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Isolation & Characterization of Nitrogen Fixing, Phosphate solubilizing Fungi from the Coastal areas around Bay of Bengal

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Abstract: ***Rhizospheric soil microorganisms play an important role in regulating the concentration of carbon, Nitrogen, phosphorous, Sulphur in nature. Phosphorous is one of the major essential macronutrient needed for any kind of living cell like plants because they are the component of protein, nucleic acid etc. Although large amount of Phosphorous is present in soil but they are in the form of insoluble phosphate. In this study our aim is to isolate and characterize some phosphate solubilizing Fungi that can fix atmospheric nitrogen & therefore can be employed as a biofertilizer. The study site was riverside Matla, South 24 Parganas. Two fungal strains were isolated KD1 & KD2 (PSM) that possess higher phosphate solubilizing capabilities than other fungi with solubilization index ranges between 2.56-3.03.These two strains nonsymbiotically fix atmospheric nitrogen as they can grow in Burks media(nitrogen free). Morphological characterization & spore count of the organisms were taken.The ability to release soluble phosphate from insoluble tricalcium phosphate were measured by phosphomolybdate ascorbic acid method.***

Keywords: Biofertilizer, PSM, nonsymbiotically, Phosphomolybdate, Ascorbic acid.

I. INTRODUCTION

The principle mechanism for mineral phosphate solubilization is the production of organic acids and acid phosphatases play major role in the mineralization of organic phosphorus in soil. It is generally accepted that the major mechanism of mineral phosphate solubilization is the action of organic acids synthesized by soil microorganism. Solubilization of insoluble P by microorganism was reported by Pikovskaya (1948). During the last two decades knowledge on phosphate solubilizing microorganism increased significantly (Richardson 2001; Rodriguez and Fraga 1999).

Several strains of fungal species have been described and investigated in detail for phosphate –solubilizing capabilities (Glick 1995;He et al. 1997). Such microorganisms have been isolated using cultural procedures with species of Aspergillus and Penicillium fungi being predominant. In soil P solubilizing fungi constitute 0.1-0.5% of the total population. They are generally isolated from rhizosphere and non- rhizosphere soils, rhizoplane, phyllosphere and rock P deposit area soil and even from stressed soils using serial plate dilution method or by enrichment culture technique (Zaidi et al. 2009).

Moreover, fungi in soils are able to traverse long distance more easily than bacteria and hence ,may be more important to P solubilization in soils (Kucey 1983). Generally, the P-solubilizing fungi produce more acids than bacteria and consequently exhibit greater P- solubilizing activity (Venkateswarlu et al.1984).

Among filamentous fungi that solubilize phosphate ,the genera Aspergillus and Penicillium (Fenice et al. 2000 ; Khan and Khan 2002, Reyes et al .1999,2002) are most representative although strains of Trichoderma (Altomare et al. 1999) and Rhizoctonia solani (Jacobs et al.2002) have also been reported as P solubilizers.A nematofungus Arthrobotrys oligospora also has the ability to solubilize phosphate in vivo as well as in vitro (Duponnois et al. 2006).

Among the yeasts, only a few studies have been conducted to assess their ability to solubilize phosphate these include Yarrowia lipolytica (Vassilev et al. 2001), Schizosaccharomyces pombe and Pichiafermentans. Of those identified, many are commonly found in agricultural soils such as Penicillium sp.,

Mucor sp. And Aspergillus sp. which has been shown to increase plant growth by 5-20 % after inoculation (Gunes et al.2009). In this study two fungi from rhozospheric soil were isolated from riverside Malta and tested for phosphate solubilizing abilities by growing them in Pikovskaya media. These strains were also tested for nitrogen fixing capabilities by growing them in nitrogen free Burks media.

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II. MATERIALS AND METHODS

A. Collection Of Soil

For the purpose of assessment and identification of phosphate solubilizing fungi, Soil samples collected from 4 regions of the riverside of Matla, 24 Parganas, West Bengal. Due care was taken during sample collection. Samples were collected from depth of 3 to 6 inches. The soil sample was collected in sterilized sampling bottle. Different Soil samples were homogenized to make a single sample.

B. Isolation of Phosphate solubilizing micro fungi

For isolation and screening of microbial strains, 1 gram of soil sample collected from sampling site was dissolved in 10 ml of distilled water. After vortexing, the soil suspension was diluted serially to 10^{-1} to 10^{-6} NA spreaded in Czapecdox agar media. Different fungal colonies were isolated & tested for further analysis.

C. Detection Of Phosphate Solubilizing Fungi

In order to detect whether the isolated fungal samples are able to solubilize inorganic phosphate, all isolated colonies were streaked on both Burks media enriched with tricalcium phosphate & incubated for 4 days. Appearance of growth in Burks media with halo zone indicates that they are phosphate solubiliser & nitrogen fixer. Two colonies were chosen as they have higher solubilizing index.

D. Morphological Characterisation By Lactophenol Cotton Blue Staining

The isolate will be characterized for its lactophenol cotton blue staining characteristics as per the standard procedure. Morphological characteristics of isolates viz. shape, size, elevation, surface form, margins and surface texture, colour were observed for their characterisation. We will also use different biochemical reactions to characterize these isolates.

E. Spore Count Using Haemocytometer

For spore count, strains KD1 and KD2 were centrifuged at 8000rpm for 5 mins.10µl sup was taken on haemocytometer and observed under microscope at 40X.

F. Phosphate Solubilization Index (SI)

We assessed the phosphate solubilisation by solubilising index(SI).A clear zone around a growing colony indicated phosphate solubilisation.SI was calculated as the ratio of the total diameter (colony+ halozone)to the colony diameter.

$$SOLUBILIZATION\ INDEX = \frac{\text{colony diameter + halozone diameter}}{\text{colony diameter}}$$

G. Determination Of Rate Of Soluble Phosphate Release

Soluble phosphate estimation were measured by Phosphomolybdate Ascorbic acid method. All 2(KD1 &KD2) cultures were inoculated in 2 separate Pikovskaya (PVK) broth of 200 ml and kept in a shaker at 28-30^{0}C. We collected samples of the PVK broth before inoculation (0 hours recording). 1.5ml of overnight culture was collected in eppendorf tube and kept in spin win about up to 10 minutes at 1000 rpm.

Then soup was collected in new eppendorf tube and pellet was discarded. The same procedure was repeated for next 5days. On 6th day, we prepared a solution of 20ml of 2.5% Ammonium molybdate, 20 ml of 6 N H_2SO_4 and 20ml 10% Ascorbic acid. These three are mixed and 40 ml of H_2O is added to the mixture.

Now 4 ml of the above prepared reagent and 1 ml soup collected at different hours over the last 6 days were mixed and 1 tube contains only the control. The set of test tubes was incubated for 1 hr. at normal room temperature. After 1 hr. of incubation OD is measured at 829 nm.

Then standard curve of NaH_2PO_4 was prepared and concentration of phosphate was determined from the standard curve.

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III. RESULTS

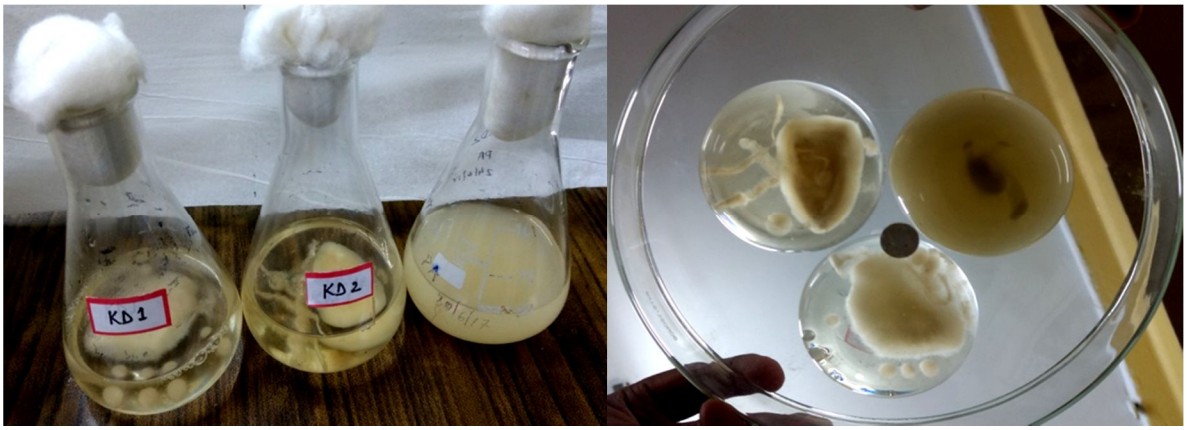


Figure no:1 &2 . KD1 & KD2 inoculated in pikovskaya (PVK) broth and incubated for 24 hrs

A. Colony Characteristics as a Result of Subculture in Burk's Medium

The 2 samples were streaked on Burk's media and incubated for 24 hrs at 37 ˚ C . Then the obtained colonies from both the sample were stained by Lactophenol cotton blue procedure and observed under microscope.

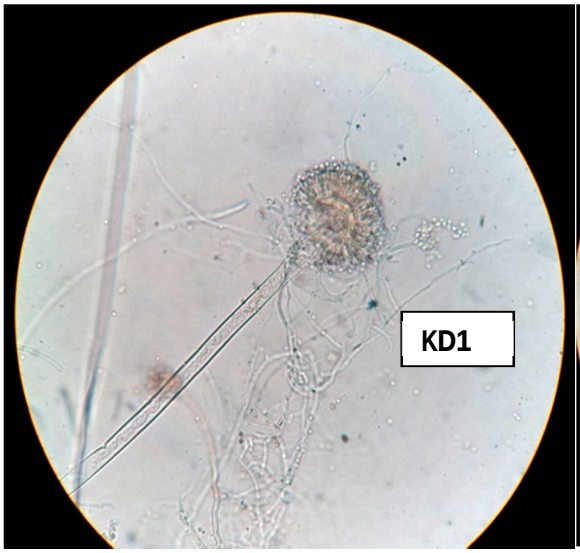


Fig:3 Kd1 Under 100x (Oil Immersion)

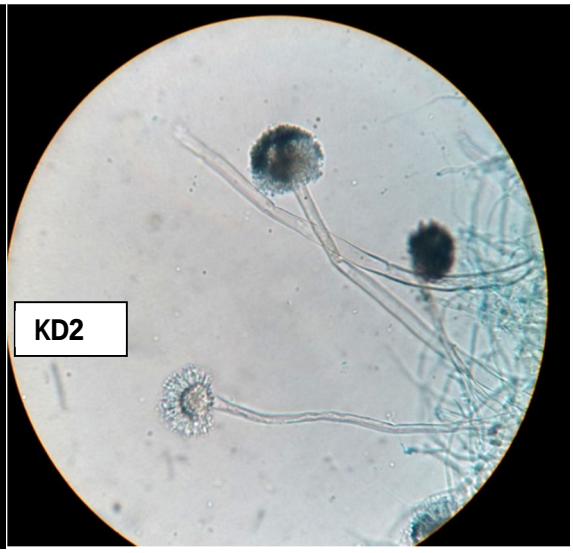


Fig:4 Kd2 Under 100x (Oil Immersion)

ISOLATES	MICROSCOPIC OBSERVATION
KD1	Large, globose , brown and biseriate conidial heads, which become radiate ,conidiophores are smooth – walled,septate metulae. Conidia globose to sub globose, brown to black ,rough- walled.
KD2	Conidial heads short ,columnar and biseriate .conidiophore short ,brownish, smooth walled.conidia globose and rough walled .

Table:-1. Microscopic Character of KD1 & KD2

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B. Spore Count By Using Haemocytometer

	Big squares (3 x3) haemocytometer count			
Spore count	Square 1	Square 2	Square 3	Square 4
KD1	18	10	6	5
KD2	22	14	22	24

Table 2.Haemocytometer count

$$\text{Spore Density (spores/ml)} = \frac{\text{average cells x Dilution factor}}{\text{Volume of a square (ml)}}$$

Spore count	Avg. cells	Dilution factor	Vol. of a square	Spores/ml
KD1	(18+10+6+4) ÷ 4	1	0.0001ml	95,000spores/ml
KD2	(22+14+22+24)÷4	1	0.0001ml	2,05,000spores/ml

Table 3: Spore count/ml

C. Zone Observation & Measurement Of Solubilizing Index(Si)

Solubilizing Index was calculated as the ratio of the total zone diameter (Colony +Halo zone) to the colony diameter

$$solubilization\ index = \frac{\text{colony diameter + halozone diameter}}{colony\ diameter}$$

	KD1	KD2
1.	2.4	2.7
2.	2.8	3.6
3.	2.5	2.8
MEAN	2.56	3.03

Table 4: Solubilization Index value

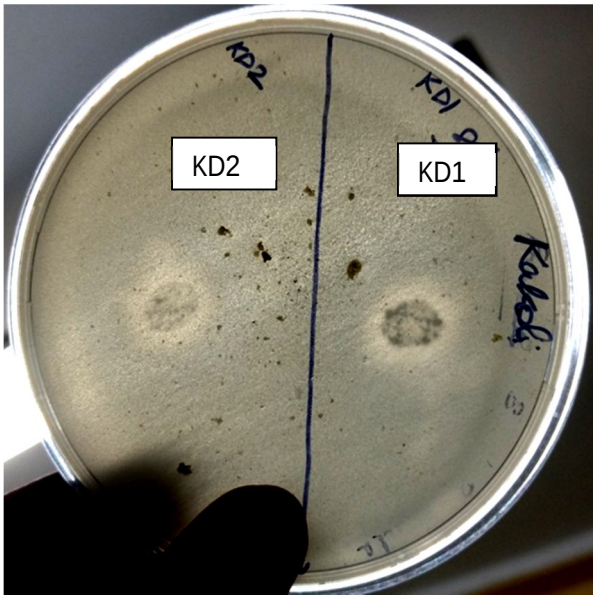


Fig:5 Phosphate solubilizing zones of KD1 and KD2 observed.

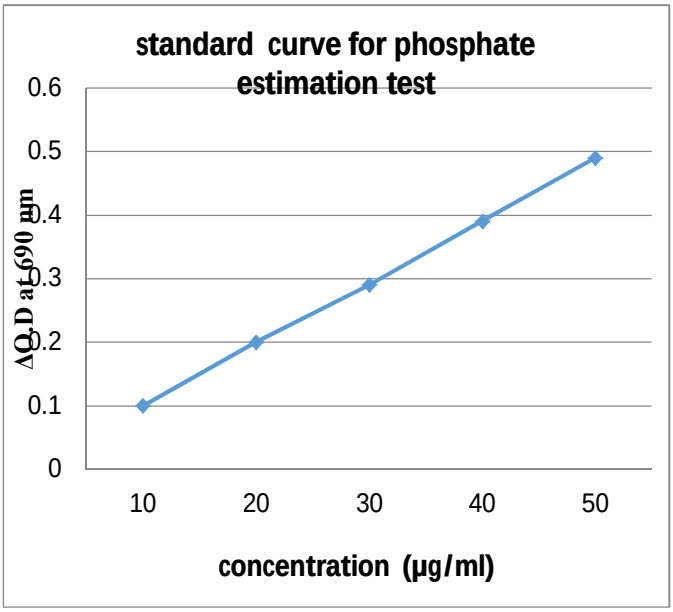


Fig:6 Standard curve for phosphate estimation

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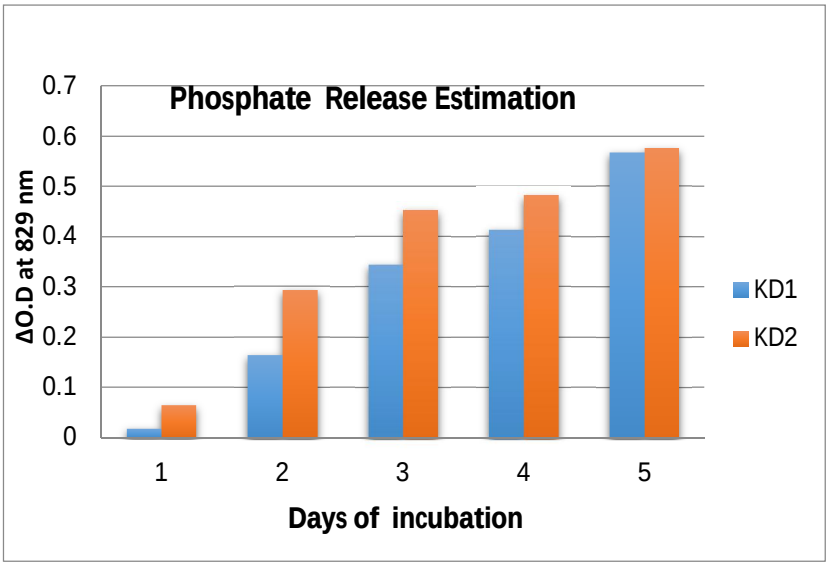


Fig:7. Conversion rate of soluble phosphorous from insoluble tricalcium phosphate

IV. DISCUSSION & CONCLUSIONS

Recently biofertilizers are gaining lot of interest in the agriculture as they are cheaper, easy to produce in large scale and devoid of adverse effects to the soil. Many plant growth promoting bacteria and fungi are being used as biofertilizer. Use of phosphate solubilising microorganisms as environment friendly biofertilizer helps to reduce the much expensive phosphatic fertilizers. Trials with Posphate solubilizing fungi(PSF) indicated yield increases in rice, maize and other cereals. Hence many PSF are now being used as biofertilizer.

As the soil of Sunderban is known to be better for cultivation compared to soils of other districts of West Bengal; we thought of isolating and characterizing different strains of phosphate solubilising and nitrogen fixing fungi in the soil of Sunderban. So we assume from this study that presence of various phosphate solubilising and nitrogen fixing fungi in the soil is one of the causes of the high fertility of the Sunderban. It is to be noted that there are other factors like presence of other plant growth promoting microorganisms which also could be the cause of high fertility. It needs to be evaluated in a larger study which requiring longer duration of time.

In this investigation, soil samples from different parts of Sunderban have been collected and processed and 4 strains have been isolated among which two strains KD1 and KD2 were found to show significant phosphate solubilising and nitrogen fixing activity as found from the clear zone in solid Pikovskaya media plates containing tricalcium phosphate and growth in Burk's media. Spores were transparent at the middle surrounded by thick black wall. KD1 and KD2 both produces spores. KD1 spore count per ml of suspension is 95,000 whereas KD2 spore count is 2,05,000 per ml.

KD1 is globose,brown,biseriate conidial heads,conidiophores smooth – walled,septate metulae ,conidia globose,brown fungi. It can solubilze phosphate, fix atmospheric nitrogen. The rate of release of soluble phosphate increases in each and every day as indicated in the graph. Among the two isolates the rate of conversion of insoluble phosphate to soluble phosphate is higher in KD2 isolates than KD1. Identification and further biochemical characterization need to be done.

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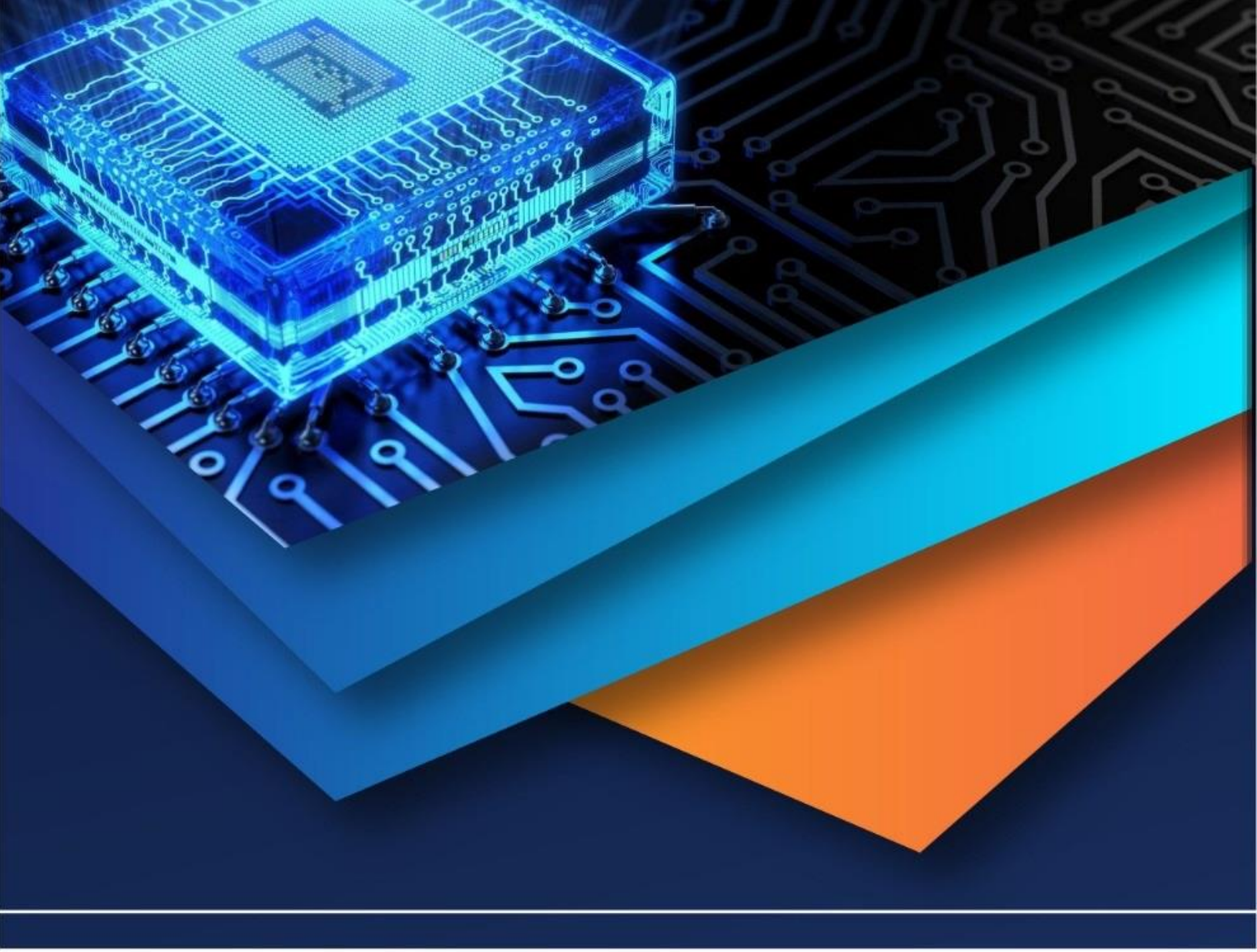
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