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Isolation and Screening of Biosurfactant Producing Bacteria from Soil and Water for their Potential Applications

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Abstract: ***Biosurfactants are amphiphilic compounds produced on the membrane of cells which tends to reduce surface tension. This study was done in order to isolate, screen for potential biosurfactant producing bacteria and its applications in plant growth and antimicrobial activity which were isolated from soil and water contaminated with used engine oil. Mineral salt medium(MSM) was used to isolate the bacterial strains. The isolated strains were screened for biosurfactant production by microplate assay, oil spread assay, blood haemolysis, CTAB agar assay, emulsification index test . The strains were followed through biochemical tests to study their composition.***
The isolated strains were run through 16s rRNA sequencing. Assessment of the properties of biosurfactant showed string antimicrobial activity against S.typhi, B.subtilis, E.coli. The biosurfactant effect on plant growth was examined and it showed improved growth in plants. The antimicrobial activity and the enhancement in the plant growth can lead to a revolution in the field of agriculture.
Keywords: Biosurfactant, Pseudomonas aeruginosa, Pseudomonas citronellolis, plant growth promoter.

I. INTRODUCTION

Past few years we have been seeing crisis of oil spillages in the oceans which have been creating havoc all over the world. This has led to keen interest towards improving the biosurfactants[11].
Organo-chemical synthesis is used to produce the dispersants that have been licenced and are kept in stockpiles around the world for use in the event of an oil spill[11] . Surfactants are a class of compounds having a diverse and significant role in various segments of the market including petroleum industry, soaps and detergent industry, environmental pollution abatement, and even in the food and beverage industry[3]. The term "biosurfactants" refers to a class of modern surfactants that are derived from microorganisms and are equally diverse in terms of structure and function. They are attracting more attention due to their biodegradability and environmentally favourable qualities[15].
Diverse micro-organisms are known to produce a number of surface-active agents primarily in order to adapt and grow on a variety of substrates among other natural functions[1]. The glycolipid class of rhamnolipids includes some of the most researched biosurfactants[2].

II. MATERIALS AND METHODS

A. Sample Collection

The water sample and soil sample were collected from the surroundings of petrol bunk vidyanagar , Hubballi in a sterile 50ml tube at 10:05am. The sample was immediately stored at 4°C till usage to prevent the microbial contamination of the sample.

B. Isolation of Microbial Consortium

The samples were enriched using inoculating in sterile Mineral Salt Medium (MSM). 1 ml of sample was inoculated in 100 ml of minimal salt medium containing (in g/L): 15g $NaNO_3$, 1.1g KCl, 1.1g NaCl, 0.00028g $FeSO_4.7H_2O$, 3.4g KH_2PO_4, 4.4g K_2HPO_4, 0.5g $MgSO_4.7H_2O$, 0.5g yeast extract at 37°C in shaker incubator (100 rpm). The medium was incubated for 24hrs and were selected based on the colony morphology on the nutrient agar and the selected microbes were gram stained (Table no.1) . The selected isolates were screened for the production of biosurfactants using the following screening methods[9].

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C. Screening of Biosurfactant Producing Isolates

Bacteria were cultivated aerobically in a 500 ml Erlenmeyer flask with 100 ml mineral salt media comprising 1.0 K_2HPO_4, 0.2 $MgSO_4.7H_2O$, 0.05 $FeSO_4.7H_2O$, 0.1 $CaCl_2.2H_2O$, 0.001 $Na_2MoO_4.2H_2O$, 30 NaCl, and crude oil (1.0 percent, w/v) .Flasks containing sterilised mineral salt medium were inoculated with a loopful of bacterial culture cultured in crude oil containing nutrient agar plates and kept in a shaker at 200 rpm and 30°C for 7 days. After 7 days of incubation, each flask's culture broth was centrifuged for 15 minutes at 6000 rpm and 4°C, and the supernatant was filtered through 0.45m pore size filter paper (Millipore). The drop collapse assay, oil spreading assay, emulsification assay, BATH assay and surface tension assay were all performed using this cell-free culture broth[9] .

D. Oil Spreading Test

The approach outlined by Morikawa et al, 2000 was used to conduct the oil spreading experiment[7]. In a nutshell, 20 ml of distilled water was placed to a plastic Petri dish, followed by 20 µl of crude oil added to the water's surface. The oil surface was then sprayed with 10 µl of cell-free culture broth. The negative control was distilled water (without surfactant), while the positive control was Triton X-100.

E. Microplate Assay

The microplate assay developed and patented by Vaux and Cottingham can be used to determine the surface activity of specific strains in a qualitative manner. A 100µl sample of each strain's supernatant is obtained and placed in a microwell of a 96-mircowell plate for this test. A grid-backed sheet of paper is used to see the plate. Only a modest amount of sample (100µl) is required. In addition, the approach can be used for automated high-throughput screening.[16]

F. Blood Haemolysis Test

In 5% Hen blood agar plates, a hemolytic assay was performed. Spot injected 50µl of bacterial culture cultured in a mineral salt medium onto blood agar plates and incubated for 48 hours at 37°C. A clear zone (hemolysis) around the colony was visually verified on the plates. A qualitative approach for determining biosurfactant production is the diameter of the clear zone. [6]

G. CTAB Agar Plate

Extracellular glycolipids or other anionic surfactants can be detected using the CTAB agar plate method, which is a semi-quantitative assay. Siegmund and Wagner were the ones who came up with the idea[14]. On a light blue mineral salts agar plate containing the cationic surfactant cetyltrimethylammonium bromide and the basic dye methylene blue, the microorganisms of interest are grown. [16]

H. Calculation of Emulsification Index (E24)

Several colonies of pure culture were suspended in test tubes containing 2 ml of mineral salt medium after 48 h of incubation; 2 ml cetaphil seed oil was added to each tube. Then, the mixture was vortexed at high speed for 1 min and allowed to stand for 24 hours. Calculation of emulsification index

Emulsification index=(Height of the emulsion layer/Total height) *10

I. Biochemical Test

Chemical Analysis of Biosurfactants was done by referring to the procedure explained by N.Mahesh[8.] (Table.no.2)

J. Sequencing

Both the water and soil sample were sent for 16srRNA sequencing. The bacteria in the soil sample was identified as *Pseudomonas citronellolis* and the bacteria in the water sample was identified as *Pseudomonas aeruginosa* .

K. Assay of Biosurfactant on Plant Analysis

Four samples were prepared using half cut water bottles with holes on the bottle for aeration. Control sample consisted of Soil with green gram, Sample A consisted of soil with crude oil and green gram, Sample B consisted of soil with crude oil and green gram along with microbes isolated from water sample introduced into the soil, Sample C consisted of soil with crude oil and green gram

along with microbes isolated from soil sample introduced into the soil. Keeping all the conditions(sunlight and water) same, all the samples were allowed to grow and the data was recorded . This was done to observe the growth of plant in every sample and how the biosurfactant microbes enhanced the growth of plants with respect to stem length, leaf length , leaf width .

L. Antimicrobial Activity of Isolated bacterium

The antibacterial activity of crude biosurfactant was tested using the agar well diffusion method. The crude biosurfactant was tested on *Salmonella typhi*, *Aspergillus niger*, *Staphylococcus aureus*, and *Escherichia coli* along with control was used as 5% ciprafloxaccin The plates were incubated for 24 hours at 37°C. The presence of a clear zone suggests that the crude biosurfactant has antibacterial action. [13]

Data Description: Nucleotide sequence
Repository: GenBank
Accession no. OP120781
OP120782

III. RESULTS

1) Oil spread test shows a positive results for *Pseudomonas aeruginosa* and *Pseudomonas citronellolis*. When the sample is added to the petri plate containing the oil and the water solution clear zone is formed. This shows that the sample as the biosurfactant property and hence it degrades the oil.
2) In 96 well microplate assay when the sample was added to the wells containing oil, a concave surface distortion is formed, this can be viewed using a backing sheet of paper with a grid. This shows that *Pseudomonas aeruginosa* and *Pseudomonas citronellolis* have surfactant nature. Fig.1(a) Fig.1 (b)
3) Blood haemolysis assay When the samples containing *Pseudomonas aeruginosa* and *Pseudomonas citronellolis* were inoculated on the blood agar and incubated at 32C for 48 hours, a clear zone was formed on the blood agar, which indicates the partial hemolysis of the blood agar. The partial hemolysis indicates the presence of biosurfactants producing organsims.
4) CTAB Agar plate assay When the cell free culture is poured into the well on CTAB agar plate and incubated for 48 hours at 37 °C, dark blue colour was formed around the wells. This indicates the presence of biosurfactants producing organsims, *Pseudomonas aeruginosa* and *Pseudomonas citronellolis*.
5) Emulsification index:

soil sample, Emulsification index=62.5%
water sample, Emulsification index=57.1%

6) Antimicrobial

The antimicrobial activity of the extracts containing surfactin produced by *P.aeruginosa* and *P.citronellolis* against various reference, environmental and clinical bacterial was then determined.
The average zone of inhibition exhibited by control against *B.subtilis* , *S.typhi* and *E.coli* was 0.5cm , 0.8cm and 0.3cm respectively. Fig.4(a) Fig.4(b) Fig.4(c)
The average zones of inhibition, the surfactin extract (WSSP) exhibited an increased antibacterial activity against *E.coli strains* (average zone of inhibition of 4cm). Fig.5(a)
The average zones of inhibition, the surfactin extract (SSSP) exhibited an increased antibacterial activity against *E.coli* strains.(average zone of inhibition of 3cm). Fig.5(b)
The average zones of inhibition, the surfactin extract (WSSP) exhibited an increased antibacterial activity against *S.typhi* strains.(average zone of inhibition of 5cm).Fig.6(a)
The average zones of inhibition, the surfactin extract (SSSP) exhibited an increased antibacterial activity against *S.typhi* strains.(average zone of inhibition of 3.8cm).Fig.6(b)
The average zones of inhibition, the surfactin extract (WSSP) exhibited an increased antibacterial activity against *B.subtilis* strains.(average zone of inhibition of 3cm).Fig.7(a)
The average zones of inhibition, the surfactin extract (SSSP) exhibited an increased antibacterial activity against *B.subtilis* strains.(average zone of inhibition of 4cm).Fig.7(b)
Graphical Respresentation of antimicrobial assay . Fig.8

7) Plant Growth Assay

When the cell free cultures of *Pseudomonas aeruginnosa* and *Pseudomonas citronellolis* were added to different samples, there was various changes seen in the plant growth. Control sample had a contamination free soil to which the green gram seeds were added(Fig.9 a) .Sample A had the soil contaminated by the engine oil to which the green gram seeds were sowed(Fig.9 b). Sample B had the contaminated soil and the cell free culture of *Pseudomonas aeruginosa* (Fig.9 c) and the sample C contains the contaminated soil and the cell free culture of *Pseudomonas citronellolis* (Fig.9d) . The growth of the seeds were observed in these samples regularly. The seeds sprouted in all the samples around 3rd day. The growth of the plant in control sample was normal whereas in the sample A the growth of the plant was very slow and in the sample B and C the growth of the plant was fast and they seemed very healthy. After 13 days the stem length, leaf length and the width was measured, the plant in control sample had a stem length of 5cm, leaf length of 3.6cm and leaf width of 1.4cm (Fig.10a), in sample A the plant was with 3cm stem length, 3.5cm leaf length and 1.1cm leaf width(Fig.10b), in the sample B the plant was with 7.5cm stem length, 4cm leaf length and 1.5cm leaf width(Fig.10c) and the sample C plant was with 6.5cm stem length, 4cm leaf length and 1.5cm leaf width(Fig.10d). This clearly shows that the sample with the biosurfactants have the plants with better growth, the stem length and the leaf length is twice the plants grown in normal control sample. So the biosurfactants acts as plant growth promotor in the contaminated soil and help in the growth of plants and also due to their antimicrobial property they help in boosting the plant immune system.

Graphical representation of plant growth assay (Fig.11)

IV. DISCUSSION

Biosurfactants are the amphiphilic compounds which can amass between phases of liquids. They are usually produced on surface of bacterial cell[4] .A wide range of microorganisms have been brought up which exhibit different class of biosurfactant property such as glycolipids, phospholipids, neutral lipids and lipopeptides[12]. Biosurfactant forms micelles complex with crude oil which leads to emulsion and thus the crude oil can be recovered by breaking the emulsion. In the similar way the biosurfactant binds with the minerals example phosphorous and forms micelles complex and helps in the uptake of minerals in plants. Psuedomonas produce glycolipid group called rhamnolipids. Rhamnolipid is primarily a crystalline acid which is composed of beta-hydroxy fatty acid connected by carboxyl end to rhamnose sugar molecule. [5] Rhamnolipid insertion inside the lipid bilayer does not strongly affect the lipid dynamics but the nature of the phytosterols could influence the effect of glycolipids on plant membrane destabilization. These subtle changes in lipid dynamics could be linked with plant defense. The rhamnolipids trigger the plant physical protective mechanism like callose deposition and stomatal closure and thus helps in plant protection.[10]

V. CONCLUSION

Biosurfactants have gained a greater interest in past years. With respect to oil and gas sector, the application of biosurfactants in degradation and in dispersant form to treat oil spills are of greater interest. Along with these application, testing on plant for comparative study of the growth gave a different angle to thought process. The antimicrobial activity and the enhancement in the plant growth can lead to a revolution in the field of agriculture. It can also thereby protect environment. In future studies , rhamnolipids , which are the key component of biosurfactant responsible for the forementioned application can be extracted and can be formulated into oil cleanser, plant growth promoter and soil quality improver products.

VI. ACKNOWLEDGMENT

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1) *Conflict of Interest:* The authors declare that they have no conflict of interest in the publication.
2) *Compliance of Ethical Standard:* This article does not contain any studies involving human or animal participants performed by any of the authors.

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FIGURES

Fig.1a

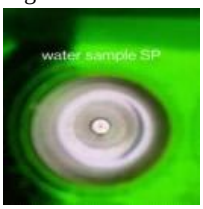

Fig.1a Microplate assay showing concave distortion of P.aeruginosa in water sample

Fig.1b

Fig.1b *Microplate assay showing Concave distortion of P.citronellolis in soil sample .*

Fig.2a

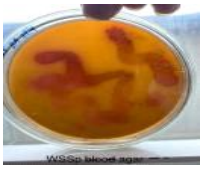

Fig.2a *Partial hemolysis exhibited by P.aeruginosa .*

Fig.2b

Fig. 2b *Partial hemolysis exhibited by P.citronellolis*

Fig.3a

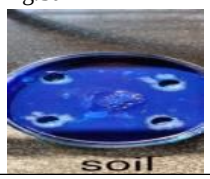

Fig.3a *CTAB agar plate assay with P.citronellolis*

Fig.3b

Fig.3b *CTAB agar plate assay with P.aerugionsa.*

Fig.4a

Fig.4a *zone of inhibition exhibited by control against B.subtilis.*

Fig.4b

Fig.4b *zone of inhibition exhibited by control against S.typhi*

Fig.4c

Fig.4c *zone of inhibition exhibited by control against E.coli.*

Fig 5a

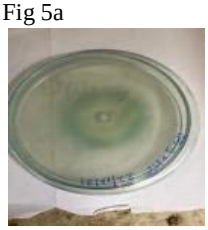

Fig.5a *zone of inhibition exhibited by P.aeruginosa against E.coli.*

Fig.5b

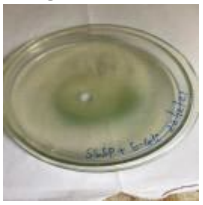

Fig.5b *zone of inhibition exhibited by P.citronellolis against E.coli.*

Fig.6a

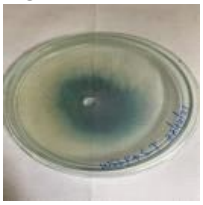

Fig. 6a *zone of inhibition exhibited by P.aeruginosa against S.typhi.*

Fig.6b

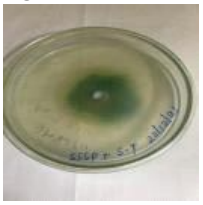

Fig. 6b *zone of inhibition exhibited by P.citronellolis against S.typhi.*

Fig.7a

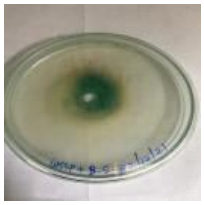

Fig. 7a *zone of inhibition exhibited by P.aeruginosa against B.subtilis.*

Fig.7b

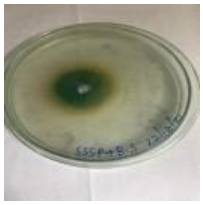

Fig. 7b *zone of inihibition exhibited by P.citronellolis against B.subtilis.*

Fig 8.

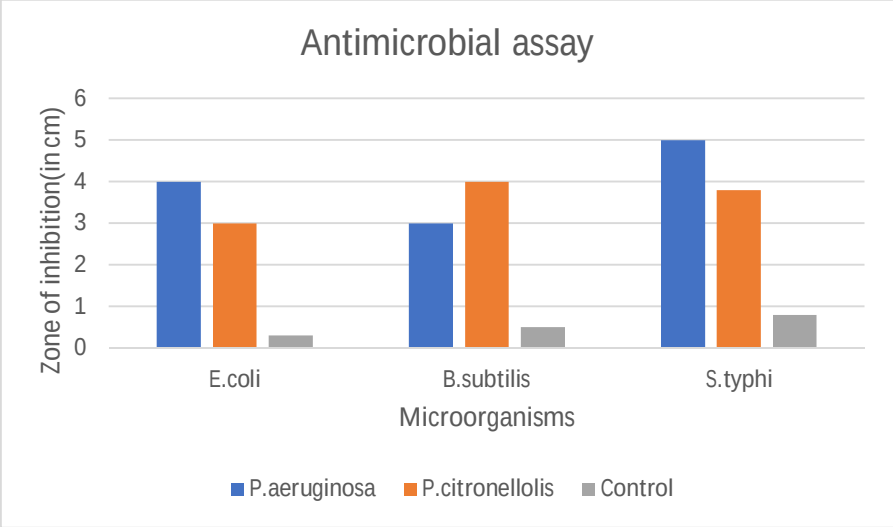

Fig. 8 *Graphical representation of antimicrobial assay.*

Fig.9a

Fig. 9a *Control sample on day 1 with uncontaminated soil and green gram.*

Fig.9b

Fig. 9b *Sample A on day 1 with engine oil contaminated soil with green gram.*

Fig.9c

Fig.9c Sample B on day 1 with engine oil contaminated soil with green gram and P.aeruginosa

Fig.9d

Fig.9d *Sample C on day 1 with engine oil contaminated soil with green gram and P.citronellolis.*

Fig.10a

Fig. 10a *Control sample on day 13 with uncontaminated soil and green gram.*

Fig.10b

Fig. 10b Sample A on day 13 with engine oil contaminated soil with green gram

Fig.10c

Fig. 10c *Sample B on day 13 with engine oil contaminated soil with green gram and*

Fig.10d

Fig. 10d Sample C on day 13 with engine oil contaminated soil with green gram and P.citronellolis.

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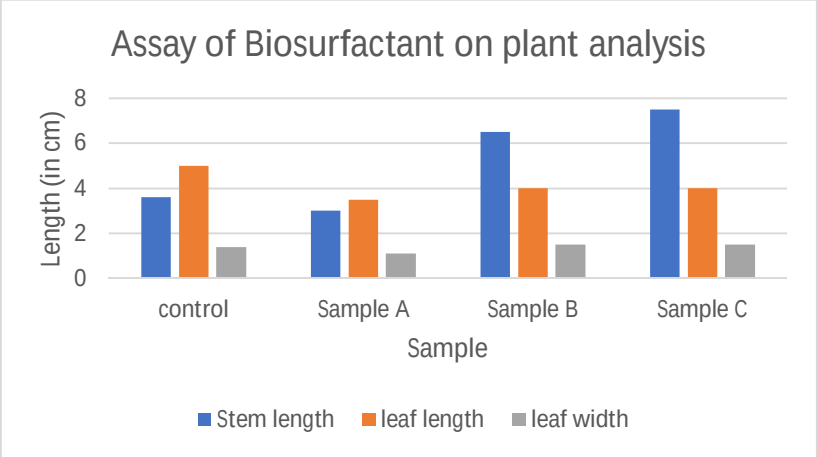

Fig. 11 *Graphical representation of plant growth assay.*

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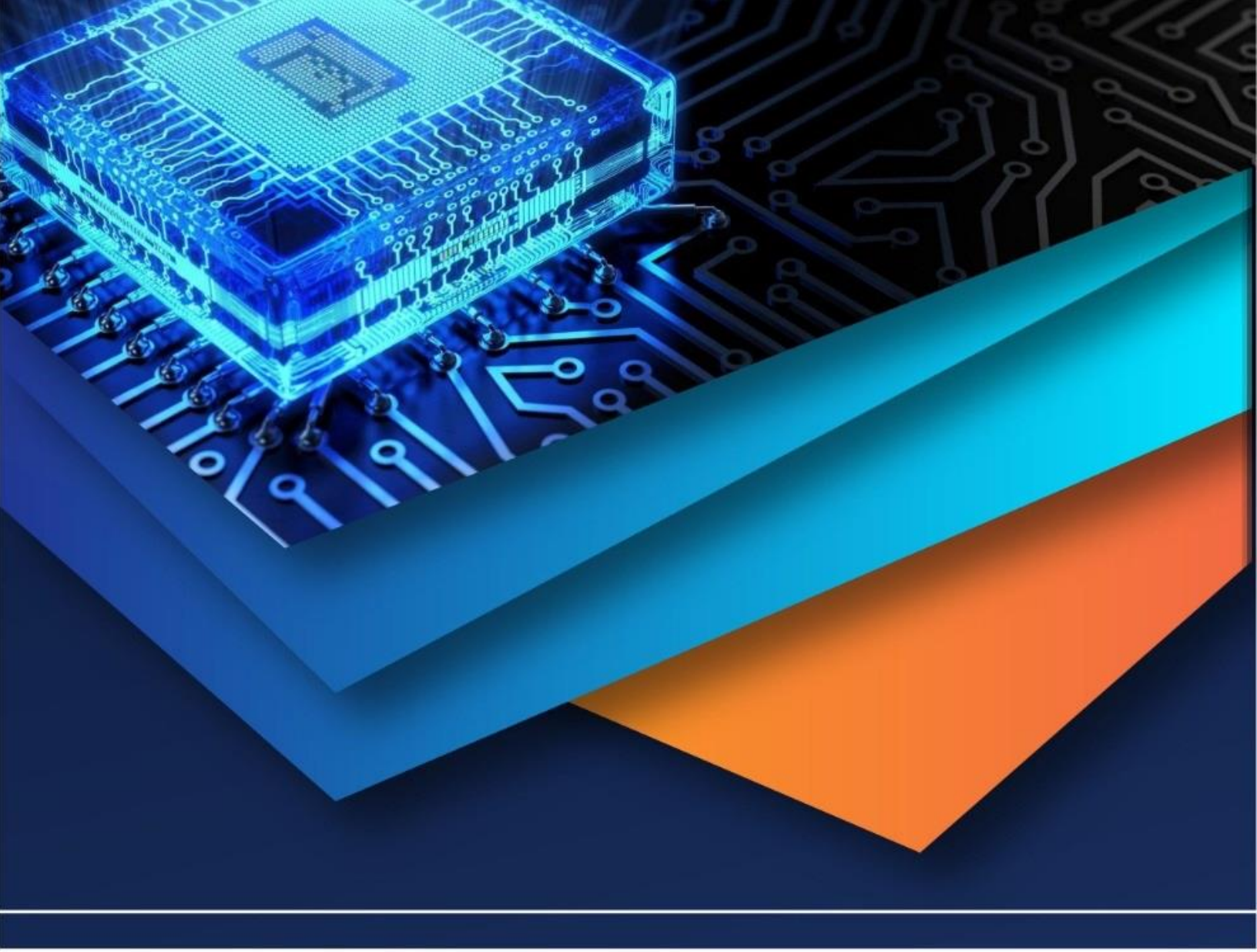

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