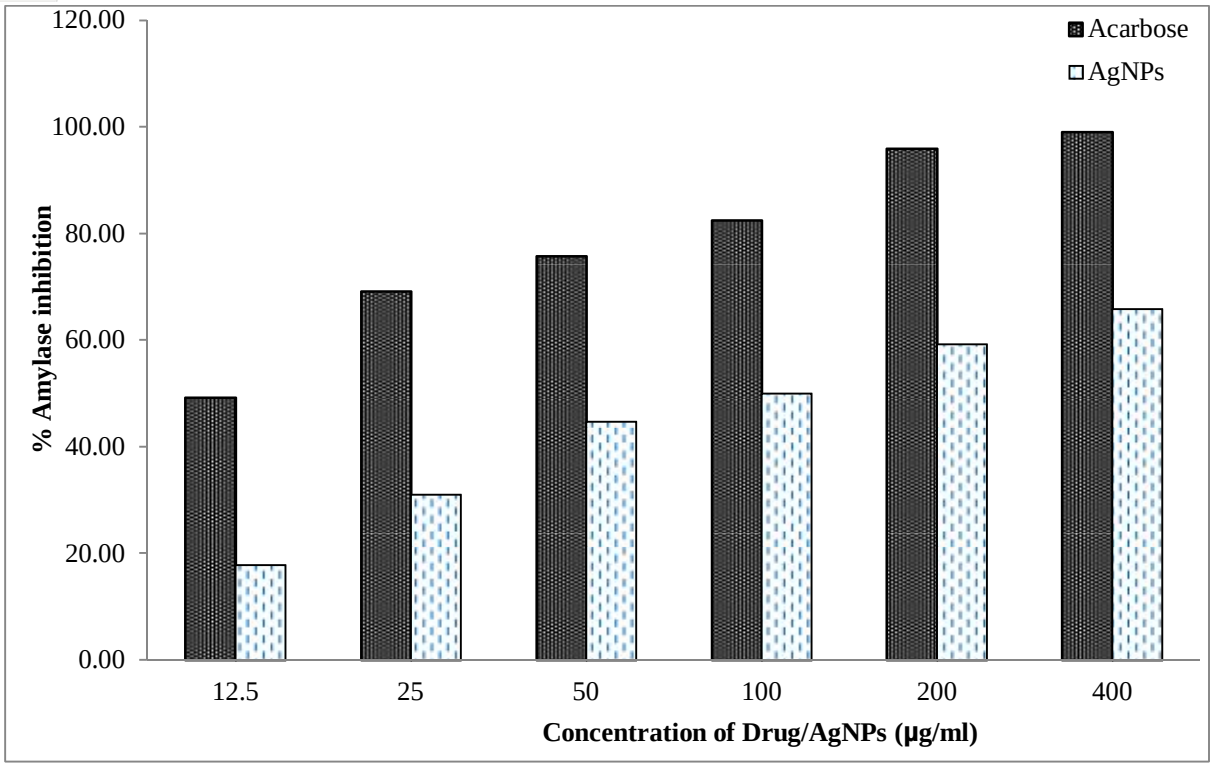

Figure 9: Alpha-inhibition potential of AgNPs of *Penicillium* species

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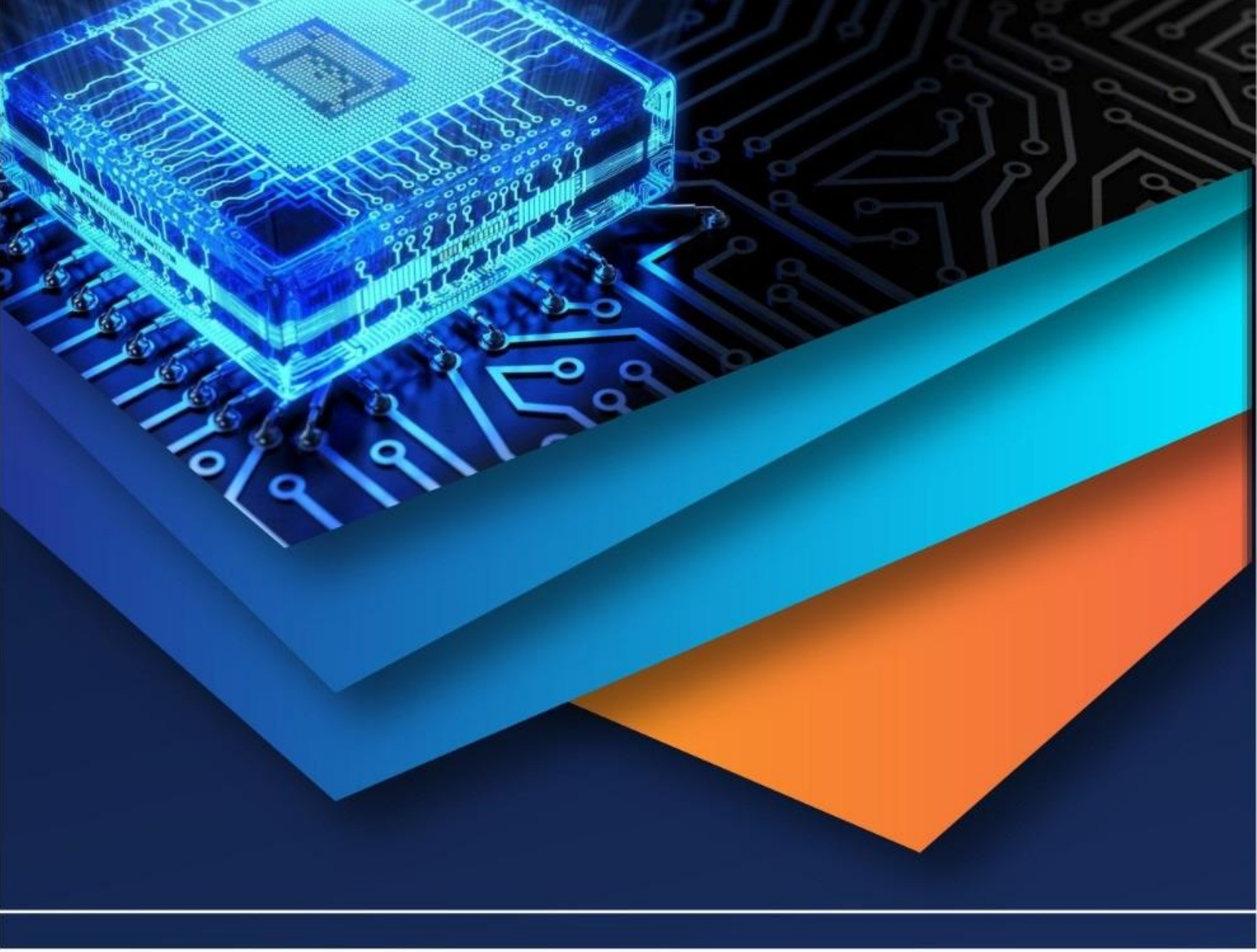

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