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Screening and Isolation of Keratinase Producing Micro Organisms

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Abstract: ***In present study 35 proteolytic isolates were isolated from various samples containing keratin rich sources from different areas in Kolhapur and Sangli region of Maharashtra state. These isolates were screened for production of proteolytic enzymes like caseinase,gelatinase by using milk agar and gelatin agar. All these isolates were gelatinase and caseinase positive.So, further used to check kertinolytic activity by studying feather degradation process.From all isolates,7 isolates named as I1, I6, P, M5-2,E2N,E4N,C9 shows keratinase activity.Out of which,isolate M5-2 show highest caseinase(10 mm zone),gelatinase(26 mm zone) activity along with 100% feather degradation(keratinase) activity.This keratinolytic isolate was further identified morphologically,biochemically and by 16s rRNA sequencing as Stenotrophomonas maltophilia YArck. The sequence is deposited at Gen Bank NCBI with gene accession no.KY941138.***
Keywords: Keratin, keratinase, proteolytic, feather meal broth, Stenotrophomonas maltophilia YArck.

I. INTRODUCTION

Keratin is naturally occurring protein. It is structural, fibrous, insoluble in nature. This protein is abundantly present in hairs, nails, wools, horns, scales, beaks, feathers, hoofs, claws and of epithelial cells in the outermost layers of the skin [4]. Keratin is difficult to degrade because of its very dense arrangement of polypeptides. It is stabilized by several intra and inter molecular hydrogen bonds, disulphide bonds and many hydrophobic interactions. Though degradation of keratin is difficult process but it is observed that various micro-organisms such as bacteria, fungi, algae, actinomycetes have potential to degrade keratin with the help of keratinase enzyme under optimum growth conditions.Microbial keratinase is group of enzymes specifically act on keratin and convert it into various amino acids.It has wide applications in various industries like detergents, foods, leather, silk and pharmaceutical industries.[3] Keratinase (E.C.3.4.4.25) belongs to class hydrolase.These are metalloproteins and efficient proteolytic enzymes. Keratinases are produced only in the presence of keratin containing substrate. It mainly attacks on the disulphide bond of keratin substrate. As tannary and poultry waste also contains major source of keratin rich material, keratinase can be used for removal of hair and feathers in tannary and poultry industry through the development of non-polluting process. Hence, the present work was directed towards the screening and isolation of keratinolytic bacteria from various keratin rich waste materials.

II. MATERIAL AND METHODS

All chemicals and reagents required for experimental work were of analytical grade, pure and purchased from Himedia Laboratory. The chemicals include nutrient agar, agar, yeast extract, NaCl, K2HPO$_4$, KH$_2$PO$_4$, MgCl$_2$ etc.

A. Sample Collection

Total 15 soil samples rich in keratin, like tannary soil, feather dumping soil, tannary effluent, hair dumping soil, poultry waste were collected from Jawaharnagar, Kasaba bawada, Shiroli, Jainapur, Ambap, Tamadalge Dist. Kolhapur and Ashta ,Bisur, Madhavnagar Dist.Sangli . All these soil samples were packed in sterile polythene bags.It was properly labelled and kept under storage at R.T. till further use.

B. Preparation of Feather Meal Powder

Native chicken feathers were treated according to the procedure described by Tork for preparation of feather meal powder[10].Then it was stored at room temperature till further use.

C. Enrichment and Isolation of Keratinase Producing Micro-Organisms

Enrichment of Keratin rich soil samples were carried out using feather meal broth with increasing substrate (feather)concentration from 1%-8% and incubated at room temperature for 24 hrs. Feather meal broth of P^H 7.5 contains NH$_4$Cl-0.5gm,NaCl-0.5,K$_2$HPO$_4$-

0.3,KH_2PO_4-0.4,$MgCl_2$-0.1,Yeast extract-0.1,Feather powder 10 gm/lit, D/W.-1 lit. [1]

After proper enrichment,feather meal agar was used for isolation of keratinolytic bacteria by four quadrant streaking technique.The colonies with different morphology were picked and purified using same medium.

D. Screening of Isolates for Proteolyticactivity

The proteolytic activity was studied by using sterile skimmed milk agar and gelatin agar plates. Simultaneously, keratinase producing bacteria were confirmed on keratin agar plate. The colonies showing highest keratin hydrolysis ability with maximum clear zone were picked up and further purified by repeated transfer on same medium and then maintained on respective medium.

E. Degradation of Chicken Feather by Keratinase Producers

For studying the biodegradation of keratinous material source of the keratinous waste(chicken feathers) were added to the medium as a sole source of carbon and nitrogen. This source was added separately to the medium at 1%w/v. After inoculation of organism,flask was incubated at room temperature on rotory shaker at 160 rpm for 2-3 days.The percent of keratinous waste degradation was then determined. [6]

F. Determination of Degree of Degradation(DD)

The residual feather was washed, dried and weighed to calculate DD by using following formula

$$DD(\%) = \frac{(TF-RF) \times 100}{TF}$$

Where, TF is total feather and RF is residual feather.

G. Morphological, Cultural and Biochemical Characterization of Isolate M5-2

From the caseinase, gelatinase and keratinase activities of all isolates, isolate M5-2 showing maximum proteolytic and keratinolytic activity was considered as keratinolytic isolate. The M5-2isolate was then studied by morphological, cultural and biochemical characteristics as described in the Bergy's Mannual of Determinative Bacteriology. Then it was further identified by 16 s r RNA sequencing and deposited in Gen Bank, NCBI.

III. RESULTS

A. Sample Collection

Total 15 keratin rich soil samples were collected and stained to observe microbial load and then subjected for enrichment.

B. Preparation of Feather Meal Powder

Total 50 gm of feather meal powder was prepared.

C. Enrichment and Isolation of Keratinase Producing Micro Organisms

The increase in the number of microorganisms in the enrichment was confirmed by Gram staining (Fig.1).

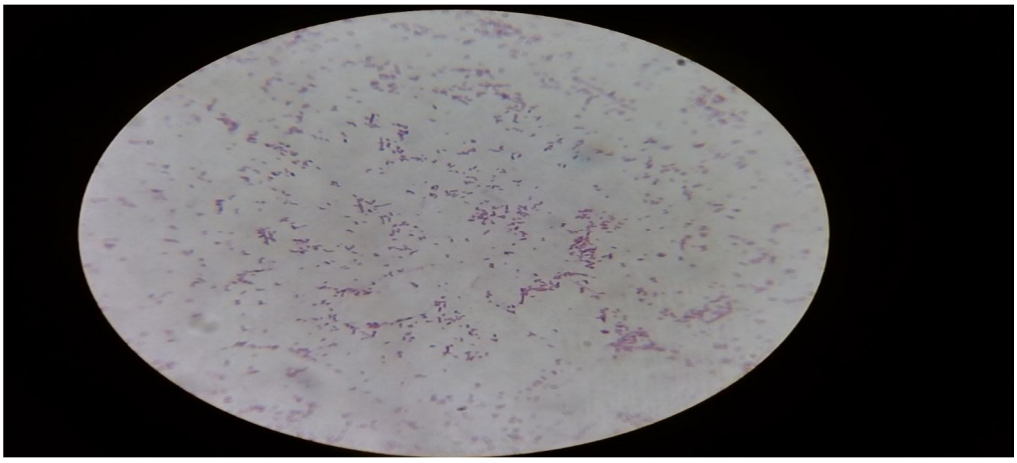

Fig.1.Microscopic Observation of Gram Staining of 8% Enriched Sample.

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From 8% enriched samples, total 35 keratinolytic isolates were isolated and purified on Feather meal agar plates.(Fig 2)

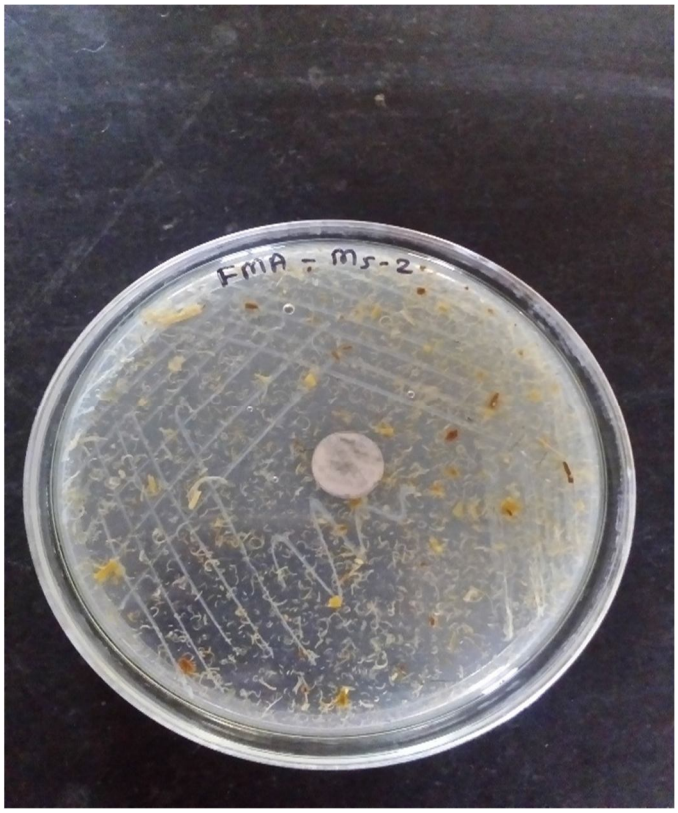

Fig.2.Screening of isolates on feather meal agar for proteolytic activity

D. Screening of Isolates for Proteolytic Activity

The proteolytic activity of isolates were confirmed by observing clear zone around growth on milk agar and gelatin agar plates (Fig. 3). It was observed that, all 35 isolates were proteolytic in nature. Out of all proteolytic isolates,7 isolates show maximum zone of clearance on milk and gelatin agar plates than that of the remaining isolates. (Table 1)

Table 1

Isolates showing maximum caseinase and gelatinase activity.

Sr.No.	Isolate	Diameter of zone of clearance on milk agar (mm) (Caseinase activity)	Diameter of zone of clearance on gelatin agar (mm) (Gelatinase activity)
1	I 1	07	20
2	I 6	10	18
3	P	09	17
4	**M 5-2**	**10**	**26**
5	E2N	09	24
6	E4N	08	23
7	C 9	08	25

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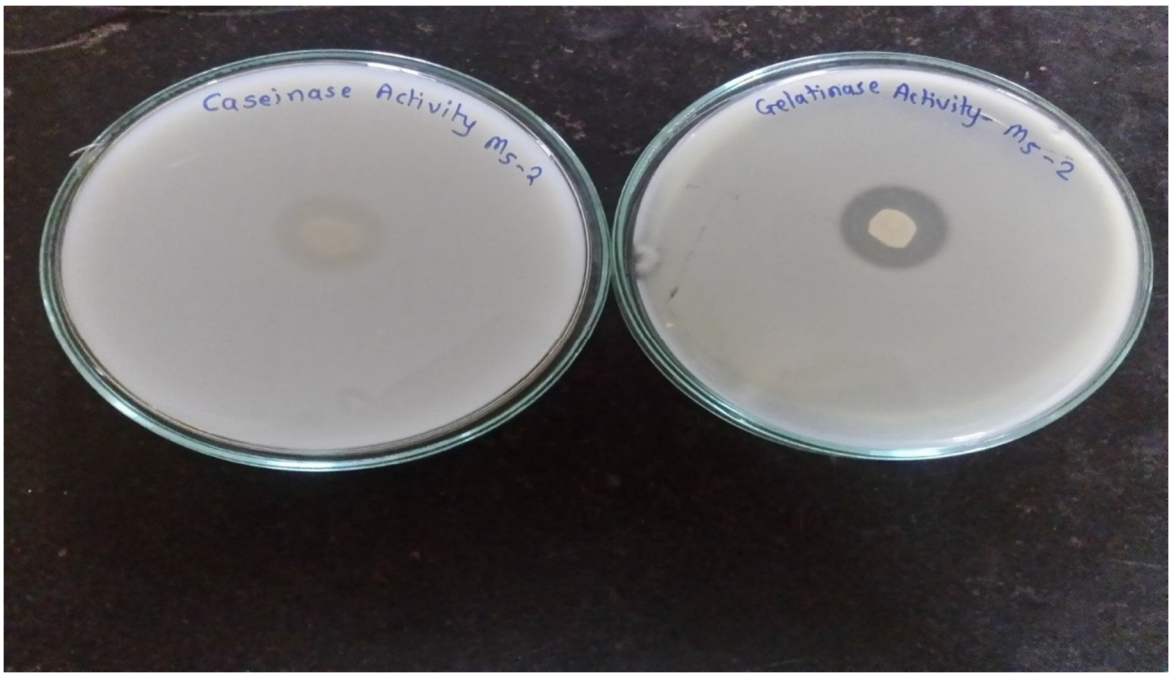

Fig.3.Caseinase Activity and Gelatinase Activity by Isolated Bacteria. - M5-2.

As M5-2 isolate was found to be more proteolytic, its keatinase activity was confirmed on pure keratin agar plate and clear zone was observed around growth after addition of trichloroacetic acid (TCA)solution.(fig. 4)

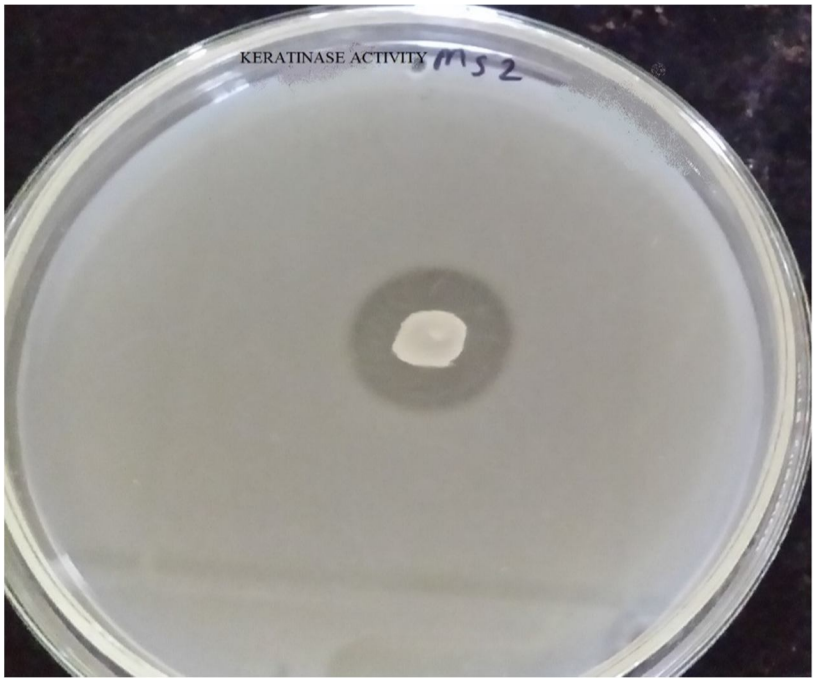

Fig. 4 Keratinase activity of M5-2

E. Degradation of Chicken Feather by Keratinase Producers

Among 7 isolates,M5-2 isolate demonstrated highest feather degrading activity(100%) after3 days incubation at R.T.where degradation of all feather barbules and almost all feather raches were observed(Table-2)

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Table 2-Isolates showing maximum keratin degradation

Sr.No.	Isolate	Initial weight of feather (gm)	Final weight of feather (gm)	Degree of degradation (%)
1	Control	1.0	1.0	0.0
2	I 1	1.0	0.31	69
3	I 6	1.0	0.49	51
4	P	1.0	0.34	66
5	**M 5-2**	**1.0**	**0.00**	**100**
6	E2N	1.0	0.22	78
7	E4N	1.0	0.43	57
8	C 9	1.0	0.28	72

(a) control flask (b)Degradation by M5-2

Fig.5. Degradation of Chicken Feather.

F. Morphological, Cultural and Biochemical Characterization of Isolate M5-2

The isolate M5-2 found to be actively motile, Gram negative short rod occurring singly (Fig.5).It formed pale yellow coloured colonies on feather meal agar plate. The colonies were regular in shape,moist and smooth. (table 3).It shows caseinase, gelatinase, keratinase, catalase, nitrate reduction,urease, Lysine decarboxylation tests positive.(Table 4)

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Table 3 Colony characters of the isolates M5-2.

Colony characters of organism (M5-2) grown on Feather meal agar incubated at room temperature for 24 hours shows-

Size	Shape	Colour	Margin
1 mm	Circular	Pale yellow	Entire
Surface	Elevation	Consistency	Opacity
Smooth	Low convex	Moist	Opaque

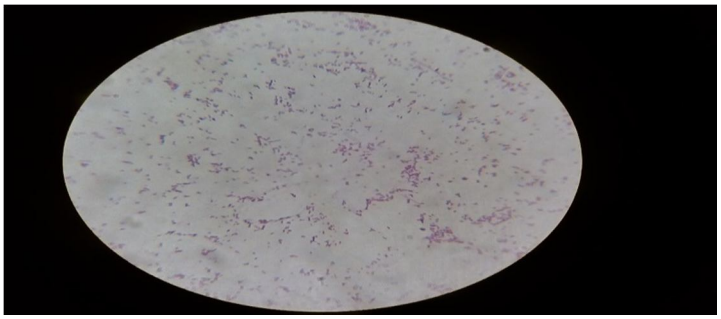

Fig.6. Microscopic Observation of Gram Staining of M5-2 Isolate.

Table 4 – Biochemical test results of isolate M5-2

Sr.No.	Biochemical Test	Result
1	Methyl Red Test	Positive
2	VogesProskauer Test	Negative
3	Citrate utilization Test	Negative
4	Indole Test	Negative
5	Nitrate reduction	Positive
6	H_2S Production	Negative
7	Lysine utilization	Positive
8	Phenyl alanine Deamination	Negative
9	Arginine Hydrolysis	Positive
10	Lactose fermentation	Negative
11	Glucose fermentation	Negative
12	Oxidase	Negative
13	Catalase	Positive
14	Caseinase	Positive
15	Gelatinase	Positive
16	Urease	Positive
17	Keratinase	Positive

After biochemical analysis, DNA of isolated bacteria was extracted and further identified by 16 s r RNA sequencing and deposited in Genbank NCBI .The identified micro-organism is Stenotrophomonas maltophilia YArck and have gene accession no.KY941138.

The Phylogenetic analysis was done using sequence aligned by CLUSTALW in MEGA 6 software. (Fig.6).

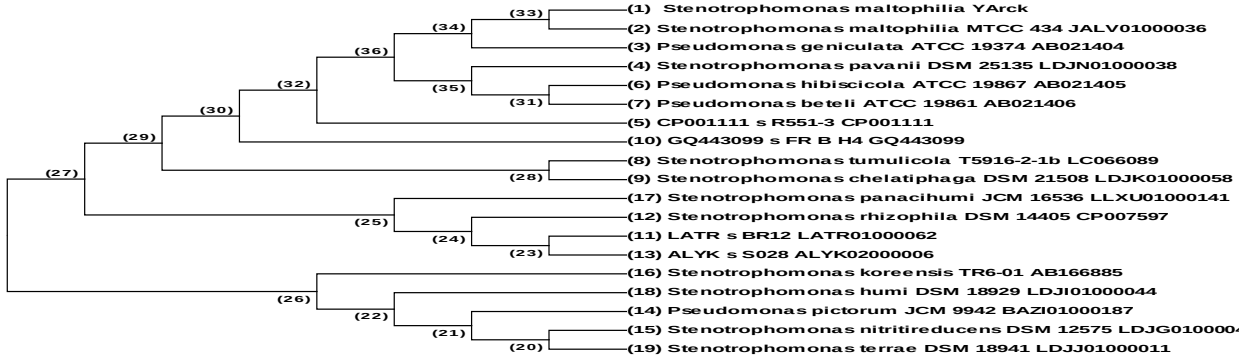

Fig7.Phylogenetic tree analysis of *Stenotrophomonas maltophilia*YArck.

IV. CONCLUSION

In present research work isolation and identification of keratinolytic organism with high keratinolytic activity was successfully carried out. From the 35 isolates, the keratinolytic micro-organism identified as *Stenotrophomonas maltophilia* YArck and have gene accession no. KY941138.It has maximum proteolytic activity. It also degrades native feather completely at room temperature within 72hrs, it indicates that,it has maximum keratinase activity and can be utilized for recycling of keratin rich industrial wastes.By using such organis.m, recycling of poultry and tannary waste for environmental protection through the development of nonpolluting process will be possible.It will be also helpful for clearing obstructions in sewage system during waste water treatment and ecofriendly dehairing process in leather industry.

V. ACKNOWLEDGEMENT

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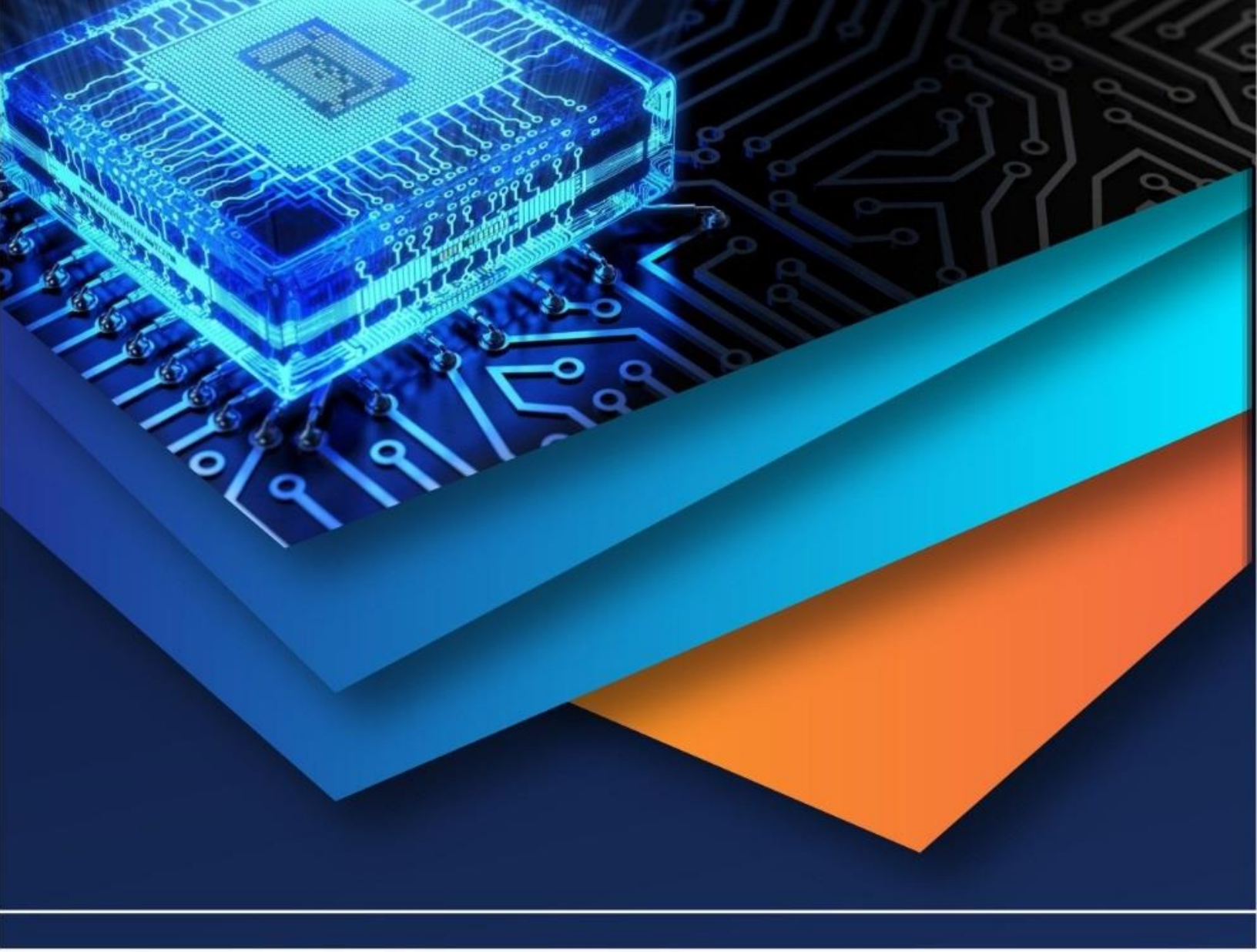

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