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Comparative Studies on Methods of Tannase Assay

Dhruvil Brahmbhatt[1], H. A. Modi[2]
[1,2]*Department of Life Sciences, School of Science, Gujarat University, India*

Abstract – Five bacterial cultures were isolated from the tannery effluent and were screened by plate assay method. Three different methods mainly Colorimetric, UV spectrophotometric and Spectrophotometric methods were selected for the assay of tannase. These methods are selected on their mechanism and principle, feasibility, economical aspect and citations. Colorimetric method measures the liberated glucose; UV spectrophotometric method determines the esterase activity while Spectrophotometric method estimates the released gallic acid formed after the hydrolysis reaction, thus these methods estimate esterase as well as hydrolytic activity of enzyme. A comparative report was made between these methods and their characteristics and limitations were briefly mentioned. All the assays enabled estimation of tannase activity and have their own advantage and limitations. However, from the present study, modified spectrophotometric method is preferred due to its sensitivity and accuracy also it is easy to understand and perform but is expensive and requires preparation of exclusive solutions. In the present study, reports of these methods are presented so that it becomes easy to classify and select a method for tannase assay.
Keywords - Screening, Tannase, Tannase assay, Hydrolysis, Colorimetric and Spectrophotometric method

I. INTRODUCTION

Tannase (E.C. 3. 1. 1. 20) is classified under family of Hydrolases, especially acting on carboxylic ester bonds. The systematic name of the enzyme is Tannin acyl hydrolase or Tannin acetyl hydrolase. Tannase is an inducible enzyme, catalysing the hydrolysis of ester and depside bonds of hydrolysable tannins and gallic acid esters [18]. Tannase sequentially acts on hydrolysable tannins (e.g. tannic acid) to cleave depside bonds followed by hydrolysis of ester bonds which liberates glucose and gallic acid [11]. Tannase is an extracellular enzyme available from fungi, bacteria, some yeast, higher plants and some animal sources as well. The present study concentrates on tannase obtained from the bacterial source, bacterial isolates that are screened from naturally available tannin rich environment having inherent ability to degrade tannins and yield tannase. The enzyme activity is studied by three methods and their features are reported. Tannase has wide industrial applications, in different food and feed, beverage, cosmetics, chemicals and brewing industries, in preparations of gallic acid, in instant tea, acorn wine, coffee flavoured soft drinks, clarification of beer and fruit juices, detanification of food and increasing the nutritive property of feed provided to cattle and also in bioremediation by cleaning up the tannins from effluents of industries specially from leather industries. Recently, bacteria producing tannase have been associated with colon cancer allocating the possibility of bacterial tannase as biomarker for colon cancer [2, 17, 18].

Since its discovery in 18th century there are various workers who have carried out different methods for estimating tannase activity. Several Titrimetric [4, 10, 14], Photometric [26], Colorimetric [5, 7], Visual methods for bacterial tannase and plate assays [19, 21], UV- spectrophotometric [1, 8, 9, 12, 22] and chromatographic [3, 15] methods to quantify enzyme releasing glucose and gallic acid have been proposed. In recent times, Sharma *et al*., 2000 [25] established a spectrophotometric method for assay of tannase. Colorimetric methods by Mondal *et al*., 2001 and Nishitani and Osawa, 2003 [13, 28] were also reported. Some methods are non-specific or present problems in determining an endpoint accurately. In addition, conditions of enzymatic reaction vary. Some others are long and tedious, requiring an extended reaction time or sophisticated equipment. Often, non-readily available substrates are also used. Such methods have been previously reviewed by [1, 3, 16, 18, 27].

Since there are so many methods concerning the assay of tannase, an attempt in the present study is made to evaluate, classify and select a suitable method for estimation of tannase activity. Comparative study between three methods, two being relatively old and one new which would include colorimetric, UV- spectrophotometric and spectrophotometric methods respectively. These methods are selected based on their feasibility, economical aspect, citations and reproducibility. The selected bacterial isolates were evaluated through each of these three methods, their results and advantages or limitations are discussed and a comparison between these methods is studied and reported.

II. MATERIALS AND METHODS

A. Screening and Isolation of tannase producing bacteria

The tannery effluent waste was collected from Central Leather Research Institute (CLRI), GIDC, Ahmedabad. The waste sample was diluted up to 10^{-5}dilution. These dilutions were subjected to nutrient agar (HiMedia, India) plates containing 0.5% tannic acid (Merck, India) as a substrate and sole source of carbon having pH 7.0. These plates were incubated at 37° C for 4 days and colonies surrounded by dark zone of hydrolysis were observed [19]. Composition of tannic acid agar plate (TAA) (gL^{-1}) is as follows: Peptone 5.0, Sodium chloride salt 5.0, Beef extract 1.5, Yeast extract 1.5, agar 15, tannic acid 5.0. Further, the isolated colonies were again subjected to the TAA containing plates with an addition of an antifungal compound to prevent the fungal contamination. The zone forming bacteria were screened by plate assay method and the selected cultures were preserved on nutrient agar slants and regular sub-culturing was done after every 30 days.

B. Inoculum Preparation

All the bacterial isolates were inoculated in nutrient broth which is used as a minimal medium for growth. 20 mL of the minimal medium was prepared and the cultures were allowed to grow till 48 hours in shaking condition at 37° C. 2% (v/v) (i.e. 2mL) volume of these bacterial suspensions were used as inoculum for enzyme production carried in 100 mL of medium.

C. Enzyme Production

Enzyme production was carried in 250 mL Erlenmeyer flask containing 100 mL of production medium. The medium contained (gL^{-1}): Peptone 5.0, Sodium chloride 5.0, Beef extract 1.5, Yeast extract 1.5 and was supplemented with 0.5% tannic acid i.e. 5.0 gL^{-1} having pH 7.0. The medium was sterilised at 121° C at 15 lbs for 15 min. After sterilisation, the flasks were cooled down to room temperature, following to that 2% (v/v) i.e. 2 mL of bacterial suspension was added as inoculum in the medium and incubated in orbital shaker (REMI, India) at 100 rpm till 120 hours at 37° C.

D. Tannase assay

1) Miller Method: This is a colorimetric method where tannase breaks tannic acid which acts as a substrate, forming gallic acid and liberating glucose. Tannase activity was determined by estimating the reduced glucose liberated using 3, 5-dinitrosalicylic acid reagent [7]. Standard curve was studied by preparing dilutions up to 1000 µg/ mL of glucose solution. The activity was determined by using the supernatant or filtered cell-free broth obtained after centrifugation at 8000 rpm at 20° C for 15 min and pre-incubating it with 0.1M acetate buffer (pH 5.0) containing 0.5% tannic acid as substrate. 1 mL of crude enzyme was reacted with the equal amount of substrate which is dissolved in the buffer and incubated for 30 min at 37°C followed by the incubation in boiling water bath for another 15 min to deactivate the enzyme-substrate activity. From this 2 mL system, 1 mL was withdrawn and reacted with 3, 5- dinitrosalicylic acid reagent and finally the system was diluted by adding 10 mL distilled water and absorbance was measured at 540 nm (Visiscan 167, Systronics, India) on spectrophotometer. The formula to calculate activity is: Enzyme activity (U/mL) = Microgram of glucose produced/ V x T, Where microgram of glucose can be obtained from the standard graph, V is the aliquot of enzyme sample and T is the time of hydrolysis [2]. All the tests were performed in triplicates. *One unit of tannase activity is defined as the amount of enzyme releasing 1µmol $min^{-1}$of glucose under assay conditions*.

2) UV spectrophotometric Method: This method is based on change in ultra violet absorption. The enzyme activity is determined by the hydrolysis of the ester bonds of tannic acid. The assay method commences by adding 0.5 mL of crude enzyme to 2 mL of 0.35% (w/v) tannic acid dissolved in 0.05M citrate buffer (pH 5.5) solution. 20 µL of the reaction mixture was withdrawn from the total system and 2 mL of ethanol solution was used to stop enzyme reaction. Absorbance on UV spectrophotometer (*UV-Vis* spectrophotometer 117, Systronics, India) was noted as t_1 at 310 nm immediately after adding ethanol and as t_2 after 10 min of incubation at 37° C. Formula for the calculation of enzyme activity by this method is: Enzyme activity (U/ mL) = 114 x Change in absorbance/ Difference in time (t_2-t_1)[22]. All the tests were performed in triplicates. *One unit of tannase activity is defined as amount of enzyme required to hydrolyse 1µmol of ester in 1 min per mL under assay conditions*.

3) Spectrophotometric Method: The method is based on formation of the chromogen formed after reaction between gallic acid (Sigma Aldrich, India) and rhodanine (HiMedia, India). Tannase acts on methyl gallate (HiMedia, India), which acts as a substrate and releases gallic acid as one of the products. Rhodanine reacts only with gallic acid, forming chromogen which is measured by spectrophotometer at the absorbance of 520 nm [25]. However the method is modified in the present study. Standard curve of gallic acid was performed by preparing concentrations ranging from 0.125 mM to 4 mM. The esterase activity of tannase was determined by using methanolic rhodanine (i.e. rhodanine dissolved in methanol). The protocol begins from obtaining cell free enzyme or crude enzyme sample by performing centrifugation at 10,000 rpm for 10 minutes at 15° C. Supernatant from the centrifugation step was filtered aseptically using sterile filters of 0.2 µm pore size (Merck Millipore,

India). 100 µL of enzyme sample was treated and pre-incubated at 37° C for 10 min with 100 µL of 0.025M methyl gallate prepared in 0.05M phosphate buffer (pH 6.5) solution. 150 µL of methanolic rhodanine (0.667% w/v rhodanine in 100% methanol) was added to the mixture and incubated for 5 min at 30° C. Following this, 100 µL of KOH (0.5M) was added and mixture was diluted to 5 mL by adding double distilled water. After the incubation of another 10 min at room temperature, absorbance at 520 nm was measured on Systronics Visiscan 167 spectrophotometer. A set of blank and control tubes were prepared simultaneously, where 100 µL of 0.05M phosphate buffer (pH 6.5) was added in blank tube and 100 µL of enzyme sample was added after the addition of KOH in the control tube only. The enzyme activity was calculated from change in absorbance: $\Delta A_{520} = (A_{test} - A_{blank}) - (A_{control} - A_{blank})$ as given. All the tests were performed in triplicates. *One unit of tannase activity is defined as the amount of enzyme required to release 1 µmol of gallic acid per minute under standard assay conditions*.

III. RESULTS AND DISCUSSION

A. Screening and Isolation of tannase producing bacteria

From the tannery effluent waste, around 13 different types of bacterial isolates were obtained out of which 5 isolates were selected based on their zone of hydrolysis on the TAA plates. Five bacterial isolates (labelled from BT 1 to BT 5) produced dark zone of hydrolysis thus confirming their tannin degrading property (as shown in Fig. 1). Microscopic examination revealed that all the organisms are Gram negative short rods. The bacterial growth pattern was optimum after 48 hours of incubation. Reports on isolation of bacteria from tannery effluents of different regions and countries are known for the purpose of biodegradation of tannins polluting environment [20, 23] and also molecular diversity of bacterial isolates from tannery effluent is studied [24]. However, present report emphasis on utilising the isolates for enzyme production as studied by *Jayaraman and Sivashanmugam* in 2011 [6].

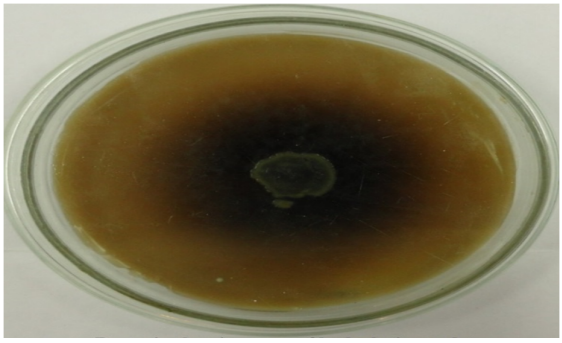

Fig. 1: Bacterial isolate showing zone of hydrolysis on TAA plate

B. Miller Method

From the results below (as shown in Fig. 2) it was clear that the enzyme activity of isolate BT 5 giving 25.73 U/ mL was optimum after 96 hours followed by isolate BT 1 giving 25.46 U/ mL of activity after 120 hours of incubation for enzyme production. The graph below represents the enzyme activity of the isolates at different hours along with standard errors. The method determines the amount of glucose liberated in the production medium and so it gives such high values for enzyme activity. The method is quite simple, economical and easy to perform. However, it's not reliable and is more time consuming than the other assays. Another limitation is that it is not specific for liberated glucose and may also give false results when interfered by other reducing sugars.

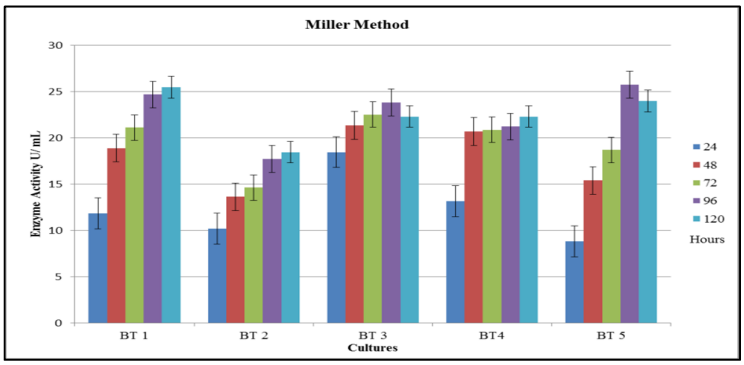

Fig. 2: Enzyme activity by Miller Method

D. UV spectrophotometric Method

Enzyme activity of isolate BT 5 was 3.12 U/ mL after 96 hours of incubation whereas BT 1 was 3.02 U/ mL after 120 hours of incubation is represented below (as shown in Fig. 3). The results were relatively similar to *Miller's* Method as same isolates gave maximum activity. The graph below represents the enzyme activity of bacterial isolates which was estimated by hydrolysis of ester bonds of tannic acid along with the standard errors. The method is preferred by many workers for determining tannase activity. The main advantage of this method is that it is simple, rapid and sensitive and requires minimum amount of sample. However, it requires ethanol which becomes a limiting factor, as it is expensive and one of the major drawbacks is that it cannot detect intracellular tannase.

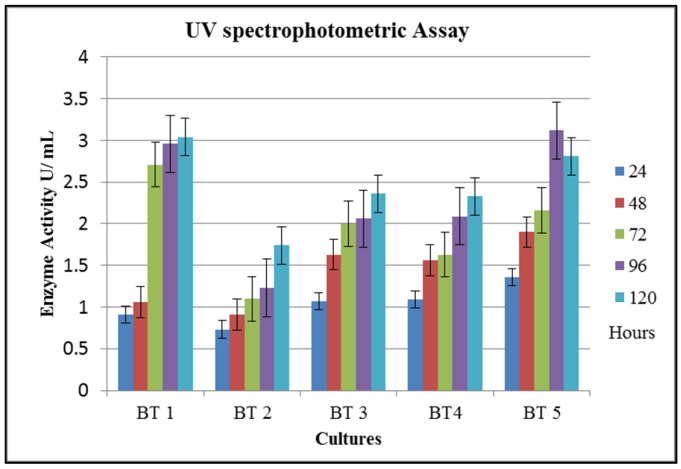

Fig. 3: Enzyme activity determined by UV spectrophotometric method

E. Spectrophotometric Method

The method is based on the formation of a chromogen formed by reaction between the gallic acid released and rhodanine. Bacterial isolate BT 5 gave enzyme activity of 6.09 U/ mL after 96 hours of incubation and activity of BT 1 was 5.68 U/ mL after 120 hours of incubation. The graph (as shown in Fig. 4) below shows enzyme activity by evaluating the released gallic acid along with the standard errors. Bacterial isolates BT 5 as well as BT 1 were consistent in their activity for all the assays. This method is comparatively new than the other methods and is widely preferred by workers for estimating the tannase activity for their cultures. The main advantage of this method is, it is relatively new, highly sensitive, rapid, and easily reproducible and it estimates the esterase activity of the enzyme. However one major drawback is that the chemicals required are relatively expensive and exclusive.

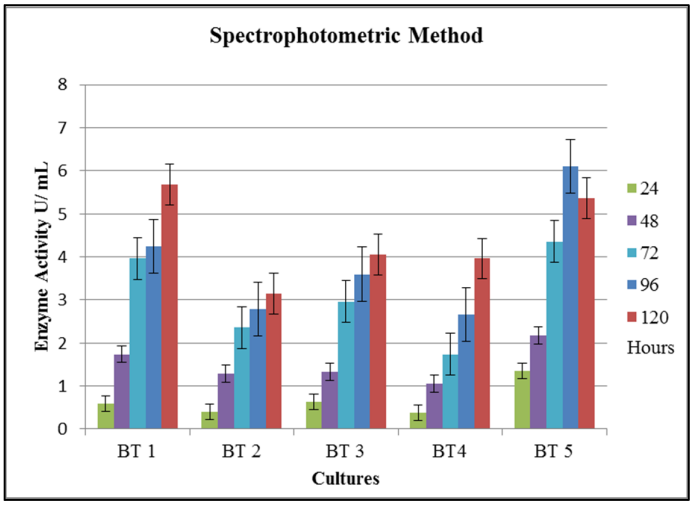

Fig. 4: Enzyme activity determined by Spectrophotometric method

F. Comparative study

The three different methods for estimating tannase activity were selected based on their different principles and their preference by various workers in the field. The graphical representation (as shown in Fig. 5) is done to display the enzyme activities of

selected five isolates after 96 hours of incubation for enzyme production. Miller method shows high value because it estimates the liberated glucose in reduced form after the enzymatic reaction, while the other two methods are based on principle of their esterase and hydrolytic activity of the enzyme respectively. Spectrophotometric method is a relatively new method compared to the other two and hence is preferred by many scientists all over the globe because it is more sensitive and accurate and can be used for crude enzyme studies, in studying the characterization of tannase, in screening the selected isolates and in kinetic studies of enzyme. The characteristic features as well as limitations of these methods are briefly written in Table I. given below.

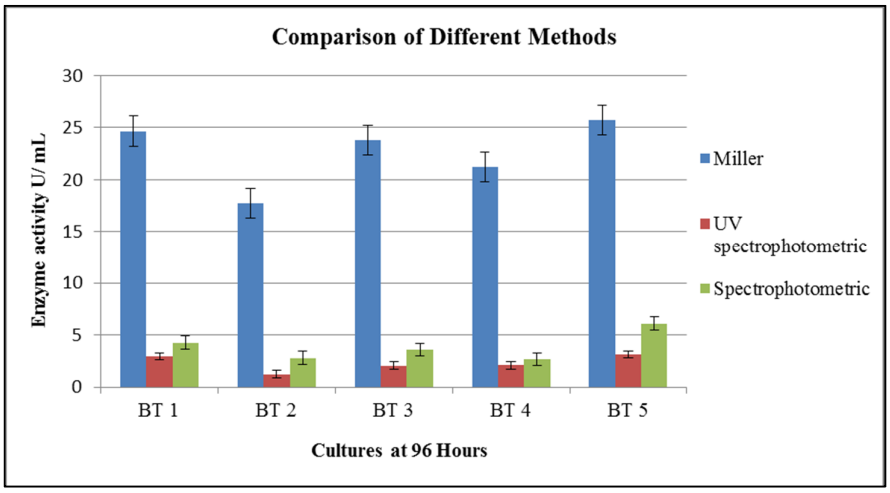

Fig. 5: Comparison of Different Methods for Different isolates at 96 Hours

TABLE I. CHARACTERISTICS OF DIFFERENT METHODS

Methods	Features/ Advantages	Drawbacks/ Limitations
Miller Method	Simple, Economical, easy to perform	Not specific, Time consuming and Not reliable
UV spectrophotometric	Simple, Rapid, Sensitive and requires minimum amount of sample	Requirement of ethanol, Requires sophisticated instrument, tedious and cannot detect intracellular enzyme
Spectrophotometric	Highly sensitive and accurate, relatively new, easy to understand and calculate	Exclusive chemicals (rhodanine), (preparation of different solutions) and expensive

IV. CONCLUSION

Tannery effluent was used to isolate the bacteria having natural ability to degrade hydrolysable tannins by producing tannase. These isolates were screened by plate assay method which showed zone of hydrolysis on TAA plates, five isolates were selected and their activity was studied by three different methods. These methods work on different principles and mechanisms and so Colorimetric, UV spectrophotometric and Spectrophotometric methods were chosen. All the isolates were screened through these assays and out of all, isolate BT 5 gave the finest results in all the assays after 96 hours of incubation period. Miller Method estimates the liberated glucose from the enzymatic reaction; UV spectrophotometric method estimates the esterase activity of tannase by detecting the hydrolysis of ester bonds while Spectrophotometric method detects the liberated esters in form of gallic acid by the enzyme reaction.

In the present study, different characteristics and limitations of these techniques are briefly reported and a comparative study is carried out among the selected isolates. Even though there are many methods for assay of tannase activity with each having its advantages as well as limitations, there is a need for a method which could be ideal in all aspects with minimum limitations. According to the analysis in the present work, the modified spectrophotometric method was easy, highly sensitive and reliable as well as relatively new, hence it was preferred. But, the limitation is that it becomes expensive due to exclusive chemicals. However, it is recommended that before selecting a method, it should be thoroughly understood along with its calculation part, its requirements and its merits and demerits.

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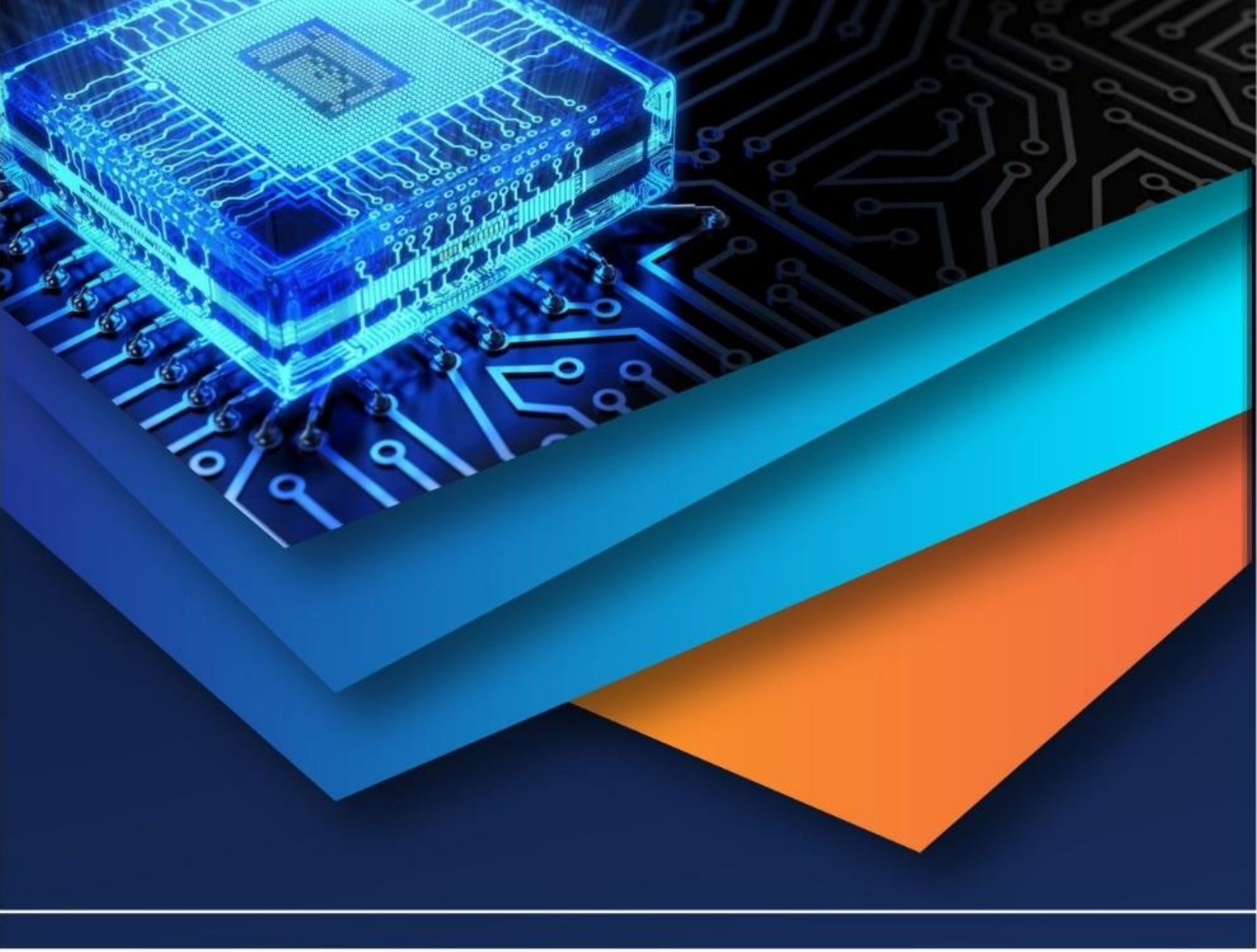

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