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Isolation, Identification, Chemical and Biological Synthesis of silver nanoparticles andits Antimicrobial Activity

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Abstract: ***Extracellular biosynthesis of silver nanoparticles (AgNPs) by Aspergillus Fumigatus isolated from Soil was reported in the present study. The biosynthesis of AgNPs was monitored by ultraviolet-visible spectroscopy and its antimicrobial activity, Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC). The isolated fungus was identified by observing morphological characteristics and Lactophenol cotton blue staining and confirmed as Aspergillus fumigatus from the report given by National Fungal Culture Collection of India (NFCCI). Based on the study, it is concluded that the AgNP's which is produced by biological methods is showing the antimicrobial activity.***
Keywords: Silver Nanoparticles, Minimum Inhibitory Concentration, Minimum Bactericidal Concentration, Aspergillus fumigatus

I. INTRODUCTION

Nanobiotechnology was born as a hybrid discipline. It is a combination of two branches, biotechnology and nanoscience [01]. The size of Nanoparticles ranges from 1–100 nm in diameters. They have unique properties such as a surface effect, an optical effect, a quantum size effect, and a macroscopic quantum tunneling effect [02]. Nano is a Greek word meaning extremely small, and its use to indicate one billionth of a meter or 10^{-9} meter [03]. The term Nanotechnology was coined by Professor Norio Taniguchi of Tokyo, Science University in the year 1974 [03]. Nobel metal nanoparticles such as gold, silver, platinum and palladium have been most effectively studied [04]. Silver (Ag) is the metal of choice of preparation of NPs and has potential applications in the field of biological system [05].

A. Synthesis of Nanoparticles

There are different methods of synthesizing nanoparticles, which includes chemical, physical, and biological methods [02]. Chemical and physical methods have traditionally been used to synthesize nanoparticles, but as "green" approaches increase in popularity, nanoparticles are increasingly being produced by nontoxic and ecofriendly methods [02].
Synthesis of nanoparticles by biological methods includes the use of plants, bacteria, and fungi. Fungi have more metabolic diversity because of which it have become one of the main biological candidates for synthesizing nanoparticles [02].
A novel biological method for synthesis of silver nanoparticles was proposed by Mukherjee and coworkers [06], the two step mechanisms were suggested. The first step involved trapping of the Ag^{+} ions at the surface of the fungal cells. In the second step, the enzyme present in the cell, reduce silver ions [07].

B. Synthesis of Nanoparticles by Fungi

It has been reported that fungi are extremely good and ideal candidates for the production of AgNP's, because of their ability to secrete large number of enzymes [08]. Many research papers reported the mycosynthesis of silver nanoparticles from fungi, such as *Aspergillus niger* [09], *Aspergillus flavus* [08], *Aspergillus fumigatus* [10], *Trichoderma harzianum* [11], *Penicillium citrinum* [12], *Fusarium oxysporum* [12], *Alternaria alternata* [13], *Rhizopus stolonifer* and *Mucorpplumbeus* [12].
The use of fungi in producing metallic nanoparticles has received significant interest as they offer certain advantages over the use of bacteria for the synthesis of nanoparticles. The ease of scaling up and downstream processing, the economic feasibility and the presence of mycelia offering an increased surface area, are important advantages to consider [14].

Fungi have potential to provide relatively quick and ecologically 'clean' metallic nanoparticles. Application of fungi for production of silver nanoparticles is potentially existing because their ability to secrete large number of proteins [15]. Silver nanoparticles production has been the focus of research by the scientific community, and rightly so. AgNP's have great potential in a number of industries such as antimicrobials and electronics [14]. Mycelia free spent medium of the fungus, *Cladosporium cladoporiodes* was used to synthesize silver nanoparticles extracellularly [17].

Earlier the exact mechanism for the synthesis of AgNPs was not known, but later it was hypothesized that the silver ions required the NADH-dependent nitrate reductase enzyme for their reduction, which was secreted by the fungus in its extracellular environment. The presence of NADH-dependent nitrate reductase enzyme in extracellular cell filtrate of the fungus used for the synthesis of nanoparticles has been confirmed [18].

Fusarium oxysporum has also been shown to produce Cadmium sulphide (CdS), lead sulphide (PbS), zinc sulphide (ZnS) and molybdenum sulphide (MoS) nanoparticles, when the appropriate salt is added to the growth medium [14].

II. MATERIAL AND METHODS

A. *Isolation of Fungi and Maintenance of Fungal Culture*

The fungal culture of *Aspergillus fumigatus* was isolated from spentwash mixed soil collected from Bhenda factory, Newasa, India, in the month of December 2017.The culture was inoculated on sterilized Sabouraud agar plates and incubated at 37^0C for 48 hrs. The culture was maintained by repeated sub-culturing on sterilized Sabouraud agar plates.

B. *Identification of Isolated Fungi*

Using cultural and morphological characteristics the fungal isolates were identified. The technique of Oyeleke and Manga was also adopted for the identification of the isolated fungi using cotton blue in lactophenol stain. The identification was achieved by placing a drop of the stain on clean slide with the aid of a mounting needle and then a small portion of the aerial mycelia from fungalcultures was taken anda drop of lactophenol was added.Usingtheneedle the mycelium was well spread on the slide. The cover slip was then gently placed with a littlepressureso as to eliminate the air bubbles. Then the slide was mounted and viewed with the light microscope undertheobjectivelense power10x and 40x respectively [19]. The isolated fungal culture was further identified and confirmed by National Fungal Culture Collection of India (NFCCI).

C. *Chemical Synthesis of Nanoparticles*

For the synthesis of chemical nanoparticles 1mM aqueous solution of $AgNO_3$ was prepared in 100 ml of distilled water. This solution was boiled and 1% 5mL solution of trisodium citrate was added drop by drop to it and mixed vigorously. After 1 hour heating solution was removed and cooled at room temperature.

D. *Biosynthesis of Silver Nanoparticles*

1) *Production of Biomass:* The 48 hours old culture of *Aspergillusfumigatus* was aseptically inoculated in sterile 100 ml Sabouraud's broth in 250 ml conical flask. The inoculated broth was incubated at 37^0Cfor 72 hours in shaking incubator adjusted at 150 rpm. After incubation the biomass was harvested by sieving through a plastic sieve. Harvested biomass was washed threetimes with sterile double distilled water to remove media component from the biomass [20].
2) *Synthesis of silver Nanoparticles:* For the biological synthesis of nanoparticles, 20 gm wet weight of biomass was inoculated into 100 ml of sterile double distilled water and incubated at 27^0C in shaking incubator adjusted at 150 rpm for 48 hours. After incubation, the biomass was filtered by Whatman filter paper no. 1 and cell filtrate was used for synthesis of nanoparticles. The 50 ml of cell filtrate was mixed with 10 ml of 10 mM solution of $AgNO_3$ in 100 ml conical flask, while, the cell filtrate without $AgNO_3$ solution was kept as a control. Both the flasks were then incubated in shaking incubator for 24 hours at 27^0C at 150 rpm in dark condition to avoid photochemical reaction. The AgNP's were purified by drying the reaction mixture in Hot air oven at 45^0C temperature for 48 hours [20].

E. *Characterization of Silver Nanoparticles*

1) *UV Visible Spectroscopy Analysis:* The reaction mixture was mixed with $AgNO_3$ solution, reduction of silver ions and their development in the silver nanoparticles was monitored by visual observation (color changes from yellowish to brown) and by UV visible spectroscopy analysis, which was done by UV visible double beam spectrophotometer of Shimadzu Ltd. within the range of 200- 800 nm [21]

2) *Antimicrobial Activity:* Silver nanoparticles were assayed for antibacterial activity by well diffusion method against selected human pathogens. The colonies of 24 hours old culture was used, 3-5well isolated colonies from pure growth of test organisms were transferred to 5 ml saline suspension to achieve the density of 10^8 cells/ml of McFarland standards during each test. The 25 ml of sterile MHA medium was poured into sterile petri plate and allowed to solidify. The 0.1 ml of bacterial inoculum was spread evenly with sterile glass spreader. A standard cork borer of 6 mm diameter was used to prepare 4 uniform wells on the surface of MHA plate. 100 µl of each solution was added in the well. The solution was, a) Biologically synthesized AgNP's b) Chemically synthesized AgNP's c) 20 µg/ml of streptomycin solution (as a positive control) d) Sterile double distilled water (as a negative control).Then the plates were kept in refrigerator (4^0C) for prediffusion. After 1 hour the plates were removed and incubated at 37^0C for 24 hours in incubator. The assay was performed in triplicates. The zone of inhibition of AgNP's against test organisms was measured in mm. [21]

F. Determination of Minimum Inhibitory Concentration (MIC)

The MIC is the lowest concentration of silver nanoperticles that visually inhibits 99% growth of inoculated bacteria. Minimum inhibitory concentration (MIC) of silver nanoparticles was determined by standard micro dilution method against selected test organisms. The test organisms were inoculated in sterile MHB. Different concentration of silver nanoparticles*viz.,* 5µg/ml, 10µg/ml, 15µg/ml, 20µg/ml, 25µg/ml, 30µg/ml, 35µg/ml, 40µg/ml, 45µg/ml and 50µg/ml were tested for MIC which leads to the inhibition of bacterial growth. The MIC was examined by observing the turbidity in each tube after 24 hours of incubation at 37°C and was analyzed by spectrophotometer at 600nm, to check whether the tested nanoparticles inhibit the growth of test organism or not. Negative control tubes are also maintained for each test organisms without adding Ag-NPs. [21].

G. Determination of Minimum Bactericidal Concentration (MBC)

The MBC end point is defined as the lowest concentration of silver nanoparticles that kills 100 per cent of the inoculated bacterial population. To determine the MBC of synthesized silver nanoprticles, the viability of test bacteria was determined in the culture tube showing no visible turbidity in MICs. A loopful culture from each tube was spot inoculated on the center of sterile MHA plates separately. The inoculated plates were incubated at 37°C for 24 hour to determine the MBC. The MBC was examined by observing the growth on each MHA plate after 24 hours of incubation at 37°C [21].

III. RESULT AND DISCUSSION

A. Identification of Isolate

In the present study, biosynthesis of AgNP's was carried out using fungal species isolated from the soil sample. The isolated fungus was identified by observing morphological characteristics and Lactophenol cotton blue staining and confirmed as *Aspergillus fumigatus* from the report given by National Fungal Culture Collection of India (NFCCI).

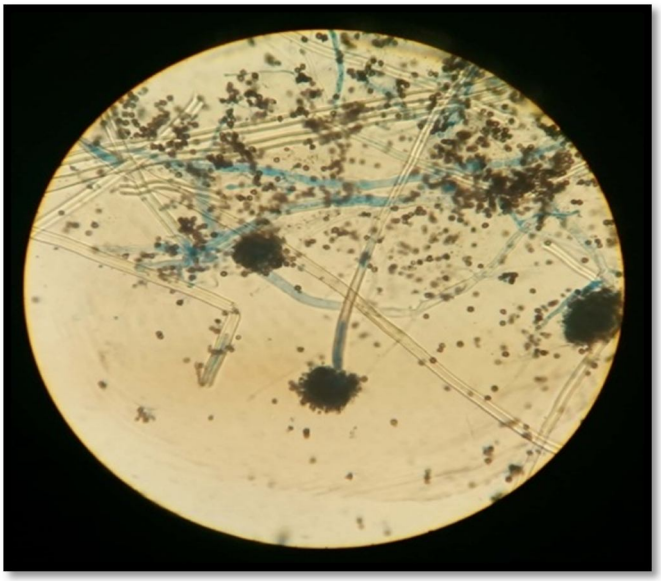


Fig no: 1 Lactophenol Cotton Blue Staining

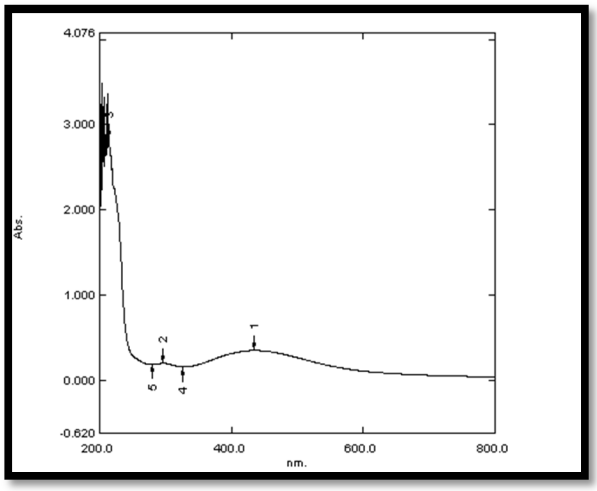
B. Biosynthesis of Silver Nanoparticles

In biological synthesis, the $AgNO_3$ solution (10 ml of 10mM) was mixed in the cell free extract of *A. fumigatus*. The rapid color change was observed in the solution from pale yellow to dark brown. This change indicated the biological synthesis of silver nanoparticles. It is well-known that due to excitation of surface plasma resonance of reduced silver nanoparticles it exhibit yellowish brown color in water. [10,21]. Ratnasri et.al., studied the synthesis of silver nanoparticles from Aspergillus fumigatus and it was confirmed by observing the color change from pale yellow to dark brownish yellow color [10]. Bansal et al., (2004) observed that the fungal species secrete an enzyme which reduce silver ion and form silver nanoparticles [21].

Fig No: 2 Biosynthesis of silver nanoparticles

C. UV-visible Spectrophotometer Analysis

This is one of the most widely used techniques to confirm the synthesis of nanoparticles. $AgNO_3$ solution was added in cell filtrate of *A. fumigatus*, after 24 hrs, cell filtrate was subjected to optical measurement by UV visible spectrophotometer. The maximum absorbance of Silver nanoparticles is known to exhibit in the range of 410 - 450 nm in UV-Visible spectrophotometer. The cell filtrate containing silver nanoparticles showed a maximum absorbance spectrum at 435nm.[10,04]. Ratnasari P.V. et.al., synthesized silver nanoparticles by using *A. fumigatus* and synthesis was confirmed by using UV-Visible Spectroscopy in the range of 320-560 nm and they found that the maximum absorbance spectrum was at 420nm [10]. Shubhangi Moharekar et.al., synthesized silver nanoparticles by using *Aspergillus niger* and it was confirmed by UV-Visible Spectroscopically and the maximum absorbance was found at 420nm [20].



Figno: 3 UV-visible spectra of Ag-NPs synthesized by *Aspergillusfumigatus*

D. Antimicrobial Activity

The antibacterial activity of silver nanoparticles was evaluated against two Gram positive i.e. *Bacillus subtilis* and *Staphylococcus aureus* and two Gram negative organisms *Escherichia coli* and *Pseudomonas aeruginosa* and for antifungal activity, the fungal strain used were *Asperigillus niger* and *Aspergillus flavus.* The results are shown in table no 7. The maximum zone of inhibition was observed against *Pseudomonas aeruginosa* i.e. about 21 mm in diameter whereas the minimum zone of inhibition was observed with *Escherichia coli* and *Bacillus subtilis* i.e about 12 mm and 12 mm in diameter respectively.

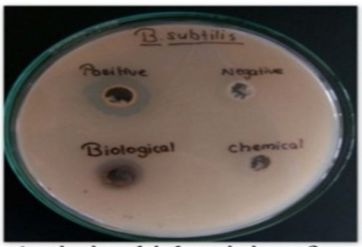
Staphylococcus aureus showed intermediate zone of inhibition i.e. 14mm in diameter. *Aspergillus flavus* showed the maximum zone of inhibition i.e. 16mm in diameter and *Aspergillus niger* showed the minimum zone of inhibition i.e. 10mm in diameter. The zone of inhibitions against bacteria was compared with chemically synthesized silver nanoparticles and streptomycin. Synthesized AgNPs independently showed efficient antimicrobial activity against Gram-positive and Gram-negative bacteria as well as fungal strains compared with chemically synthesized silver nanoparticles and streptomycin

Table No: 7 Antimicrobial activity of Silver nanoparticles

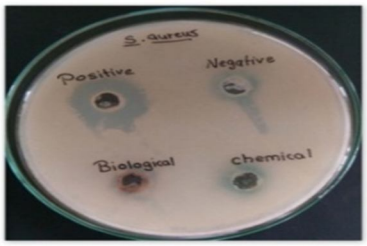
Test organisms	Zone of inhibition(mm)			
	Biological Synthesis of AgNP's	Chemical synthesis of AgNP's	Positive control (Streptomycin) (20µg/ml)	Negative control (distilled water)
B.subtilis	12	-	21	-
S.auerus	14	14	23	-
E.coli	12	-	30	-
P.aeruginosa	21	11	20	-
A.niger	10	-	24	-
A.flaus	16	-	26	-

E. MIC and MBC.

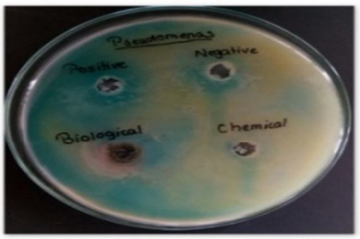
Silver nanoparticles were subjected at different concentration of silver nanoparticles to study the minimum inhibitory concentration



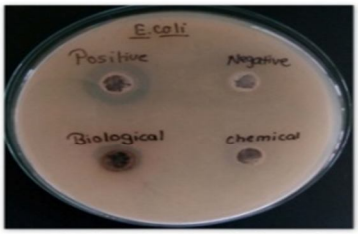
Antimicrobial activity of AgNPs against *B.subtilis*



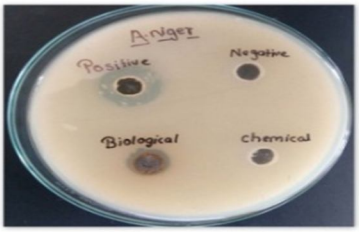
Antimicrobial activity of AgNPs against *S.auerus*



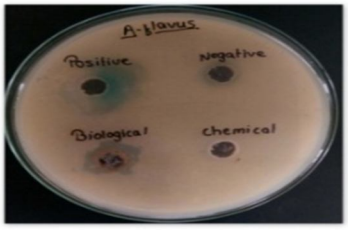
Antimicrobial activity of AgNPs against *P.aeruginosa*



Antimicrobial activity of AgNPs against *E.coli*



Antimicrobial activity of AgNPs against *A.niger*



Antimicrobial activity of AgNPs against *A.flavus*

(MIC) against the selected test organisms. Ag-NPs exhibited highest inhibition activity against *Pseudomonas aeruginosa* and it was revealed by MIC value which was found to be 10µg/ml. MIC tube showing no visible turbidity was selected for further MBC determination to find out the break point of silver nanoparticles. It revealed that at the 10µg/ml concentration of Ag-NP's 100% inhibition was observed for the bacteria *Pseudomonas aeruginosa*. The value of MBC was found to be 20µg/ml. These results clearly indicate that to treat infectious disease caused by Gram negative bacteria, *Pseudomonas aeruginosa* biosynthetic silver nanoparticles could provide an eco-friendly and safer alternative for the conventional chemotherapeutic agents. [22]. Zarei *et al* observed that antibacterial effect of silver nanoparticles against Gram negative pathogens showed MIC and MBC value of 3.12µg/ml and 6.25µg /ml respectively. To know the interaction of nanosilver with bacteria several studies have been investigated. But the actual bactericidal mechanism of silver nanoparticles is not clear and still confused. It was found that, on the membrane of treated microbes the majority of the nanosilvers were localized. Sulfur and phosphorus molecules containing amino acids inside or outside of bacterial cell membrane protein are the key element of the antimicrobial effect and it is assumed that silver ion has high affinity towards it. Because of this the osmotic stability gets affected which in turns leads to bactericidal activity. It was also suggested that silver nanoparticles releases silver ions which can interact with phosphorus moieties in nucleic acid which leads to inactivation of DNA replication. It can also react with sulfur containing proteins which leads to the inhibition of enzymes required for bacterial metabolisms. To treated organisms, this may causes bacteriostatic or bactericidal effects [22].

IV. CONCLUSION

In nanotechnology, biologically synthesized silver nanoparticles are most prominent. Today the important aspect of nanotechnology is to develop a reliable process for the synthesis of silver nanoparticles. Biological methods are currently gaining importance and are reliable because they are ecofriendly, cost effective and do not involve any toxic material during synthesis. In the biosynthesis of silver nanoparticles by using *Aspergillus fumigatus*, we reported that it is a simple, ecofriendly and low cost approach. The synthesized silver nanoparticles were characterized by UV-Visible Spectrophotometer which confirmed the maximum synthesis of silver nanoparticles at 435nm. Antibacterial activity of biologically synthesized silver nanoparticles was tested against two Gram positive i.e. *Bacillus subtilis* and *Staphylococcus aureus* and two Gram negative organisms *Escherichia coli* and *Pseudomonas aeruginosa* and antifungal activity was tested against *Asperigillus niger* and *Aspergillus flavus* by agar well diffusion method. We also studied the Minimum Inhibitory Concentration (MIC) which was found to be 10µg/ml. Minimum Bactericidal Concentration against the *Pseudomonas aeruginosa* was 20µg/ml.

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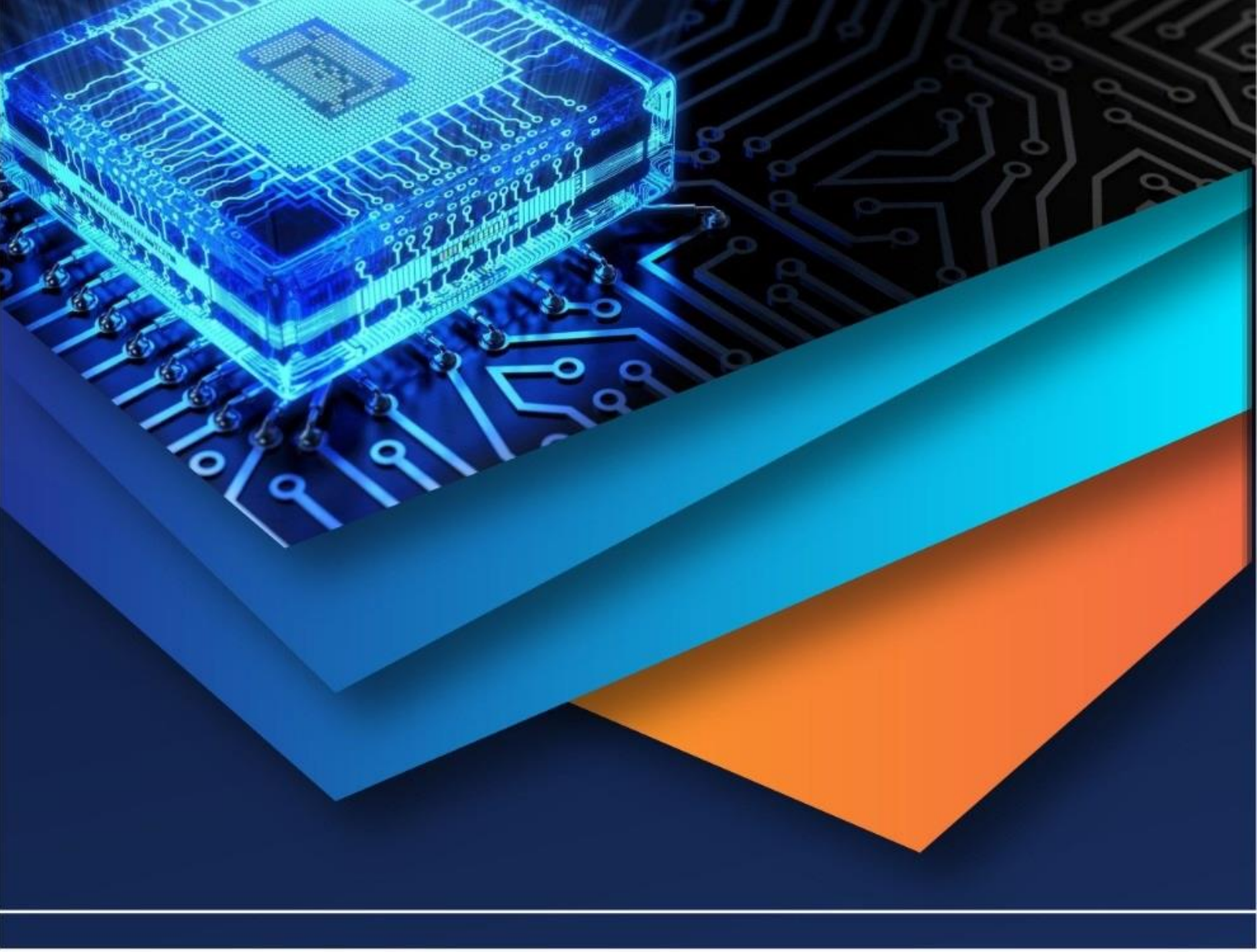

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