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Antimicrobial & Growth Promoting Activity of Pigments from Bacteria Isolated from Ganga Water

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Abstract: ***Many microorganisms, including algae, fungi and bacteria produce pigments. For some, these serve a similar purpose: photosynthesis. For others, these pigments play a protective role, for example to absorb UV radiation or to quench free radicals of oxygen. Other pigments have been suggested to have antibiotic properties. The bacterial pigment xanthomonadin offers protection against photodamage (Rajagopal et al., 1997). In Rhodobactersphaeroides, carotenoids are essential constituents of the photosynthetic apparatus and are assumed to prevent theformation of singlet oxygen by quenching of triplet bacteriochlorophylla (Jens Glaeser and Gabriele Klug , 2005). In this study water sample were taken from Ganga River to isolate some pigment producing bacteria so that we can see the role of pigments in other organisms such as fungi & bacteria & to characterize them. Here we want to check the antimicrobial properties of the pigments on other organisms such as bacteria & fungi & also whether they can promote growth of other organism or not.***
Keywords: Pigments, Free radicals, UV radiation, singlet oxygen, antimicrobial properties,

I. INTRODUCTION

With the increase in awareness in toxicity of synthetic colors, demand for pigments in the form of natural color in food, industry, cosmetics are increasing. Natural dye can be obtained from ores, insects,bacteria, plants, animals etc. These natural pigments may be nontoxic, nonhazardous to environment. Among them microbial pigments are superior as because they are fast growing, less space required, no criteria for seasonal growth & most important easy to manipulate.Studies on chromogenic bacteria has started very early on Bacillus polychromogens by Chamot and Thiry in the year 1900, on Flavobacteriumlasseuri by Lasseur in 1913, on Serratiaanoium by Duran Reynolds and Clausen in 1937; on Pseudomonas by Kluyveret al. in 1939.

Microorganisms synthesizes pigment to protect itself from injurious effect of light rays of visible and near ultraviolet range. These pigments are synthesized as secondary metabolites and not often found in all types of organisms. Pigments come in a wide variety of colors, some of which are water soluble.Micro-organisms which have the ability to produce pigments in high yields include species of Monascus, Paecilomyces, Serratia, Cordyceps, Streptomyces and yellow-red and blue compounds produced by Penicilliumherquei and Penicilliumatrovenetum, Rhodotorula, Sarcina, Cryptococcus, Monascuspurpureus, Phaffiarhodozyma, Bacillus sp., Achromobacter, Yarrowia and Phaffia also produce a large number of pigment . Microorganisms produce various pigments like carotenoids, melanins, quinones, flavins, prodigiosins and more specificallymonascins, violacein or indigo.

This research work will provide information not only on the types of pigmented bacteria but also about the pigments that are produced by them.

If the pigments show antimicrobial property, they shall find their application in agricultural and industrial fields especially as protective agent against plant diseases or as a weather coat for metal sheets / outside wall paints, then it will be of prime importance for the isolation of these type of bacteria.

II. MATERIALS AND METHODS

Isolation of pigment producing bacteria:- In order to isolate some pigment producing bacteria, water sample from the riverside Ganga were taken on a sterile conical flask & serial dilution were made upto 10^{-8} . Then serial dilution and standard plate count method were performed on Nutrient Agar plates. Plates were incubated in an incubator for 24 hours at 37˚C. Four colonies- I_1-Bluish Green, I_2-Brown, I_3-Yellow, I_4-orange were chosen for further analysis

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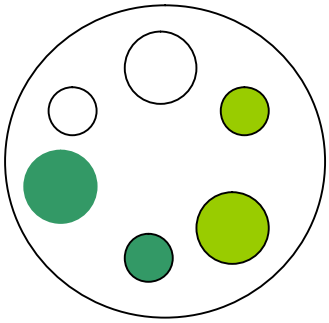
A. Characterization Of Pigments Produced By Bacteria

1) *Extraction Of Pigment:* 24 hour cultures of the each sample were taken in sterile eppendrof tubes & centrifuged at 10,000rpm for 10 minutes. The supernatant were collected containing the pigments. Then each pigment sample is passed through 0.22micrometer Millipore filter to prevent the presence of any bacterial cell in the pigment mixture.
2) *Biochemical Characterization Of Pigments:* Pigments were biochemically characterized on the basis of -Diffusibility through agar ; pH of the pigments; Solubility test in water, alcohol(90%), Acetone, Methanol, Chloroform,benzene, Petroleum ether. Stability test was per performed by (a) Storage in dark, (b) Storage in light , (c) High Temperature.
3) *Spectrophotometric Analysis Of Pigments:* UV visible analysis of spectrum for 4 pigments were done. Absorption maxima of the pigments were taken by using both UV & Visible light starting from 180nm to 780nm.
4) *Determination Of Antimicrobial Property Of Pigments:* In order to determine if the pigments had any antimicrobial effect (i.e.Antibacterialand antifungal),100□l of 24 hrs fresh culture of *Bacillus subtilis* and *Escherichia coli*(test organisms) were spread on NA plates. Now pigment was diluted at the ratio – 1:20, 1:50, 1:100. 3 wells were cut on each plate at uniform distance. The pattern of addition of pigments are shown in figure(a). After addition the plates were kept at incubator for 24hrs at 37^{o} C& zone of inhibition were measured accordingly. Similar experiment were conducted on fungal test organism by using *Penicilliumsp, Rhozopussp* in Czapecdox agar media. These plates were incubated at 28^{o} C for 48 hours.



Fig(a)

'C' → Control (Sterile water)
Crude→ Pigment (without dilution)
1:100, 1:50, 1:20→ are the dilutions of pigment volume added to each well → 50 □l

5) Determination Of Growth Promoting Activity Of Pigment

a) Aim: To determine if pigments have any growth promoting activity or not each of the four bacterial pigmented soup were tested against 24 hrs fresh cultures of *Escherichia coli* , *Bacillus subtillis* **.** The pattern of addition of culture and pigments are tabulated below:

	LB □□□□□□□□□□l)	Test organism □□l)	Pigment □□l)	Sterile distilled Water □□l)	Total Volume
Control	4800	100	----	100	5 ml
Experiment	4800	100	100	---	5ml

After addition, the tubes were transferred to shaker incubator at 37^{o}C for 24 hrs. The O.D. of these cultures was measured by spectrophotometer at 590 nm wavelength.

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III. RESULTS

A. *Pigment Characterization*

1) *Biochemical Characteristics*

Table 1: The diffusibility and pH of the pigments

Colour of pigment	Produced by bacteria	Diffusible/non diffusible in agar	pH of the pigment
Bluish Green	I_1	Diffusible	9.0 (Alkaline)
Brown	I_2	Diffusible	11.0 (Alkaline)
Yellow	I_3	Non Diffusible	9.5 (Alkaline)
Orange	I_4	Non Diffusible	10.0 (Alkaline)

Table2: The effect of light and temperature on the pigments

Colour of pigment	Stability		
	Dark(at RT) (observed for 7 days)	Light (observed for 7 days)	Temperature (121^oC)
Bluish Green	Stable (The bluish part settles at the top of the test tube, remaining solution is green)	Unstable	Turns slightly dark green
Brown	Stable	Stable	Stable
Yellow	Stable	Stable	Stable
Orange	Stable upto 72 hrs	Unstable	ND

ND- Not Determined

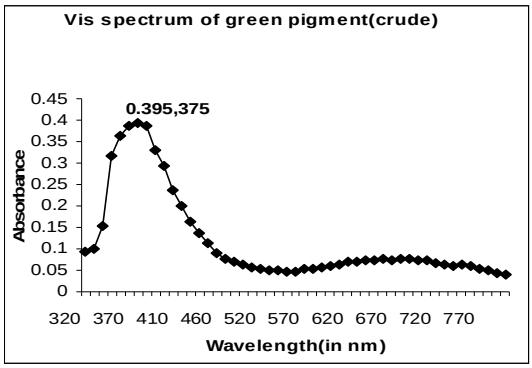
Table 3: The solubility of pigments in various compounds

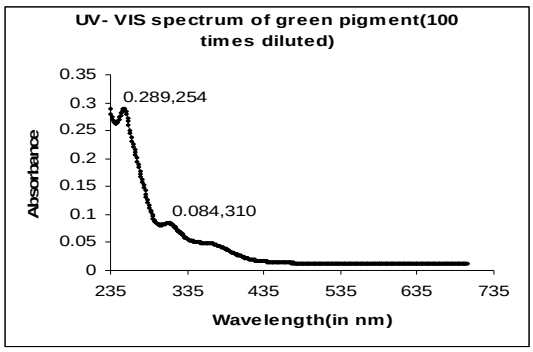
Colour of pigment	Solubility								
	Water	Acetone	0.2(M) HCl	0.1(M) NaOH	Ethanol	Methanol	C_6H_6	$CHCl_3$	Pet ether
Bluish Green	√	√	√(colour changes to pink)	√	√	√	√	√	√
Brown	√	√	√	√	√	√	√	√	√
Yellow	partially	√	√	√	√	√	I	I	I
Orange	partially	√	√	√	√	√	I	I	I

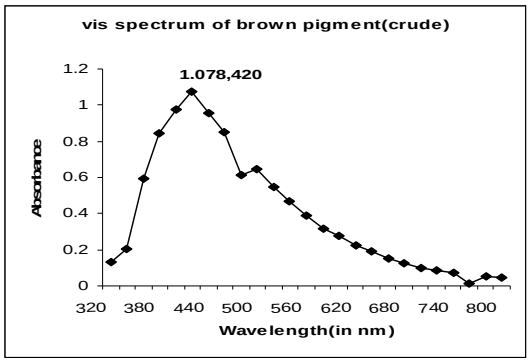
2) *Spectrophotometrical Analysis*

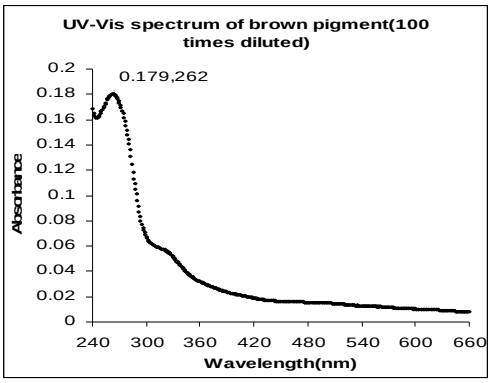
Table 4: The absorption spectra of all the pigments at UV- Vis range

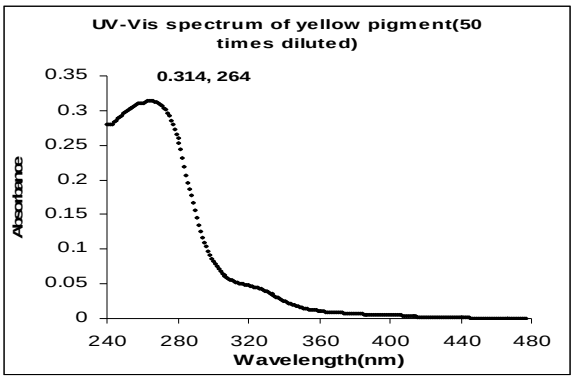
Colour of pigment	Absorption maxima(*)		
	UV range (200-400 nm)	Visible range (400-800 nm)	Comments
Bluish Green	254*, 310, 375	-	No peak observed at the visible range
	In 0.2M HCl 266*	-	-
	In Methanol 248*, 325	-	-
Brown	262*	420*	A peak observed at the visible range
Yellow	264*, 370	-	No peak observed at the visible range
Orange	252*, 316	-	No peak observed at the visible range

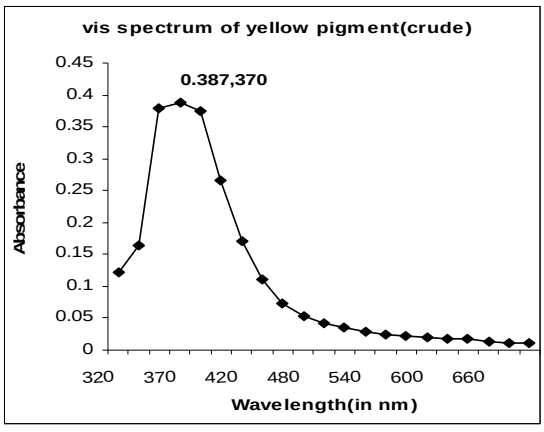


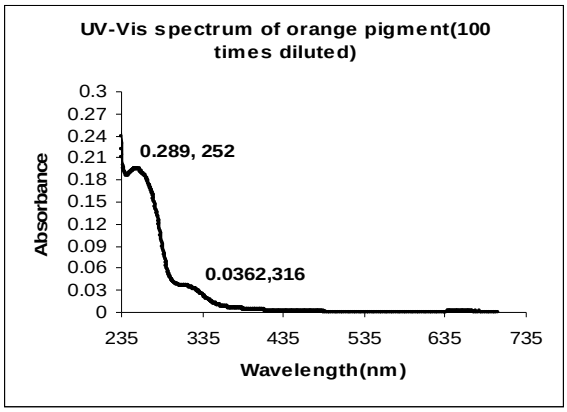


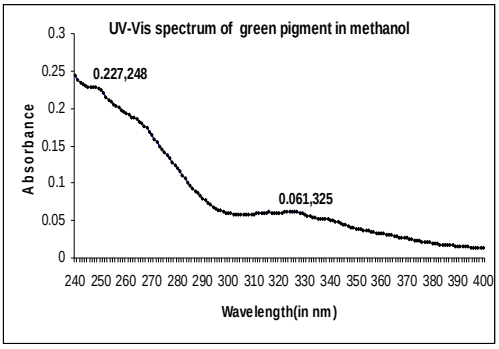


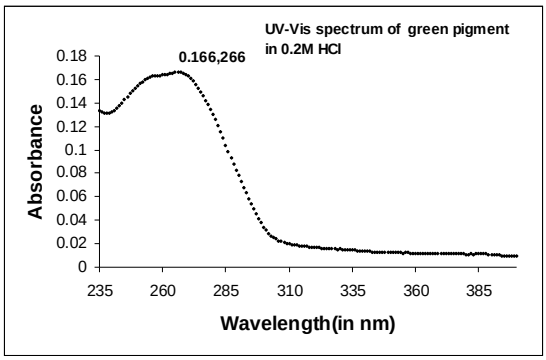












The diffusible green pigment and brown pigmentwas extracted from liquid bacterial cultures by centrifuging the cells to pellete down and pigments remaining in the supernatant. Non diffusible pigments like the yellow and orange were also extracted from broth cultures by the similar procedure. Alkaline bluish green pigment turned pink in 0.2M HCl solution and was stable upto 96 hrs at room temperature and for 13 days under refrigerated conditions. The green colour got decolourised after 96 hrs in 0.1M NaOH. The brown pigment is very much stable under all conditions of high temperature, light and dark. The yellow pigment got decolourised after 21 hrs at 0.2 M HCl. The orange pigment turned pinkish orange in 0.2M HCl. Bluish green pigment turned light blue in acetone and chloroform solution.

All the pigments showed maximum absorption at the UV range. Diluted solutions of pigments showed no peak at the visible range. The absorption maxima of pigments at the vis range is concentration dependent. The purification of the pigments is necessary to further characterize the biochemical properties.

3) Antibacterial & Antifungal Property

Table 5 : The antibacterial effect of pigments

Bacterial pigments	*Escherichia coli*	*Bacillus subtilis*
Green (crude)	x	√ (Zone of diameter =19.0mm)
1:20	x	X
1:50	x	X
1:100	x	X
Yellow	x	X
Brown	x	X
Orange	x	X

Table-6:The antifungal effect of pigments

Bluish green (crude)	22.0mm	37.0mm
1:20	X	x
1:50	X	x
1:100	X	x
Brown (crude)	√	22.0mm
1:20	X	x
1:50	X	x
1:100	X	x
Yellow(crude)	X	31.0mm
Orange (crude)	Not found	x

x: Not having activity

Only Green pigment (Crude) shows antibacterial activity against Bacillus subtilis (the zone of diameter is 19.0 mm) but not against E.coli cells. The brown and yellow have no antibacterial activity against E. coli & B. subtilis cells but have antifungal activity on

Rhizopus sp. The orange pigment did not show anti bacterial or anti fungal activity. The crude green and the brown pigments also showed antifungal property against Penicillium sp. and F9 isolate(may be Penicillium, Plate . The green pigments is more effective against fungus then the brown pigment as both the crude and diluted green pigments showed antifungal property against Penicillium sp.

4) Growth Promoting Activity

Table 7: Effect of pigments on the growth of other organisms

Bacterial pigments	E .coli	B. subtilis
Bluish green	×	×
Brown	√	√ (brown colour got decolorized)
Yellow	×	√
Orange	×	×

Table 8: Growth promoting activity of pigments shown by OD readings of cells at 590 nm against LB medium as blank.

Bacterial pigments	Organism used	OD readings (Organism in LB media) Control	OD readings (Organism in LB media + pigment)	Increase in OD values
Brown	E.coli	1.195	1.355	0.160
	B. sublitis	0.634	1.183	0.549
Yellow	E.coli	1.195	1.269	0.074
	B. sublitis	0.634	0.791	0.175

The growth promoting activity of brown and yellow pigments were observed for gram positive (*B. subtilis*) but not on gram negative(*E. coli*)bacteria as observed by the increase in the OD values, which indicated the increase in growth of the organisms after the addition of the pigments. The brown colour got decolourised when added to *B. subtilis* culture. Further research studies are required to understand the mechanism of the growth promotion activity of the two pigments.

IV. DISCUSSION & CONCLUSION

Four types of pigmented bacteria (I_1, I_2, I_3, I_4) were isolated. The pigments were extracted by growing the cells in broth and pelleting the cells down, pigment remaining in the supernatant The pigments were also extracted by scraping cells from solid agar plate and suspending them in organic solvents. Then centrifuging the suspension, cells remaining as the pellet and the pigments in supernatant solution. Green pigment was soluble in water, got diffused through agar, stable in dark, alkaline in nature, got changed to pink colour in acid, soluble in solvents like alcohol, acetone, chloroform, showed maximum absorption at 254 nm(UV range). Brown pigment was soluble in water, got diffused through agar, was alkaline in nature, stable in dark and light observed for 7 days ,soluble in solvents like alcohol, acetone, chloroform, showed maximum absorption at 262 nm(UV range) and 420 nm(Visible range).
Yellow pigment partially soluble in water, did not diffused through agar, was alkaline in nature, stable in dark and light observed for 7 days, soluble in solvents like alcohol, acetone, but insoluble in benzene, chloroform, pet ether. It showed maximum absorption at 264 nm(UV range). Orange pigment was partially soluble in water, did not diffused through agar, was alkaline in nature, soluble in solvents like alcohol, acetone, but insoluble in benzene, chloroform, pet ether, showed maximum absorption at 252 nm(UV range)
Green pigment showed antibacterial property against gm (+) eve bacteria like B. subtilis but not against gm(-)eve bacteria. It also showed antifungal property against Penicillium and Rhizopus sp. The pigment did not show any growth promoting property for B. subtilis and E. coli. Brown pigment did not showed antibacterial property against gm (+) eve bacteria like B. subtilis and against gm(-)eve bacteria like E. coli. It ashowed antifungal property against Penicillium sp and Rhizopus sp. The pigment showed growth promoting property for B. subtilis (brown colour got decolourised) and but not for E. coli. Yellow pigment neither showed antibacterial property against gm (+) eve bacteria like B. subtilis and nor against gm(-)eve bacteria like E. coli but have antifungal property against Rhizopus sp. The pigment showed growth promoting property for B. subtilis only. The application of pigments as a protective agent against microbes borne plant diseases may be applied to benefit plant production. The application of pigments having antibacterial and antifungal properties can be used commercially as weather coats in paints for metal sheets and outside walls.

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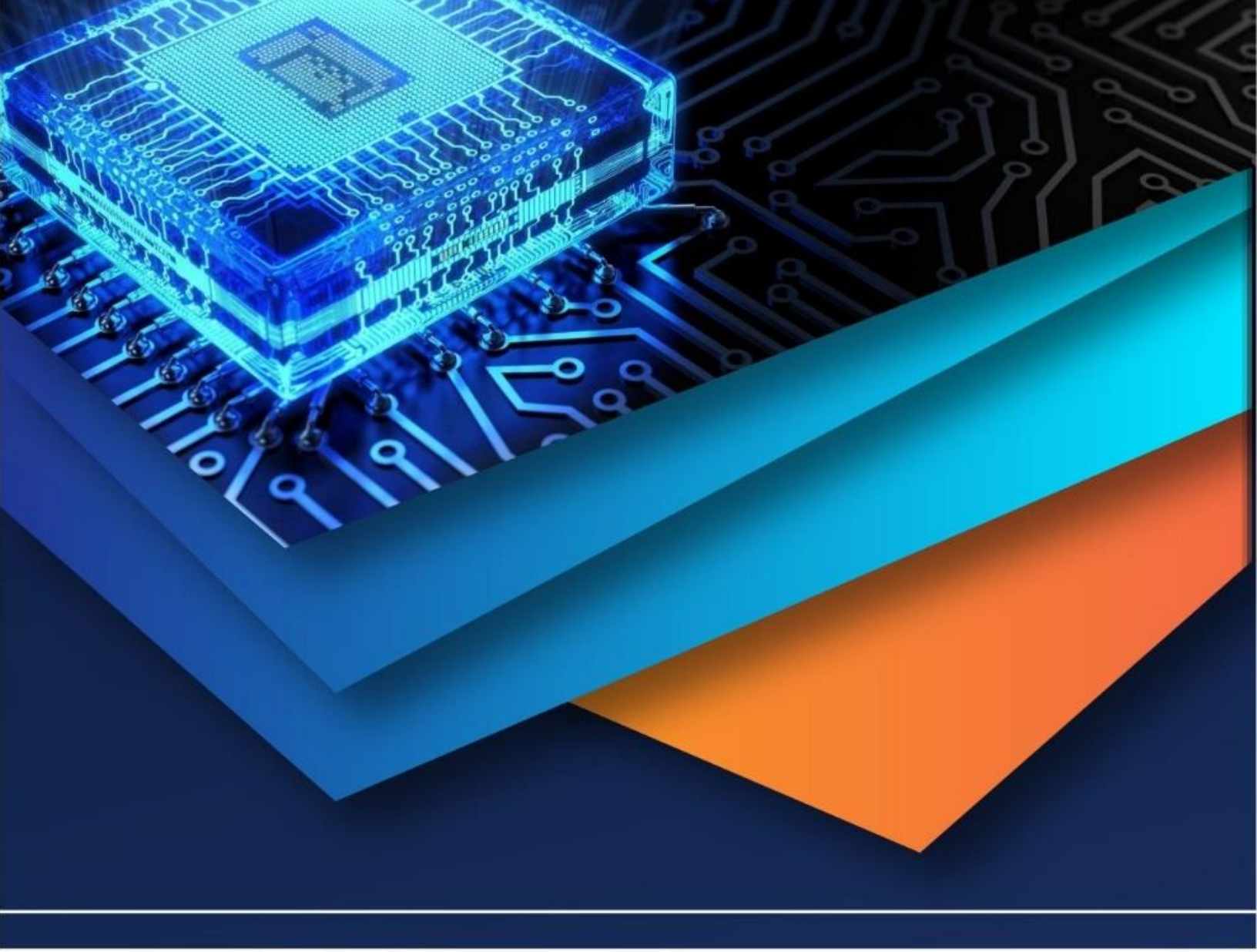

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