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Study of Xylanolytic Microorganisms by using Xylan Extracted from Natural Waste

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Abstract: The novel approach used in current research is use of extracted xylan from natural wastes like wheat bran and Pistia sp for screening of xylanolytic microorganisms. Xylan recovered from extraction of wheat bran was 22.2% and Pistia sp was 23.7%.A total of 36 isolates were obtained from different soil samples collected from Kolhapur District. Out of these, B2 isolate showed 27mm diameter of zone of clearance on medium containing pure xylan, while on medium containing Pistia sp and wheat bran showed 27mm and 28mm respectively. This B2 isolate was identified as Massilia timonae after 16s rRNA sequencing and the new strain was named as Massilia timonae B2YR.Its 16s rRNA nucleotide sequence was deposited in GenBank under accession number KY942185. Along with xylanases the organism also produces cellulase and amylase enzyme.
Keywords: Xylan, Xylanases, wheat bran, Pistia sp, 16s rRNA, Amylase, Cellulase

I. INTRODUCTION

Xylan the major component of hemicelluloses accounts for 1/3rd of the renewable carbon on earth. [3] Xylan is polymer of β-1,4 linked D-xylose residue with substitutions of L-arabinofuranose, D-glucouronic acid and 4-o-methyl-D-glucouronicacid at 2' and 3'position [7], [8], and [9]. Complex structure of xylan is hydrolyzed by group of enzyme collectively termed as Xylanases. Endoxylanases (Endo-1, 4β-xylanases), β-xylosidase, and side chain cleaving enzyme esterase's. [4] Xylanases have wide range of industrial and biotechnological application. They are commercially used in food and feed. Xylanases are used as emulsifier, sweetener, preservatives etc. in food and as animal feed in feed industry. [2] Xylanases are also used in paper and pulp industry as bioleaching agent. [17] Recently xylanases are used to increase sugar recovery from agriculture residue for biofuel production. [5] Now a days increasing demand for xylanases is for production of xylooligosachharides which is an emerging prebiotic. [14]

II. SCOPE OF WORK

The enzyme production is mainly limited due to cost of substrate used for growth of microorganism and production of enzyme. Hence to lower the production cost it is necessary to use substrate which are naturally and readily available. Some of these wastes like agricultural, agro-industrial waste and inedible crops can be used as substrate for production of xylanases agro-industrial waste like wheat bran, sugarcane bagasse and inedible crops like the weed *Cyperus iria*, *Cyperus rodents*, *Echornia* sp, *Pistia* sp.

The present work describes extraction of xylan from natural resources like wheat bran and *Pistia* sp as a substrate and its utilization for screening of xylanolytic microorganisms and also to study ability of organisms to produce other enzymes such as cellulases and amylases.

III. METHODOLOGY

All higrade chemicals were used for the work. Extracted xylan is used in the work and its results were compared with pure xylan obtained from Hi media.

A. Sample Collection

1) *Soil Sample Collection*: Various soil samples were collected from saw mill industries and garden from Kolhapur district. The soil sample were packed in sterile polythene bag and kept under storage till further use.
2) *Substrate Collection:* Wheat bran was collected from local houses and bought to laboratory for further use. *Pistia* sp was collected from Takala pond, Kolhapur, washed in running tap water and bought to laboratory.

B. Preparation of Substrate

Wheat bran obtained from houses was washed 2-3 times in distilled water. Water was decanted and wheat bran was dried in oven. The dried wheat bran was powdered in mixer grinded and passed through sieve using 60 mesh and stored at low temperature till further use. Similarly weed *Pistia* sp collected from pond were washed, sun dried, powdered and stored at low temperature.

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C. Extraction of Xylan from Natural Resource

The natural resource used here were Wheat bran and *Pistia* sp for extraction of xylan using modified protocol of Hauli.[6]

1g of natural resource was added to 10 ml of 4% NaOH and kept at temperature 85°C for 3 hrs. Above mixture was then centrifuged at 15000 rpm for 15 minutes and the supernatant was collected. Collected supernatant was neutralized by acetic acid to pH 6. Neutralized supernatant was filtered through filter paper and filtrate was collected. Xylan was then precipitated by addition of 3 volumes of 95% ethanol to collected filtrate. Precipitated xylan was collected by filtration.

The xylan recovered from the extraction process calculated as follows

$$\text{Xylan\%} = \frac{\text{dry weight of extracted xylan}}{\text{weight of sample used for extraction}} \times 100$$

This recovered xylan is used as substrate i.e., carbon source.

D. Confirmation of Xylan Extracted by TLC and FTIR Analysis

The xylan extracted from natural waste was confirmed by performing Thin Layer Chromatography. Spots of extracted xylan and commercial xylan from Birchwood (Hi media) reconstituted in Distilled water were loaded and ascending Thin layer chromatography was carried out in solvent system containing n-Butanol: Aceticacid: Water (2:1:1). The detection was carried out by spraying the chromatogram with developer containing 0.2% orcinol in mixture of methanol: H_2SO_4 in the ratio of 90:10. The plate was dried in oven at 100°C so as to develop the color. The extracted sample was also subjected for FTIR analysis with comparison with commercial xylan from Birchwood (Hi media).

E. Enrichment

Enrichment of soil sample was carried out using natural resources like wheat bran and *Pistia* sp as whole carbon source. The enrichment was carried out in the increasing substrate concentration from1% - 8% prepared in modified Horokoshi broth in 10 ml KNO_3-0.025, KH_2PO_4-0.01, $MgSO_4$-0.001, $CaCl_2$-0.001, NaCl-0.05, wheat bran or *Pistia* sp (1% - 8%).0.1g of soil sample was inoculated in sterile broth and incubated at room temperature for 24 hrs.

F. Isolation of Xylanolytic Microorganisms

Growth obtained in 8% enrichment was streaked on sterile modified Horokoshi medium containing 1% wheat bran or 1% *Pistia* sp. The plates were then incubated for 24 hrs at room temperature. The isolated colony obtained was further purified on Nutrient Agar.

G. Screening and Confirmation of Xylanolytic Activity

Well isolated and purified colony obtained was spot inoculated on three separate sterile modified Horokoshi medium containing 0.5% pure xylan, 0.5% xylan extracted from wheat bran, 0.5% xylan extracted from *Pistia* sp, incubated at room temperature for 24 hrs. After incubation iodine was poured over the medium to select xylanolytic microorganisms.

H. Phenotypic Characteristics

The selected isolate was identified on the basis of morphological, cultural, biochemical properties and 16S rRNA sequencing. The 16s rRNA nucleotides are deposited at GenBank, NCBI.

I. Study of Cellulase and Amylase activity.

The isolate was also checked for its ability to produce other enzymes like Cellulase and Amylase. This isolate was spot inoculated on Carboxy methyl cellulose agar and Starch agar respectively.

IV. EXPERIMENTAL RESULTS

A. Sample Collection

A total four soil samples packed in sterile polythene bag from saw mill industries from Bagal chowk, Mudshingi, Vikram Nagar and Badhule were bought to the laboratory used and stored at room temperature.

B. Substrate Preparation

100 gm of wheat bran and *Pistia* sp after processing were powdered and stored till further use.

C. Extraction of Xylan

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During extraction the dry weight of xylan extracted from 1g of wheat bran and *Pistia* sp were 0.222g and 0.237g respectively. Thus the recovery of xylan obtained from both the resources according to formula

$$\text{Xylan\%} = \frac{\textit{dry weight of extracted xylan}}{\textit{weight of sample used for extraction}} \times 100$$ is 22.2% and 23.7% respectively (Table 1) .

Table 1
Summary of Xylan Extraction

Natural resource used	Amount of resource	True recovery of xylan
Wheat bran	1g	22.2%
Pistia sp.	1g	23.7%

D. Confirmation of Extracted Xylan by TLC and FTIR Analysis

1) *TLC Study:* The pure xylan and xylan extracted from wheat bran and *Pistia* sp when subjected for TLC it was found that all the three had same rate of flow. As seen in fig 1

Figure 1: TLC of lane 1xylan extracted from wheat bran (Xwb),lane3 2 xylan extracted from *Pistia*(Xpis) lane 3 pure Xylan (xylan from birhwood ,Hi media).

2) *FTIR Analysis:* The FTIR study showed (Fig 2 and 3) that both extracted and commercial xylan, have absorption bands at 3200cm^{-1} -3450 cm-1 and also at1100cm^{-1}-1190 cm-1 which can be attributed to the OH stretching characteristic of glycosidic groups and to CC and COC stretching in hemicelluloses, respectively. Absorption band near 1350 cm^{-1} - 1420 cm^{-1} is detected and it is assigned to the CH bending vibration present in cellulose and hemicellulose chemical structures. The prominent band at 1010cm^{-1} - 1040 cm-1 is also associated with hemicelluloses and is attributed to the C–OH bending. Finally, a band at 897 cm-1, is typical of glycosidic linkages between the sugar units in hemicellulose. [1]

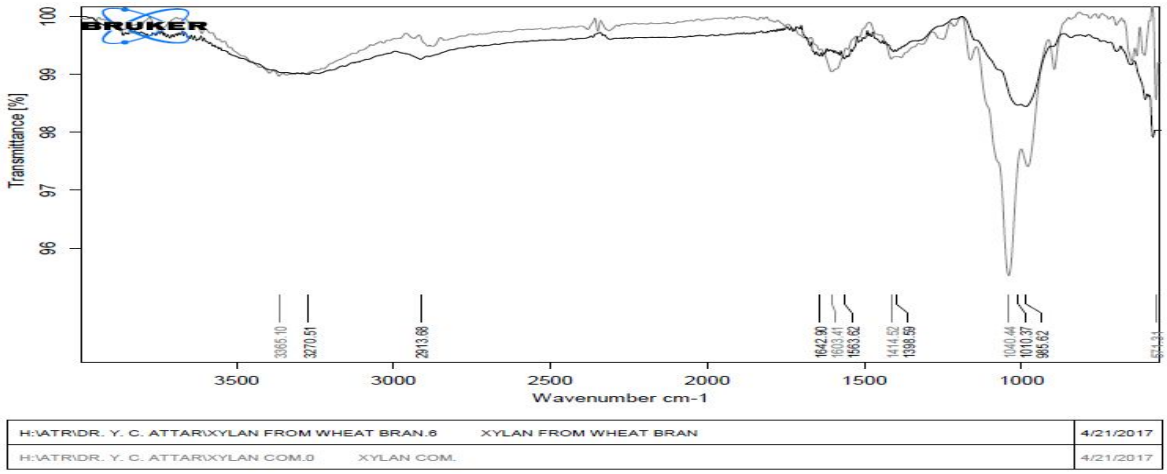

Figure. 2 FTIR analysis of xylan extracted from wheat bran and commercial xylan.

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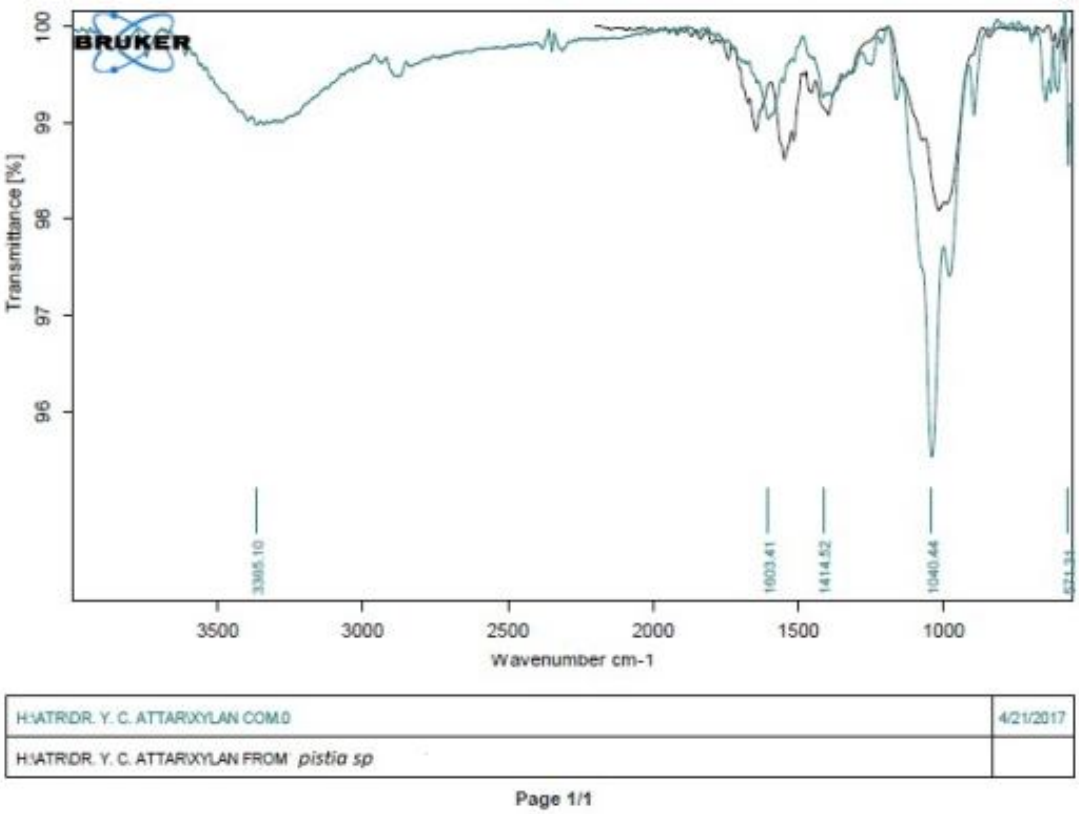
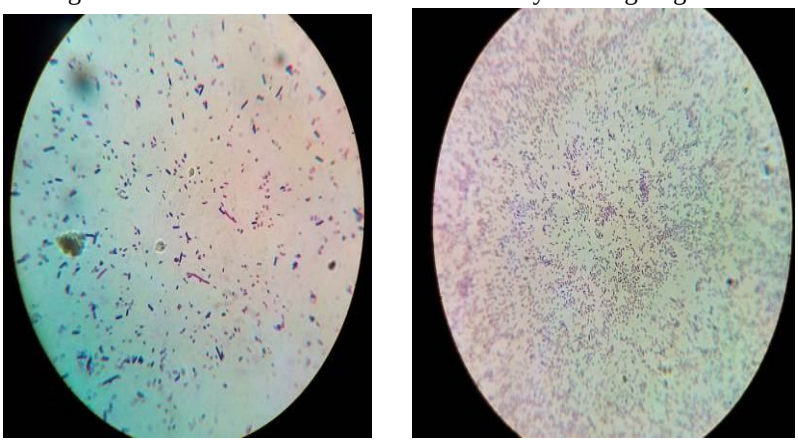


Figure 3.FTIR analysis of xylan from *Pistia* sp and commercial Xylan.

E. Enrichment

The increase in the number of microorganisms in the enrichment was confirmed by staining. Fig 4



(a) (b)

Figure 4. a) Staining of 4% enrichment sample.b)Staining of 8% enrichment sample.

F. Isolation of Xylanolytic Microorganisms

After incubation a total of 36 isolates were obtained from the various samples. (Table 2). From Bagal chowk sample 15 isolates were obtained which were labeled as SM1 to SM15, from Vikram nagar soil sample 5 isolates labeled as V1 to V5, from Mudshingi soil sample 8 isolates which were labeled as M1 to M8 while from Badhule soil sample also 8 isolates were obtained labeled as B1 to B8.

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Table 2 Soil Sample And Number of Isolates

Sr. no	Soil sample	Number of isolates
1	Bagal chowk	15
2	Vikram nagar	5
3	Mudshingi	8
4	Badhule	8

G. Screening and Confirmation of Xylanolytic Activity

The iodine was poured over the medium to observe xylanolytic microorganism's. The xylanolytic microorganism showed a zone of clearance around the growth. The diameters of clear zone produced by the microorganisms were measured. (Table 3). A total of 5 organims showed xylanase activity. From these B2 isolate showed maximum zone of clearance of 27mm on medium containing xylan from *Pistia* sp, 28mm on medium containing xylan from wheat bran while 28mm on medium containing pure xylan (fig 5).

Table 3
Diameter of Zone of Clearance Produced by Isolate

Isolates	Xylan from wheat bran	Xylan from Pistiasp	Pure xylan
SM15	10mm	10mm	10mm
V4	18mm	18mm	18mm
M5	12mm	15mm	12mm
B2	27mm	27mm	27mm
SM2	20mm	21mm	21mm

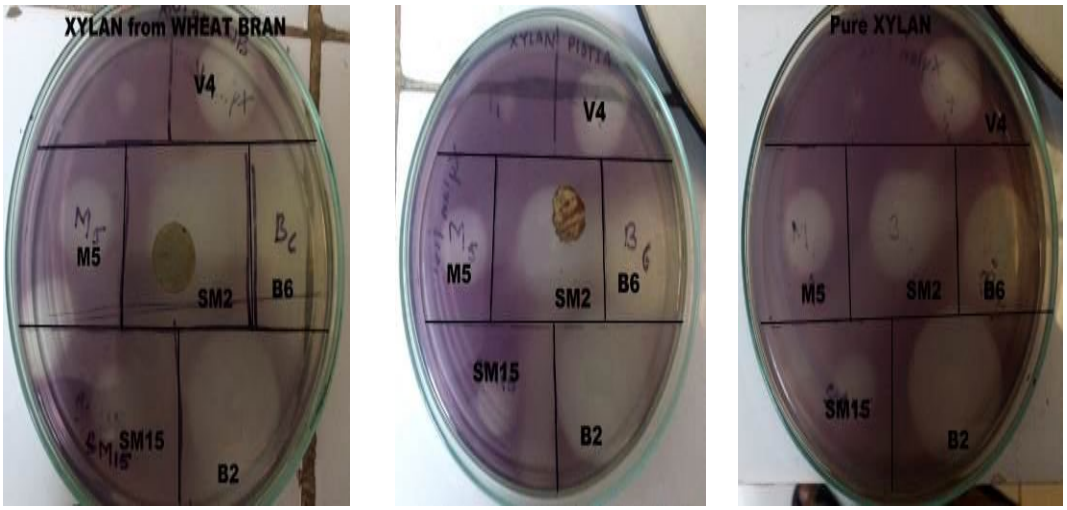

Fig a Fig b Fig c

Figure 5. (a) Isolates showing clear zone on medium containing Xylan from wheat bran. (b). Isolates showing clear zone on medium containing Xylan from *Pistia* sp. (c) Isolates showing clear zone on medium containing pure Xylan

H. Phenotypic Characteristics

The cultural and morphological characteristic of the potent organisms B2 showed that it is Gram negative bacilli, motile, non

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sporulating, catalase positive in nature (Table 4). The 16 S rRNA 1076 bp nucleotide sequence analysis studies showed that organism belong to *Massilia timonae* the strain was named as *Massilia timonae* B2YR.The 16 S rRNA nucleotide sequences are deposited at GenBank under accession number KY942185.The Phylogenetic analysis was done using sequence aligned by CLUSTALW in MEGA 6 software. The analysis shows the close relation with *Massilia timonae* CCUG 43427A (fig 6). The figures presented in bracket represent the percentage relatedness of the isolates to the organisms.

Table 4
Morphological, Physiological and Biochemical Characteristics of *Massilia timonae* B2YR KY942185

Colony characteristics	observation	Biochemical test	Result
Size	2mm	Glucose	Weakly +
Shape	Circular	Xylose	+
Color	Pale white	Maltose	+
Margin	Entire	Indole	-
Surface	Smooth	Methyl red	-
Opacity	Opaque	Voges Prausker	-
Elevation	Flat	Citrate utilization	+
Consistency	Moist	Nitrate reduction	+
Gram nature	Gram negative short rods	Urease test	Late +
Motility	Actively motile	Lysine decarboxylation	+

+ Acid production, - negative test.

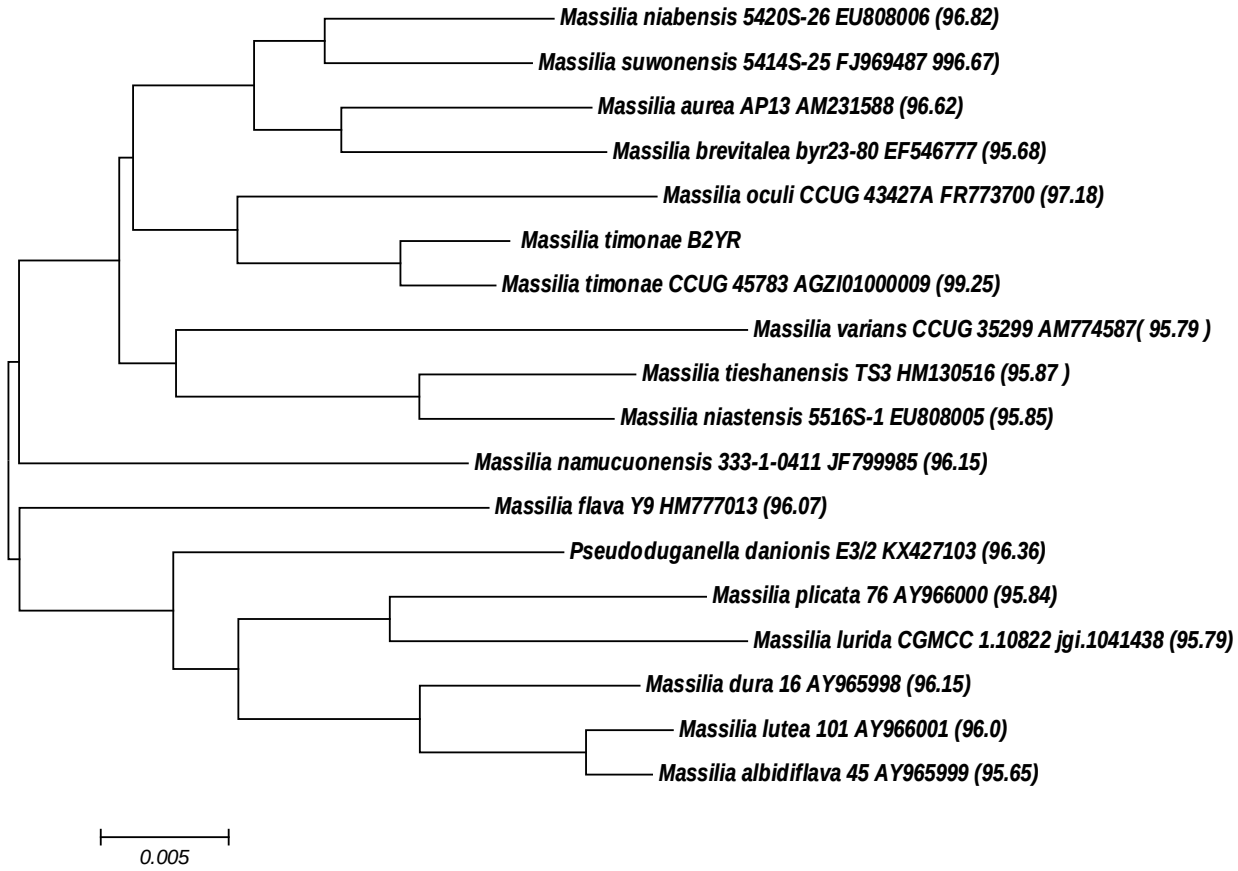

Fig6. Phylogenetic Analysis of *Massilia timonae* B2YR KY942185

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I. Confirmation of Cellulolytic and Amylolytic Activity

The growth of obtained on carboxy methyl cellulose medium and starch medium was flooded with iodine to observe zone of clearance around growth a diameter of 20 mm and 8mm was observed on respective medium confirming the cellulolytic and amylolytic activity.

V. CONCLUSION

This is the first report were extracted xylan from natural waste wheat bran and *Pistia* sp is used for screening of xylanase producers. The extracted xylan is confirmed with commercial xylan by TLC and FTIR analysis. The organism *Massilia timonae* B2YR KY942185 not only produces enzyme Xylanase but also produces Cellulase and Amylase. This ability of organism to produce multiple enzymes can be exploited to control environmental pollution, by using natural wastes for production these enzymes. Thus, the approach of using natural resource can lower the production cost.

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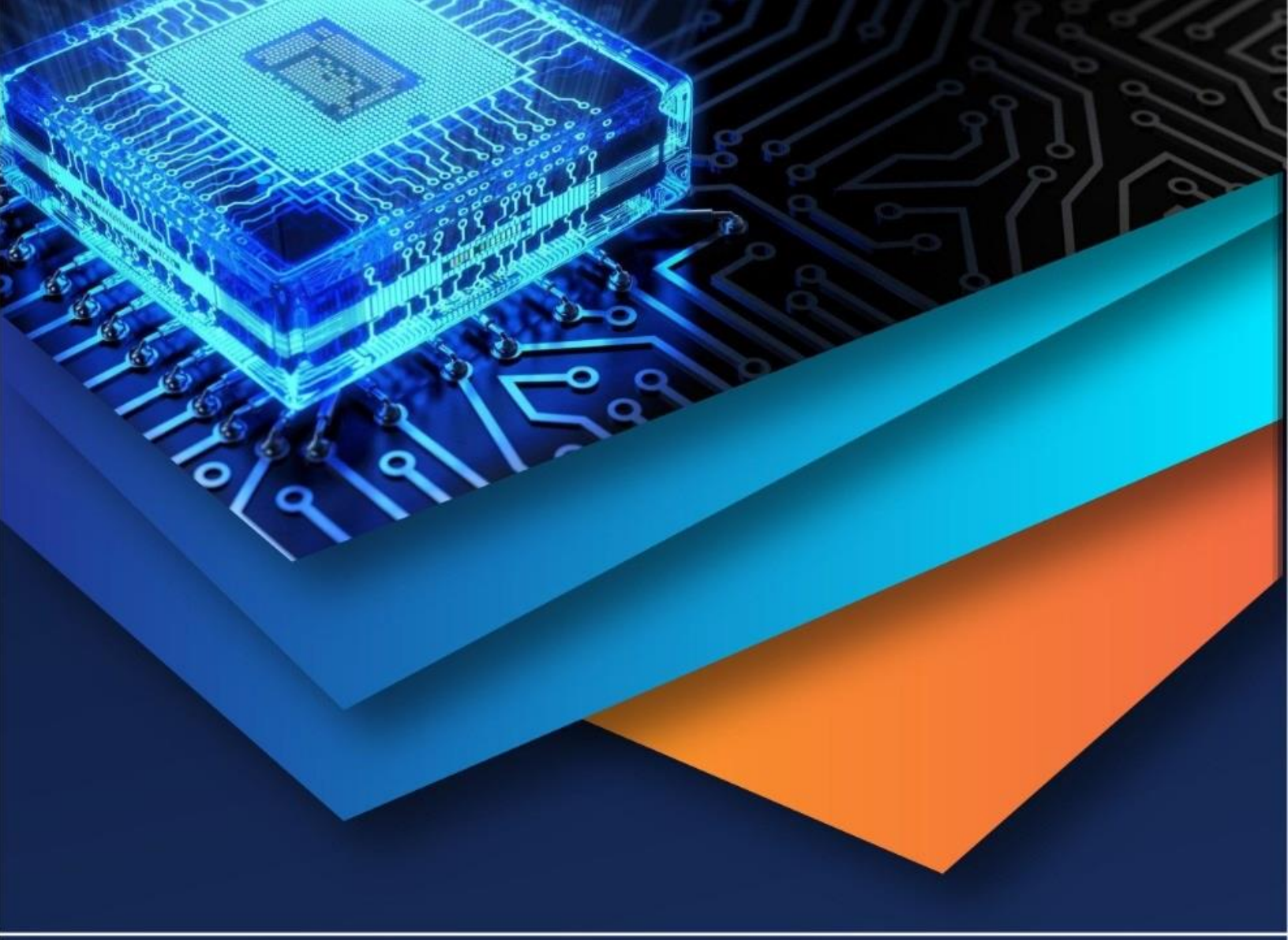

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