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A Study on Isolation of Phosphate Solubilizing Bacterial (PSB) Strain and fungi from salinity affected soil and production of Biofertilizers

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Abstract: ***The use of phosphate solubilizing bacteria (PSB) as biofertilizers has concurrently increased phosphorous uptake in plants and improved yields in several crop species. A laboratory study was conducted to isolate, identify and characterize the phosphate solubilizing bacteria from salinity affected area of Amravati district (Daryapur, Bhatkuli, and Anjangaon). (Aspergillus spp, Penicillium spp. and trichoderma spp.) have the more solubilizing ability of inorganic insoluble phosphate than bacteria, i.e., B. cereus, B. megaterium, B. polymyxa,, two pseudomonas spp, Enterobacter spp., Hence the application of biofertilizer prepared by above mentioned fungi should be helpful to increase the crop yield in salinity affected soil by solubilizing large concentration of inorganic insoluble phosphate.,***
Keywords: Phosphate Solubilizing Bacteria (PSB), Biofertilizer, salinity affected soil, Amravti district, crop.

I. INTRODUCTION

Phosphorus (P) is one of the essential macronutrients for plant growth and reproduction. However, it is a limiting factor in many soils, because an important part of this element is insoluble (Del Campillo et al., 1999). These PSB also can stimulate plant growth by other mechanisms such as the production of phytohormones, nitrogen fixation, inhibition of phytopathogenic microorganisms, production of siderophores and ACC deaminase (Bhattacharyya & Jha, 2012). The majority of the isolated organisms are bacterial organisms, although several fungi are also known to solubilize phosphates. These bacteria and fungi have the potential to be used as biofertilizers. Their role in increasing the soil nutrient value is of utmost importance. Their application to crop fields has resulted in an increased yield of several crops, such as cereals, legumes, fibers, vegetables, oils, and other crop plants (Silini-Cherif, 2012; Viruel et al., 2011; Khalimi et al., 2012).

Biofertilizers may be defined as the preparations containing the living cells of different strains of microorganisms that help in enhancing the nutrient uptake by the plants and hence enriches the nutrient quality of the soil by their interaction in the rhizosphere when these biofertilizers applied either on the top soil or through seed inoculations (Isolation of azotobacter and cost effective production of biofertilizer by Gomare et al., 2013). Phosphorous is such an important macronutrient which is very often present in the soil in unavailable form. Many soil bacteria particularly those belonging to the genera Bacillus and Pseudomonas posses the ability to bring insoluble phosphates in the soluble forms by secreting organic acids. These acids lower the pH and bring about the dissolution of bound forms of phosphorous. These bacteria are commonly known as phosphobacteria. They can be applied either through seed or soil application. Phosphorus, both native in soil and applied to inorganic fertilizers becomes mostly unavailable to crops because of its low level of mobility and solubility and its tendency to become fixed in soil. The phosphate solubulizing bacteria are life forms that can soil. The phosphate solubulizing bacteria are life forms that can help in improving phosphate uptake of plantain on different ways (Rajasekaran et al., 2012). The substances are normally present in a form that is easily absorbed by the plant. But the use of chemical fertilizers has some harmful side effect on the environment (Usman et al.,2015).

Several reports have examined the ability of different bacterial species to solubilize insoluble inorganic phosphate compounds, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate. Among the bacterial genera with this capacity are Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Micrococcus, Aereobacter, Aspergillus, Flavo bacterium and Erwinia. There are considerable populations of phosphate solubilizing bacteria in soil and in plant rhizospheres. These include both aerobic and anaerobic strains, with a prevalence of aerobic strains in submerged soils. A considerably higher concentration of phosphate solubilizing bacteria is commonly found in the rhizosphere in comparison with non rhizosphere soil. The soil bacteria belonging to the genera Pseudomonas and Bacillus and fungi are more common (Role of Bio-Fertilizer in Organic Agriculture: A Review by Mishra et al., 2012).

Thus in this research paper the investigation has been carried in the isolation of phosphate solubilising bacteria and fungi from salinity affected soil collected from Amravti district and then grown in selective media and finally the production of biofertilizer by PSB bacteria and fungi in it.

II. MATERIALS AND METHODS

A. Collection of soil sample

Soil of about 1 kg was collected from different area of salinity affected soil in Amravti district and was put inside a plastic bag and brought to the laboratory for isolation of bacteria from it.

B. Serial dilution of soil samples

About 1 gm of composite soil was taken from the plastic bag and then the soil was diluted to 10 ml of water in a test-tube which served as stock solution. Remaining 9 test tubes were filled with 9 ml of water. Transferring of 1 ml of water from the stock solution to 9 ml of sterilized distilled water with the help of pipettes yielded 10-1 dilutions and the series continued up to 10-9 dilutions. Sterility is the hallmark of any bacteriological isolation so the entire process was carried in the laminar airflow.

C. Bacterial colony identification and external morphology study

Using the spread plate technique the bacterial colony identification and external morphology was studied for which nutrient agar media was prepared. Therefore 100 ml of Nutrient agar Media was prepared for four Petri plates. The NA media was autoclaved and then poured in four Petri dishes which were also sterilized by autoclave. Then the serial dilutions of 10-2, 10-4, 10-6, 10-8 were chosen and from that 0.1 ml of culture was transferred from each serially diluted test tubes and spread on the Petri plates by means of the spreader. Then the Petri dishes were kept in the incubator for 370 Celsius for 24 hrs for the incubation and growth of bacteria. After 24 hrs of incubation the Petri dishes were taken out of the incubators and the following bacterial external morphology were studied.

D. Pure culture isolation of bacteria

Well developed and separated colonies which were identified on the nutrient agar plates were marked and then these separated colonies were chosen and by the help of inoculating needle the colonies were transferred and streaked separately on test tubes having nutrient agar slants for the growth of the single colonies of bacterial cultures from the mixed culture of bacteria. That was grown in the Petri plates. The test tubes were marked after the strains of chosen colonies from Petri plates and were left in the incubator at 37° Celsius overnight for growth and incubation. After incubation of the pure cultures overnight different single species of bacterial culture slants developed in the test tubes which were further picked and purified.

E. Gram staining of the bacterial species strains from the pure culture slants

The pure cultures of different colonies that were obtained in test tubes were put for gram staining for more specific identification of the colonies. The gram staining was done in laminar airflow hood. For this purpose the slides were taken from slide rack. The slides were washed with ethanol. Then each colony was marked on the slides. Then with the help of inoculating needle the loopful strains were picked from each test tube and made a smear on the slides and heat fixed. The slides were then taken in the staining room for staining the smears. Then smears were stained in following steps a) First applied crystal violet on each slides. Kept for 30 secs. b) Distilled water wash. c) Iodine on the slides as mordant (1 min) then 95% alcohol washes and then washed with distilled water. d) Safranin was applied on the slides and then Washed with distilled water and f) the slides air dried. The entire gram staining technique was done following the Christian Gram technique (Collee *et al.*, 1996).

F. Screening of bacterial strains

After gram staining of the bacterial strains from the pure culture slants and microscopic studies the bacterial strains which were identified to be phosphate solubilising bacteria by studying the morphological structures were further confirmed by their ability to be grown on Pikovaskya agar media which is the most important test for the phosphate solubilising bacteria. The bacterial colonies were picked from the pure culture slants by the help of the inoculating needle and were streaked in the PKV agar media plates and were incubated at 37° Celsius overnight. In the next day the bacterial colonies showed a clear Halozone formation which confirmed them to be PSB bacterial species. For further studies these colonies were again grown in nutrient agar media and several Biochemical tests were performed.

G. Preparation of production media of phophate solubilising bacteria as starter cultures

After the screening of the PSB bacterial strains from the pure culture slants the bacterial strains were transferred to the liquid broth which was also the production media and as well as the starter culture for the growth of cells. Production media is that media in which the number of viable bacterial cells of that particular bacteria increases because that bacteria is grown in that particular media only. Thus in phosphate solubilising bacteria strains were grown in Pikovaskyas production media (Protocol followed for growth of PSB). Thus a 100ml of separate conical flasks were taken and PVK media was prepared after ph adjustments and autoclaved. Then inside the laminar airflow the pure cultures marked in the pure culture slants were transferred to the PVK production media conical flasks by the help of sterilized inoculating loop. Then the conical flasks were put in the rotary B.O.D shaker for 1 week or 7 days. The viable cell count in the production media or the liquid broth was found to come up to 109 Cfu/ml. Then for the mass production of PSB biofertilizer the inoculums from these starter cultures were transferred to larger flasks.

H. Mass production of PSB biofertilizer and preparation of inoculum

The Phosphate solubilising bacterial strains in the starter cultures were needed to be grown in large scale for which their mass production were required. So larger conical flasks of 1000 ml were taken and then again starter cultures were transferred to these larger conical flaks containing the appropriate growth media in aseptic conditions for small scale production and for large scale production again 1 liter of the starter cultures were put into the fermenter. Finally continuous agitation and proper aeration was done for about 1 week. The flasks were checked for time to time for the growth of the cell mass and that they were free of any contamination. After 1 week the cell population increased up to 109 cells/ml or 109 cfu/ml load in the larger conical flasks. Then the conical flasks were stored in cool temperatures so that they can be mixed with proper carrier materials. Moreover it is not advisable to keep the conical flasks for long time in storage because of the loss of cell load.

I. Carrier material preparation

The carrier should have the following characteristics

a) It should have high organism matter content
b) Low soluble salts less than 1%
c) High moisture content capacity.

In this experiment for the inoculation to be made charcoal, cow compost and vermicompost was used as carrier material. There are many steps for preparation of the carrier material. The steps are discussed below- First about 1 kg of dried cow dung and black coal was brought from different areas. Then by the help of mortar and pestle the entire coal was crushed to dried powdered form. After crushing also the remaining pieces were further powdered by the help of mixer and grinder. The dust form of coal as charcoal was made and to it 1% calcium carbonate and wooden charcoal or activated charcoal was mixed and neutralized so that no contaminants are present

A) Similarly the cow dung was also crushed and powdered with the help of mixer and grinder.
B) Some amount of vermicompost was also added as a carrier material.

J. Preparation of inoculum with carrier material (Mixing)

The mass produced bacterial cell cultures of PSB were taken out of storage and then the cell cultures were mixed with the sterilized carrier materials in individual beakers. The mixing of the carrier materials and the production media were in the ratio 2:1 where 1 part of production media was mixed with 2 parts of carrier material or in other words 30:60 ratios of both. It was done manually and under aseptic conditions. The cell count of that carrier mixed culture was found to be 108 CFU/gm. The biofertilizers were packed in polythene bags which are advised to be of 250 gm. Then the packets were left in room temperature for curing.

K. Media and Reagents

The nutrient agar media for isolation and slant preparation was made. It includes peptone-5gm, beef extract-3gm, NaCl-5gm, agar-18gm and distilled water-1000ml. B) Pikovaskya agar media was prepared Halozone test for PSB bacteria only. In its composition it has glucose-10gm/ml, yeast extract- 0.5gm/ml, ammonium sulphate- 0.5gm/ml, magnesium sulphate-0.1gm/ml, calcium phosphate-5gm/ml, sodium chloride- 0.2gm/ml, potassium chloride- 0.2gm/ml, manganese sulphate- 0.002gm/ml, ferrous sulphate-0.002gm/ml, Agar- 1.8gm/ml and distilled water- 1000ml. C) Pikovaskya broth was prepared for production media and for mass production of PSB bacteria. In its composition it has glucose 10gm/ml, yeast extract- 0.5gm/ml, ammonium sulphate- 0.5gm/ml, magnesium sulphate-0.1gm/ml, calcium phosphate- 5gm/ml, sodium chloride- 0.2gm/ml, potassium chloride- 0.2gm/ml, manganese sulphate- 0.002gm/ml, ferrous sulphate- 0.002gm/ml and distilled water-1000ml. The biochemical tests included methyl red test,

Vogaeus Proskaur test, indole test, citrate test, catalase test, starch hydrolysis test and nitrate reduction test. In the gram staining techniques crystal violet, Gram s iodine and safranin were used. Other reagents included Kovac s reagent, alpha napthol, hydrogen sulphide and Gram s iodine.

III. RESULTS AND DISCUSSION

On the basis of cultural character, Morphological character and biochemical character phosphate solubilizing bacteria was identifying. Following character was compare with 'BERGEY'S MANUAL' and all phosphate solubilizing bacteria and fungi were identified. Total 34 isolates was isolated from salinity affected soils in Amravati district. (Daryapur, Bhatkuli, and Anjangaon tahshil). Out of 34 isolates 21 PSB and 13 fungi were found, but only 3 fungi species showed significant zone of phosphate solubilization. Among 21, only 8 PSB bacterial culture showed variation and others were repeated. A clear halo zone was formed around the colonies after 2 days of incubation on solidified Pikovaskaya`s agar plates and All phosphate solubilizing bacteria and fungi were selected and sub cultured on Pikovaskaya`s agar plates for further studies. That is Bacillus cereus, Bacillus megaterium, Bacillus subtilis, Bacillus polymyxa, two pseudomonas spp., Micrococcus spp., Enterobacter spp., fungi (Aspergillus spp., Penicillium spp. and tricoderma spp.) Out of this isolate fungi (Aspergillus spp., Penicillium spp. and tricoderma spp.) having efficiency of Phosphate solubilization was more as compare to other isolated phosphate solubilizing bacteria that is (284, 220, 276). But Enterobacter spp. having efficiency of Phosphate solubilization was less as compare to other isolated phosphate solubilizing bacteria that is (127). Efficiency of Phosphate solubilization was determined by plate assay using Pikovaskaya's Agar Medium (Figure 1).

A. To Isolate Phosphate Solubilizing Bacteria and Fungi from salinity affected soil in Amravati District (Daryapur, Bhatkuli, and Anjangaon)

1) Colonies showing zone of clearance were observed on Pikovaskaya's agar plates.

2) The ability to solubilize precipitated phosphate was positively exhibited by Bacillus cereus, Bacillus megaterium, Bacillus subtilis, Bacillus polymyxa, two pseudomonas spp., Micrococcus spp., Enterobacter spp., fungi (Aspergillus spp., Penicillium spp. and tricoderma spp.)

3) All phosphate solubilizing bacteria and fungi were selected and subculture on Pikovaskaya's agar plates for further studies. Determination of Efficiency of Phosphate solubilization, solubilize by. Bacillus cereus, Bacillus megaterium, Bacillus subtilis, Bacillus polymyxa, two pseudomonas spp., Micrococcus spp., Enterobacter spp., fungi (Aspergillus spp., Penicillium spp. and tricoderma spp.)

4) The Phosphate solubilising bacterial strains in the starter cultures were grown in large scale for mass production. So larger conical flasks of 1000 ml were taken and then again starter cultures were transferred to these larger conical flaks ,then starter cultures which are further used in mass production of biofertilizers

B. Determination of Efficiency of Phosphate solubilization solubilize by Isolated 8 bacterial culture and 3 fungi.

% of Efficiency of PSB was calculated by using following formula

$$Efficiency\ of\ phosphate\ solubilization = \frac{Solubilization\ diameter}{Diameter\ of\ colony} \times 100$$

Sr. No.	PSB and Fungi strain	Colony Diameter	Solubilization Diameter	% Efficiency 48 Hr
1.	Bacillus subtilis	0.9	1.4	155
2.	Pseudomonas spp.	0.5	1.2	240
3.	Pseudomonas spp.	0.6	1.4	233
4.	Bacillus polymyxa	0.8	1.1	137
5.	Bacillus megaterium	1.2	1.8	150
6.	Bacillus cereus	1.2	1.6	133
7.	Enterobacter spp.	1.1	1.4	127
8.	Micrococcus spp.	1.3	3.6	276

9.	Trichoderma spp.	1.3	3.6	276
10	Aspergillus spp.	1.3	3.7	284
11	Penicillium spp.	0.5	1.1	220

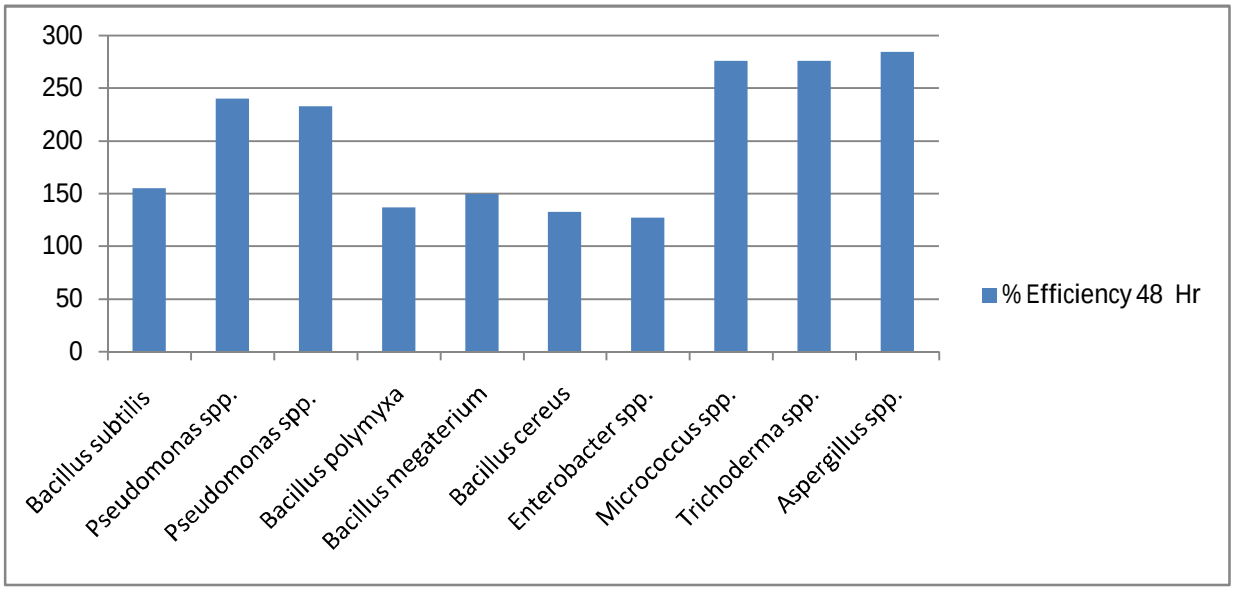

Figure: 1 Percentages of efficiency

These phosphate solubilising bacteria are very important in solubilization of insoluble phosphate to soluble phospahate by release of organic acids. Thus the biofertilizers which are mentioned in this research paper after production through the selective and optimized media and mass production and then packed to send the salinity affected area for their applications in various plant fileds. These bioinnoculants are ready to increase the nutrient supply of salinity affected soil containing plants. Production of enzyme like phosphates is other mechanism of phosphate solubilization (Rodriguez and Fraga, 1999).

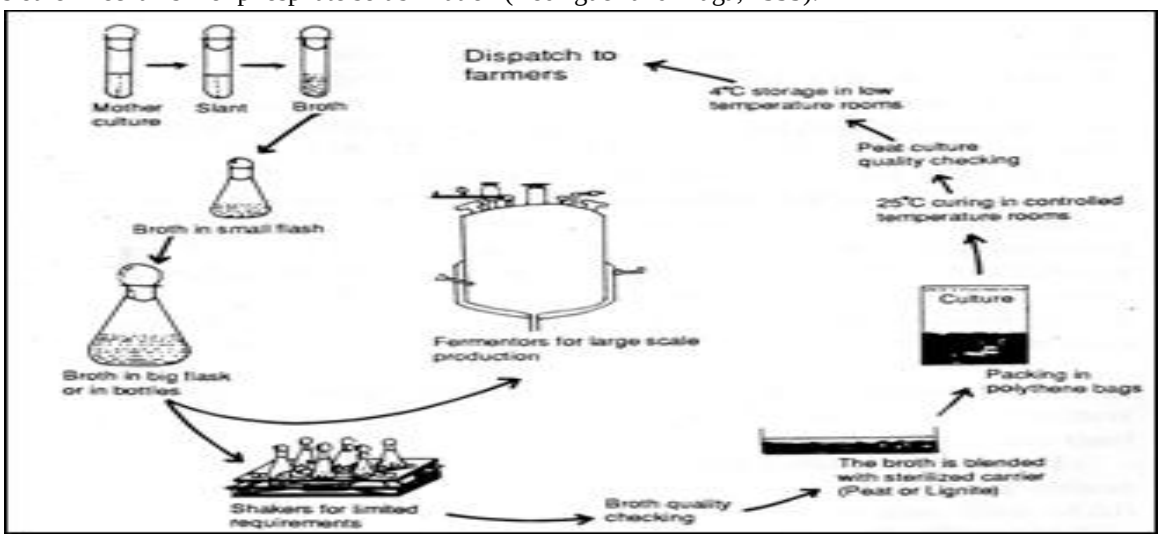

Figure: 2 Production of Biofertilizer

Aspergillus spp isolates, showed high activity of AP at 11 M of P; however, the production of this enzyme was under detection limit in excess of phosphate compared to limiting condition, which could explained that the synthesis of alkaline phosphatase by these bacteria was inducible in low Pi, while it was repressed in high concentration. These results are in concordance with solubilization activity Pikovaskaya's Agar, where Aspergillus spp. isolates were strong P solubilizer. Interestingly, Enterobacter spp. strain produced a smaller drop in pH value compared to others isolates. This might suggest that this strain is capable to solubilize phosphate by other ways than the production of organic acid. Therefore, we found a positive correlation between phosphate

solubilizing capacity and phosphatase enzyme activity. Phosphorus in soil is important for plant development, and the lack of P limits plant growth. Although chemical fertilizers are added to the soils, plants can only utilize low amounts of phosphatic fertilizers. In this case, the selection of highly efficient PSB will practically increase phosphorus in plant rhizosphere. Various PSB have been isolated from different plant roots (Yu et al., 2011; Afshan et al., 2015).

IV. CONCLUSIONS

A total of 11 strains were isolated in this study, the cheapest and easily available waste materials (Agricultural and vegetable wastes) were utilized for the production of nutrient rich phosphate solublizing biofertilizer for salinity affected soils better growth of plant. The present work revealed the presence of phosphate solubilizing bacteria and fungi of salinity affected soils from Amravati district, this study has revealed that, the phosphate solubilizing efficiency of the isolated species could be used to solublize higher amount of phosphates found in the salinity affected soils of Amravati district. From the study it was observed that the fungi (Aspergillus spp, Penicillium spp. and trichoderma spp.) have the more solubilizing ability of inorganic insoluble phosphate than bacteria, i.e., B.cereus, B.megaterium, B.polymyxa,, two pseudomonas spp, Enterobacter spp., Hence the application of biofertilizer prepared by above mentioned fungi should be helpful to increase the crop yield in salinity affected soil by solubilizing large concentration of inorganic insoluble phosphate.

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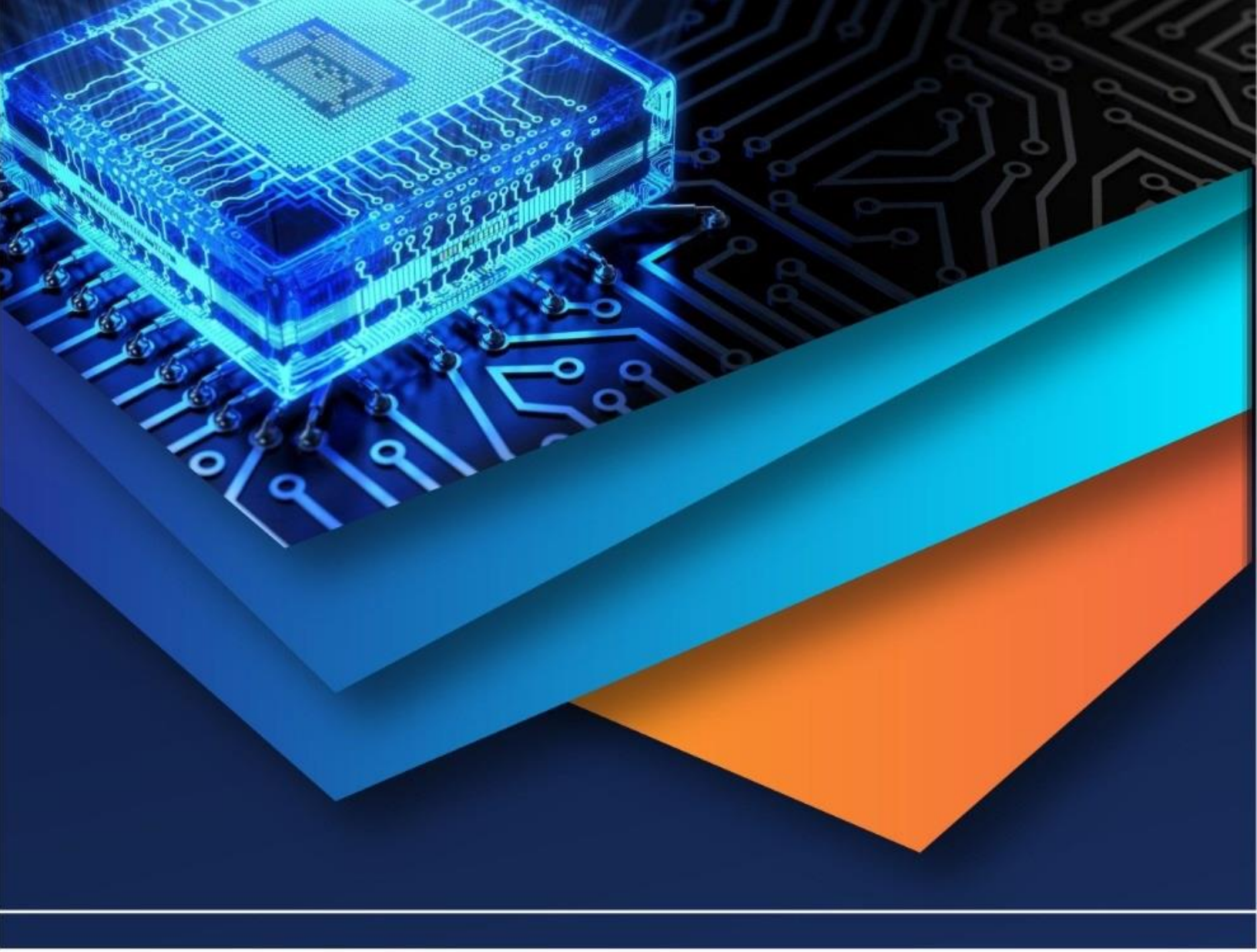

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