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Optimization and Production of Phytase by Bacillus subtilis and its Agricultural Application

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Abstract: ***In an attempt to explore extracellular phytase producing Bacteria from soil, the culture B-7 isolate was selected on the basis of formation of the clear zone on PSM plate and by assaying fermented broth. The isolate B-7 identified as Bacillus subtilis through morphological, cultural and Biochemical characterization as per Bergey's Bacteriological analysis. As the fermentation, the process is significantly influenced by various components present in the medium and its concentration. Hence, the isolate was tested in varied concentrations of components as per Plackett-Burman method. The maximum production of phytase was observed at 72 hours of incubation at RT and at pH 6.5. The most suitable medium is wheat bran extract medium shows 81.30 U/ml. the study also shows that the di-potassium hydrogen phosphate has a negative effect on phytase production and wheat bran shows strong positive effect. Finally, the application of Phytase was carried out by an In-vitro and in-situ method on Maize, Sorghum, and wheat. In the In-vitro method, maize is the best-supported Plant as Shoot is concerned. In In-situ method Maize, Sorghum, and wheat give 12mm, 18mm, 7.5mm root length, and 13mm, 10mm, 11mm shoot length after 12 days.***

Keywords**: *Phytase, Phytate, Bacillus subtilis, Plackett-Burman, Maize***

I. INTRODUCTION

Plants require phosphorus for their growth. Besides the use of phosphate, solubilizing microorganisms that make phosphorus from the rock phosphate, use of organisms to release the phosphorous from the phosphorylated organic molecules such as Phytate, also constitute an important means[2]. This involves the use of an enzyme phytase produced by these microorganisms. Commercial production of this enzyme by cost effective methods would involve the screening of cheap substrates and optimization of the medium.

Phosphorous is an essential component of living organisms, it is found as orthophosphate, pyrophosphate, sugar phosphate, nucleic acids, and derivatives of another form of phosphate. Phytic acid (Myo- inositol hexakisphosphate) is an anhydrous storage and organic form of phosphate, which comprises up to 80% of total phosphorous of most plant products like seeds and pollen grains [3,15]. Phytic acid acts as an antinutritional factor by chelating metals (Ca+2, Mg+2, Zn+2 and Fe+2), also forming a complex with proteins, making them unavailable to the organism[11,13], thereby, affecting digestion of animals by inhibiting enzymes like amylase, trypsin, acid phosphatase and tyrosinase [5,10]. The majority of phosphorus is in its insoluble form. A low abundance of phosphorus is typical in many agricultural soils, and where phosphates are often complexed to soil constituents, making them unavailable to many organisms [5,6]. To overcome plant growth limiting phosphate deficiency, phosphate dense fertilizers are applied to crops regularly. However, plants can only absorb a limited amount of phosphates and the rest is rapidly converted into insoluble P. There is also an extensive loss of phosphates in agricultural lands via runoff and much of the phosphate ends up in water reservoirs.[6]

Phosphate is essential for the optimum growth of most bacteria and has a central role in many metabolic and energy producing pathways. Microorganisms associated with hydrolyzing organic and inorganic phosphates are known as phosphate-solubilizing bacteria (PSB)[]. Typically, inorganic phosphate solubilization is initiated as a consequence of the actions of low molecular organic acids such as gluconic and citric acid[16].Organic P compounds undergo mineralization and the resulting P will be readily available for the plants to absorb [19]. This mineralization process is facilitated by enzymes secreted by soil microbes such as phosphatases and phytases [17,18]. Since, the phosphate of phytic acid is not metabolized by monogastric animals (pigs, poultry, and fishes) due to lack of phytase, it comes to soil environment with excreta, consequently, contributes to the phosphorous mediated pollution problems [2]. Enhancement of phosphorus concentration in aquatic body forms the cyanobacterial bloom and hypoxia, causing the death of animals therein. [14]

A phytase is a group of enzymes responsible for phytate hydrolysis[1]. Common bacterial phytase (EC 3.1.3.8, Myo-inositol hexakisphosphate 3-phosphorylase) is the member of the histidine acid phosphatase subfamily that catalyzes the hydrolysis of phytic acid to inorganic phosphate and myoinositol phosphate derivatives[12]. Commonly, the phytase is an inducible enzyme that

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encompasses complex gene regulations for its synthesis [19,22]. Considering critical impacts of such enzymes in the dissolution of complex compounds into usable P, it is highly desirable to develop the bacterial inoculants with high phosphatase and phytase activity to overcome P-limiting soils[20,21].

II. METHODOLOGY

All high-grade chemicals were used for the work. Pure Phytate obtained from Sigma-Aldrich.

A. Isolation and screening of phytase producing bacteria

Rhizosphere soil samples of Legumes and poultry farm soil samples were collected from various regions. One gram of each sample was suspended in (10) ml of sterile distilled water and was serially diluted and the best dilution of each sample was spread onto PSM (Phytase Screening Medium) plates8. The agar media composed of Glucose (1.5) %, (NH4)2SO4 (0.5) %, MgSO4.7H2O (0.01)%, KCL(0.05)%, NaCl(0.01)%, CaCl2.2H2O(0.01)%, FeSO4(0.001) %, MnSO4(0.001) %, Sodium Phytate (0.5) %. The pH was adjusted to 6.5 and (1.5) % agar was added before autoclaving at (121) °C for (15) minutes. The inoculated plates were incubated at temperatures of (37) °C for (1-3) days and observed for the clear zones of hydrolysis around the colonies which gave an indication of extracellular phytase production. Each such colony was picked up and maintained till further use.

Screening for best phytase producing strain microbial colonies capable of hydrolyzing Phytate which can be recognized by their surrounding clear halo was obtained by re-plating single colonies. The halo (Z) and colony (C) diameters were measured after (3) days of incubation at (37) °C. Hydrolysis efficiency of all the isolates were determined by the formula Z-C/ C.

B. Identification of the selected phytase producing bacterial isolate

The isolate (B7) was identified based on the identification scheme in Bergey's Manual of Systematic Bacteriology. The strain was initially examined for cell morphologies and cell arrangement by gram staining, presence or absence of spores and capsules and motility using microscopy. The various biochemical tests carried out.

C. Production of phytase

The isolate was subjected to fermentation in a fermentation medium containing the following(g/L): $(NH_4)_2SO_2$-0.4g, $MgSO_4.7H_2O$-0.2g, casein-1g, KH_2PO_4-0.5g, K_2HPO_4-0.4g dissolved in 1000 ml of wheat bran extract. The pH of the medium was adjusted at 6 before sterilization. It was then inoculated with 10ml of inoculum along with the addition of pre-sterilized CaCl2 (0.2%) and incubated on a rotary shaker at 200 rpm for 3 days. After 3 days of fermentation, the fermented broth from the flask was transferred into centrifuge tubes and centrifuged at 10000 rpm for 15 minutes at 4^0c. The supernatant was then transferred into the clean flask which was used as crude enzyme solution. Along with that, the daily analysis of enzyme production was also carried out. Also, the production was carried out in Maize fall extract medium, phytase screening medium and in Maize starch medium by providing optimum environmental conditions.

D. Phytase assays

Phytase assays were routinely assayed by incubating 0.1ml of enzyme solution with 0.9ml of 2mM sodium Phytate in 0.1M Tris-HCl buffer (pH 7.0) at 37oC for 10 min. After incubation, the reaction was stopped by adding 0.4ml of 10% TCA and the released inorganic phosphate was measured spectrophotometrically at 630 nm by adding 0.4ml of 2.5% ammonium molybdate and 0.2 ml ANSA reagent.[9]

One unit of enzyme activity was defined as to liberated 1µmol of phosphate per minute per ml under the assay condition.

E. Screening of medium components

Seven components were screened for their effect on phytase production by Bacillus subtilis. The Plackett-Burman design was used for the procedure. The seven component were represented at two levels high (+) and low (-) as (shown in tables 1 and 2). The number of positive and negative signs per trial is (k+1/2) and (k-1/2) respectively. Each column comprises an equal number of positive and negative sing and each row represent a trial. Effect of each variable was determined by the following equation-

$$E(xi) = 2\left(\sum mi_{+} - \sum mi_{-}\right)/N$$

Where,

E (xi) = Concentration effect of the tested variable

mi_+ and mi_- = Phytase activities from the trials were the variable (xi) measured was present at high (+) and low (-)

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levels.

N = Number of trials

Table no.1: - The Plackett-Burman experimental design matrix

Combination Trials	Assigned variables						
	X1	X2	X3	X4	X5	X6	X7
1	+	-	-	+	-	+	+
2	+	+	-	-	+	-	+
3	+	+	+	-	-	+	-
4	-	+	+	+	-	-	+
5	+	-	+	+	-	-	+
6	-	+	-	+	+	+	-
7	-	-	+	-	+	+	+
8	-	-	-	-	-	-	-
Control	0	0	0	0	0	0	0

Control is the flask containing all ingredients in the concentration used in original phytase production medium.

Table no.2: - Variables showing medium components used in Plackett-Burman design.

Variable	Medium components	O value g/100ml	+ value g/100ml	- value g/100ml
X1	$(NH_4)_2SO_4$	0.04	0.06	0.02
X2	$MgSO_4$	0.02	0.03	0.01
X3	Casein	0.10	0.15	0.05
X4	KH_2PO_4	0.05	0.075	0.025
X5	K_2HPO_2	0.04	0.06	0.02
X6	Wheat bran	10	15	5
X7	$CaCl_2$	0.2	0.3	0.1

F. Application of Phytase

Availability of phosphorus is one of the most significant determinants of plant growth. Phytase is one of the extracellular enzymes that trigger the substantial increase in the specific phosphate availability to plants in its presence. An experiment to assess the potential of phytase to promote the growth of common crops, maize, sorghum, and wheat was performed.

1) In-vitro: The method described by D.J Mukesh Kumar et al., 2011 was used here[4].

The surface sterilized seeds were inoculated in low phosphate nutrient medium& tubes were inoculated at R.T for 4 days. Then after that 4 days old seedling were transferred to a tube consisting 10ml of low phosphate nutrient broth which was supplemented with 0.1ml enzyme & was labeled as "Test". The control tubes with the same content but without the enzyme were also kept for incubation. These tubes were then incubated for 12 days with suitable light & dark period. After 12 days, root & shoot length of the germinated seeds were measured.

2) In-situ: Garden soil was taken in glass beakers/glasses and seeds were sowed in it. They were placed near a window to provide the diurnal light cycle. A little water was added to each seed and allowed to germinate. On the fourth day, 1ml broth containing cells was added as inoculums to each seedling and the germination was allowed to continue for a further 12 days after which the results were noted in terms of root and shoot length. Un- inoculated controls were also used for each plant.

III. EXPERIMENTAL RESULTS

In the present study total of 9 colonies showed positive for phytase production. Among these, (2) were from rhizosphere soil

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samples and were designated as A1 to A2 and (7) were from poultry farm soil samples and designated as B1 to B7. All the isolates were spot inoculated to calculate and their halo (Z) and colony (C) ratio. The isolate B7 was found to produce phytase with significantly higher activity (Fig.1) and was selected for further studies.

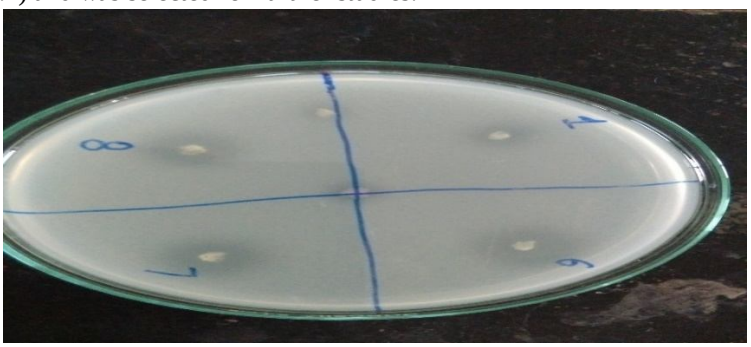


Figure. 1: Zone of Clearence On PSM Agar Plate

A. Screening of crude substrates

Table 3 and fig 2 shows the results of this study using the Bacillus subtilis isolate for the production of phytase using two locally available crude substrates wheat bran and maize fall and the synthetic medium PSM(Phytase Screening Medium) for comparison. The results clearly indicate the crude medium with wheat bran showing superior results.

Table. 3: Units (per ml extract) of enzyme produced with different crude agricultural wastes

	Wheat bran extract	Maize fall extract	Phytase screening medium
0 min (control)	14.45	14.45	14.45
24	45.16	32.52	28.00
48	64.13	49.68	40.65
72	81.30	65.94	52.39

Synthetic phytase screening medium was used for reference

On the basis of these results, wheat bran was used for the further optimization studies

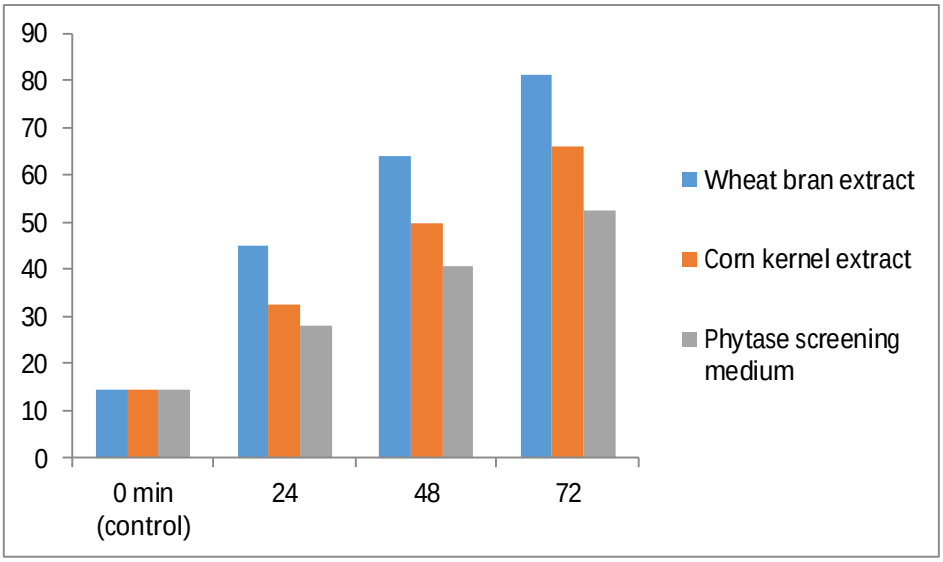


Fig.no. 2.1progress of phytase production with different crude agricultural waste (PSM is used as reference)

B. Optimization of medium components

The widely used statistical Plackett-Burman design was used in this study and results are presented in table 4.2 and figure 4.2. The

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highest production of phytase was observed in trial 1 that used upper limit concentrations of ammonium sulfate, potassium di-hydrogen phosphate, wheat bran and calcium chloride. One other combination, trial 3 also gave a higher yield of phytase in which the four components used in upper limit concentration were ammonium sulfate, magnesium sulfate, casein, and wheat bran. The common factors in these trials are seemed to be only two, ammonium sulfate the nitrogen source and wheat bran the crude substrate serving as carbon and energy source. All the remaining trials (2, 4…, and 8) gave lower production in comparison with the control, although trial six with a yield of 81.3 units was close. Trial eight with all the ingredients in lower limit concentrations acceptably gave the lower yield of phytase.

It is also evident from the same table that component five i.e. di-potassium hydrogen phosphate has a negative effect on phytase production(fig 4.3) probably because it has a major role to play as a buffer in the medium and any change in its concentration tends to affect the growth and productivity of the organism. The component which shows a strong positive effect was wheat bran which goes to support our theory of proving to be a suitable crude substrate for phytase production by the Bacillus subtilis isolate.

Table 4.- Phytase production in media using combinations as per Plackett-Burman design.

Combination Trails	X1 $(NH)_2SO_4$	X2 $MgSO_4$	X3 casein	X4 KH_2PO_4	X5 K_2HPO_4	X6 WBE	X7 $CaCl_2$	Units
1	+ 0.06	- 0.01	- 0.05	+ 0.075	- 0.02	+ 15	+ 0.3	97.56
2	+ 0.06	+ 0.03	- 0.05	- 0.025	+ 0.06	- 5	+ 0.3	50.58
3	+ 0.06	+ 0.03	+ 0.15	- 0.025	- 0.02	+ 15	- 0.1	87.62
4	- 0.02	+ 0.03	+ 0.15	+ 0.075	- 0.02	- 5	+ 0.3	72.26
5	+ 0.06	- 0.01	+ 0.15	+ 0.075	+ 0.06	- 5	- 0.1	75.88
6	- 0.02	+ 0.03	- 0.05	+ 0.075	+ 0.06	+ 15	- 0.1	81.3
7	- 0.02	- 0.01	+ 0.15	- 0.025	+ 0.06	+ 15	+ 0.3	76.78
8	- 0.2	- 0.01	- 0.05	- 0.025	- 0.02	- 5	- 0.1	37.03
Control	0 0.04	0 0.02	0 0.1	0 0.05	0 0.04	0 10	0 0.2	83.10
E(Xi)	11.06	1.12	11.54	18.74	-2.48	26.87	3.83	

Std. Deviation = 18.378

*Key:

WBE - Wheat bran extract

\+ - Upper limit concentration of component

\- - Lower limit concentration of component

0 - Composition concentration in original medium used as Reference

. E (Xi) - Concentration effect of the tasted variable.

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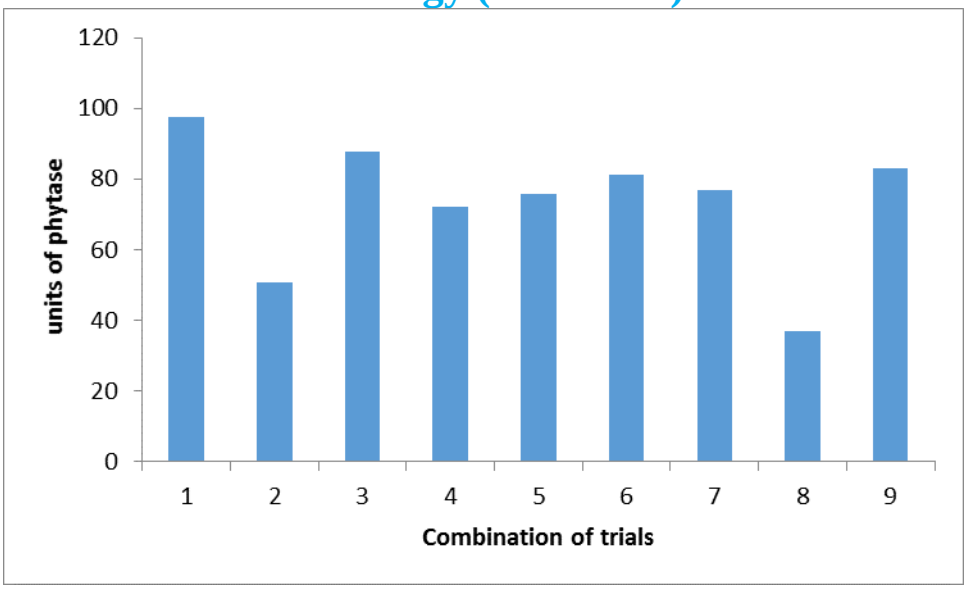


Figure 3: Graphical Representation of Data in Table 4.

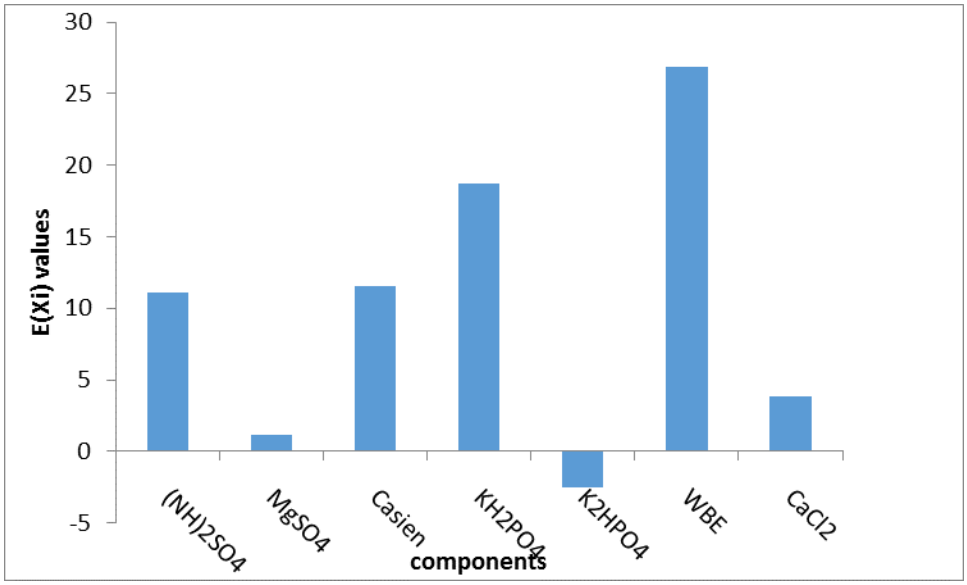


Figure 4: Significance level of component concentration effect

C. Studies on plant growth promoting activity

Phytase has been reported earlier as a plant growth promoting factor that could be used in agriculture and horticulture. Studies on this aspect were performed thru in-situ and in-vitro experiments, on three common agricultural crops- maize, sorghum, and wheat. The results(Fig.5) of in-situ studies presented in table 5 show that the phytase is a strong growth promoting substance as evidenced by the difference in root and shoot lengths between the test and control plants. Maize is the best-supported plant as far as the shoot is concerned. A similar study performed in-vitro (table 6) however gave surprising results where sorghum and wheat did not germinate in the synthetic medium while maize was stimulated almost four times above the control. Phytase, however, did induce germination in sorghum when added in the solid medium directly. But no such effect was observed on the wheat plant. A probable explanation for this observation could be that soil provides some yet unknown nutrient to sorghum and wheat that is lacking in the synthetic medium used for the study. This obviously is not the case with maize. This study was also performed on wheat using sterile soil. In this study also the wheat was stimulated by phytase to the same extend as in the unsterile soil but the comparative size of the root and the shoot were greater (10 and 13 respectively). This is probably due to a role played by some other factor –probably

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microorganisms- that are destroyed by sterilization.

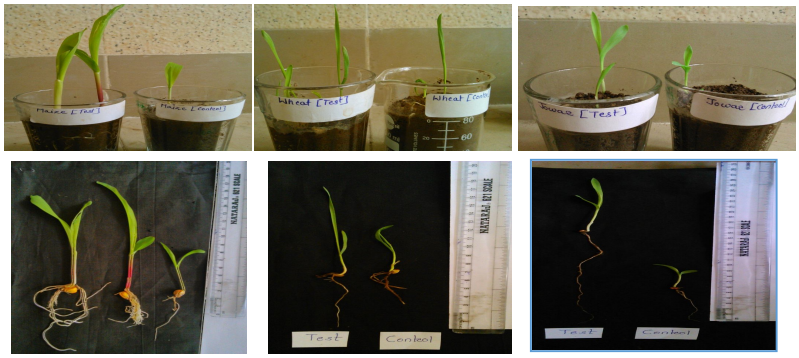


Figure 5: Sudies on Plant Growth Promoting Activityt

Table 5: Results of in-vitro studies on promotion of plant growth by Bacillus subtilis phytase

Seed used	Root length		Shoot length	
	Test	Control	Test	Control
Maize	12	7	13	5
Sorghum	18	7	10	5
Wheat	7.5	3.5	11	6.5

Table 6: Results of Insitu studies of plant growth by Bacillus subtilis phytase.

Seed used	Root length (cm)		Shoot length (cm)	
	Test	Control	Test	Control
Maize	3.6	1.0	8.2	2.0
Sorghum	No germination			
Wheat				

IV. CONCLUSION

Bacillus subtilis strain used in this study was found to be a potent phytase producer that could utilize wheat bran as its most favored carbon and energy source. The phytase produced by this organism interestingly, however, supported the maize plant more than the wheat plant in its growth. Being a GRAS organism this organism this isolate of Bacillus subtilis has a good potential for commercial exploitation after some more study on production optimization and plant growth promoting activities.

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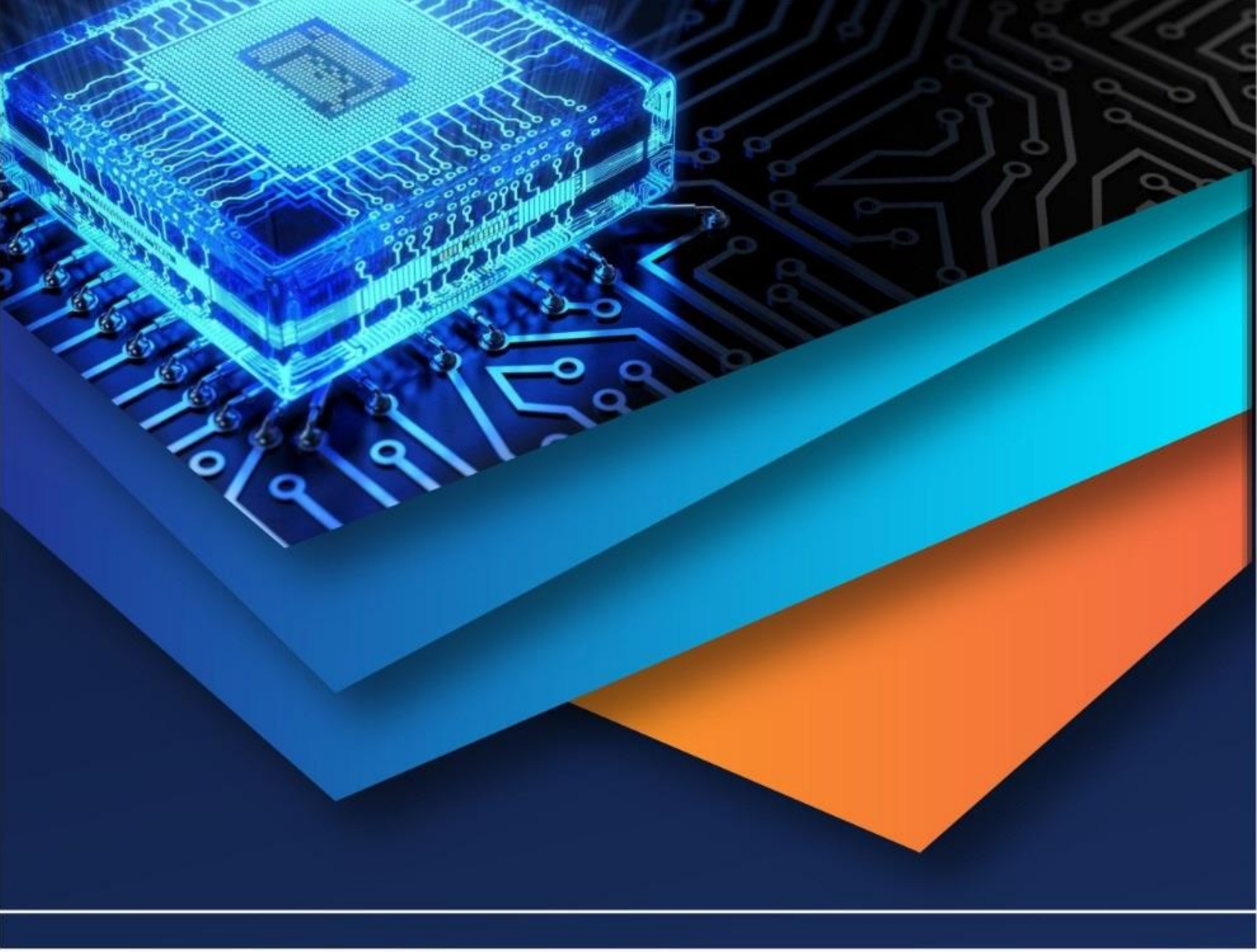
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