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Screening and Isolation of Polyhydroxy butyrate Producers

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Abstract*: A study was conducted to isolate, screen and identify bacteria for the production of a biodegradable polyester plastic called poly-β-hydroxy butyrate(PHB). It is a substitute for conventional non-biodegradable plastics and has many applications in various areas like industries, pharmacy and agriculture. The present study reports the isolation and screening and identification of PHB producers from marine water. Five isolates were screened using sudan black staining method. These isolates were then cultured in a PHB fermentation media and the product obtained was purified and quantified. The strains were characterized morphologically, biochemically and were identified by 16S rRNA sequencing. The Strain isolated from Sau showed high PHB production of 35.72% by sample 1 and 24.03% by sample 2 from Pen among the five strains. Results showed the two isolates were gram positive rods and were identified as Bacillus sp. and Enterobacter sp.
Keywords: Polyhydroxy butyrate, PHB production, screening, characterization, Sudan black staining

I. INTRODUCTION

Plastic materials have become part of our life as a basic need but it poses serious problems as it is non-biodegradable. These materials remain as waste for several years. Recycling plastic has its own limitations and has a tremendous harmful impact on the environment. The bioplastic is an ideal alternative for the environmental and waste management problems. The Biopolymer is biodegradable and is produced by living organisms which can be converted into carbon dioxide and humic materials. These are polyesters which include polyhydroxylalkanoates(PHAs), polyhydroxybutyrate (PHB), polylactides, and aliphatic polyesters. PHB is produced by many micro-organisms in response to physiological stress. It is a product of carbon assimilation and used by micro-organisms as sources of energy while other sources of energy are not available [1]. It is derived from renewable and biodegradable materials and is compostable [2].PHB is water insoluble and relatively resistant to hydrolytic degradation. This differentiates PHB from most other currently available biodegradable plastics, which are either water soluble or moisture sensitive. PHB has good oxygen permeability, ultra-violet resistance but poor resistance to acids and bases. PHB is soluble in chloroform and other chlorinated hydrocarbons and biocompatible and hence it is suitable for medical applications. PHB sinks in water facilitating its anaerobic biodegradation in sediments [3].This study aims at isolation and study of Polyhydroxybutyrate from micro-organisms from marine environment as the marine source is less exploited.

II. MATERIAL & METHODS

A. Sample collection

The water samples were collected from the areas near Mumbai and Navi Mumbai.

Fig. 1 Marine sample collection sites in Mumbai and Navi Mumbai

Water samples were collected from the areas like Juhu, Colaba, Dadar, Marine drive, Haji Ali, Bandra, Pen, Belapur, Thane, Airoli. Hot water samples were collected from Vrajreshwari and Sav. These samples were serially diluted in Saline Solution.

B. Screening of bacterial isolates for PHB production

The isolates were screened using Sudan Black Staining Method. For this method, the plates containing nutrient agar were divided into 4 parts. The bacterial isolate were spotted in duplicates and incubated at 30˚C for 24 hours. Ethanolic solution of (0.02%) Sudan Black was spread over the colonies and the plate was kept undisturbed for 30 minutes. They were washed with ethanol (96%) to remove the excess stain from the colonies. The dark blue stained colonies were the positive isolates for PHB production. These isolates were sub-cultured and maintained at 4˚C [4].

C. Fermentative PHB production

PHB production is a two-step process. The first step was cell growth in carbon rich medium where inoculum (Mixed with phosphate buffer and the absorbance was set to 0.5 at 660nm) was grown in 100 ml medium. The medium was prepared using $(NH_4)_2SO_4$, 20 g/L; KH_2PO_4, 13.3 g/L; $MgSO_4.7H_2O$, 12 g/L; Citric Acid, 1.7 g/L; trace element solution, 1.0 ml/L containing $FeSO_4.7H2O$ 10g/L; $ZnSO_4.7H_2O_2$, 25g/L; $CuSO_4.5H_2O$, 1.57 g/L; $MnSO_4.5H_2O$, 0.5 g/L; $CaCl_2.2H_2O$,2 g/L; $Na_2B_4O_7.10H_2O$, 0.23 g/L; $(NH_4)_6Mo_70_{24}$, 0.1 g/L; pH 7.2 at 29˚C at 120 rpm for 24 hours. Broth was centrifuged at 10,000 rpm for 15 minutes and the biomass was collected and weighed. The second step was cell growth in Nitrogen Deficient Minimal Medium (NDMM) consisting of Na_2HPO_4, 3.8 g/L; KH_2PO_4, 2.65 g/L; NH_4Cl, 2 g/L; $MgSO_4$, 0.2 g/L; Fructose, 2 g/L and trace minerals 1.0 ml/L, which contained EDTA, 5.0 g/L, $ZnSO_4.7H_2O$, 2.2 g/L, $CaCl_2$, 5.45 g/L, $MnCl_2.6H_2O$, 5.06 g/L, H_3BO_3, 0.05 g/L, $FeSO_4.7H_2O$, 4.79 g/L, $(NH_4)_6Mo_70_{24}$,0.4 g/L, $CoCl_2$ $6H_2O$, 1.6 g/L, $CuSO_4$ $5H_2O$, 1.57 g/L. pH, 7.0 at 30°C for 48 h (at 120 rpm)[5].

D. Extraction and Quantification of PHB

Fermentative samples were centrifuged at 10,000 rpm for 10 min and washed with acetone and ethanol. The pellet was suspended in 4% Sodium hypochlorite and incubated at room temperature for 30 min. The suspension was again centrifuged at 10,000 rpm for 10min. The supernatant was discarded and pellet was washed with acetone and ethanol. 10ml of chloroform was added to the tube by placing it in hot water bath (60˚C) to separate PHB from liquid broth. Chloroform was evaporated to obtain PHB crystals.

$$\text{PHB accumulation (\%)} = \frac{\text{Dry weight of extracted PHB (g/L)} \times 100\%}{\text{CW (g/L)}}$$

The extracted PHB from the five isolates was dissolved in 10 ml of concentrated $H_2SO_4$and the tube was heated in a water bath kept at 60˚C for 1hr to complete the conversion of PHB crystals into crotonic acid. The sample was cooled, vortexed and the amount of PHB was determined spectrophotometrically at 234nm against H_2SO_4 as the blank using 200 mg/ml crotonic acid as the standard [6]The standard curve was used for the estimation of PHB yield[7].

E. Characterization and identification of Bacteria

The morphological characteristics of the isolate were examined. The potent isolate were observed for colony characterization by observing size, shape, presence of endospores and motility of the cells were determined. The biochemical characterization was done according to the method of Bergey's Manual of Determinative Bacteriology. 16S rRNA sequencing of the strain was carried out for further identification by Chromous Biotech, Bangalore. The genomic DNA was isolated the and the 16S rRNA fragment was amplified using high fidelity PCR polymerase, the product was sequenced bi-directionally and the sequence data was aligned, analyzed and the nucleotide sequences were submitted to Gen Bank.

III. RESULTS AND DISCUSSION

A. Isolation and Screening of PHB producers

Biodegradable plastics production has to be developed in order to replace plastic from the petroleum origin. The study was based on the isolation, screening and quantification of PHB producing microbes from a marine environment as there are few reports on PHB producers from marine ecosystem. There exists tremendous biodiversity in the marine environment whose knowledge is considerable miniscule hence it was selected as the source for biodegradable plastic producers. Lee *et al*., 2000have reported the production of biopolymers from marine bacteria[8]. However, there are several reports of PHA accumulating Vibrio's from marine sediments [9,10].The PHB and PHA granule enhance the survival ability of microbes under adverse conditions by serving as the storage material and helps to cope with the stressed and imbalanced environment[11].The present study was focused on isolating, screening and identifying the polyhydroxybutyrate (PHB) producing bacteria from marine sources that can be effectively utilized for

the synthesis of bioplastics because it is easily biodegradable. Marine water samples were used as the source as marine microbes are efficient in producing versatile compounds due to the versatile environment and the nutritional stress in which they survive. Samples of 10^{-5}dilutions were cultured on Nutrient Agar Plates and incubated at 37˚C for 24 hours to isolate the marine bacterial colonies. The isolates were tested for PHB production following the viable colony screening using sudan black staining method as shown in Fig. 2. The use of Sudan black dyes was based on the idea that these synthetic organic compounds stain the plastic compounds into a bluish black color. Nine different isolates were identified positive for PHB accumulation.

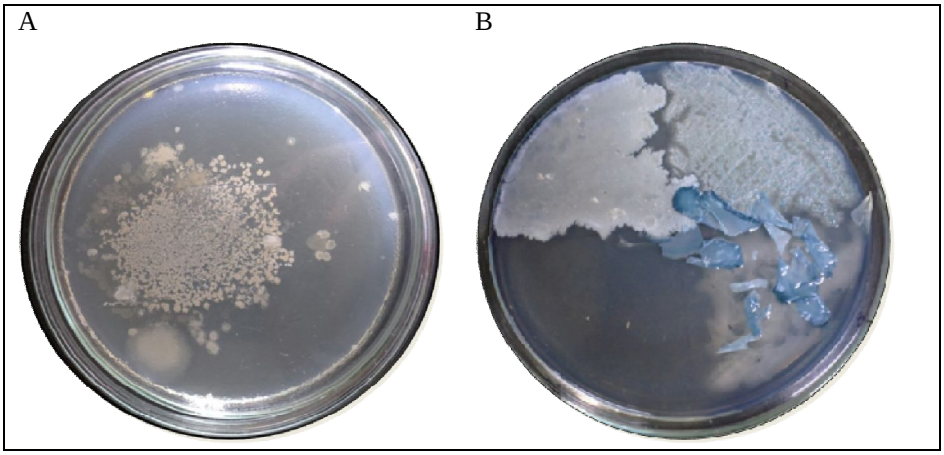

Fig. 2A. Isolation of marine sample on Nutrient agar medium; B. Screening of PHB producer using sudan black stain; the positive isolate showed the appearance of blue black droplets

B. Extraction of PHB

Five out of the nine isolates were processed for fermentative PHB production based on the intensity of uptake of sudan black stain and on the variation in their colony characteristics.PHB synthesis was carried out in two steps using Carbon-rich medium and Nitrogen deficient medium. It was found to increase from the log phase of growth and it continued until late exponential phase as the carbon source was utilized for growth and product formation. PHB produced by all the five isolates was purified using sodium hypochlorite-chloroform method. Sodium hypochlorite solution was used for the lysis of bacterial cells as PHB is accumulated intracellular. Acetone ethanol (1:1 v/v) solution was used to wash out the non-PHB cell mass as it is more specific and useful solvent (PHB unaffected) and the PHB granules were obtained after drying as shown in Fig. 3. It was quantified spectro photo metrically using crotonic acid as the standard.

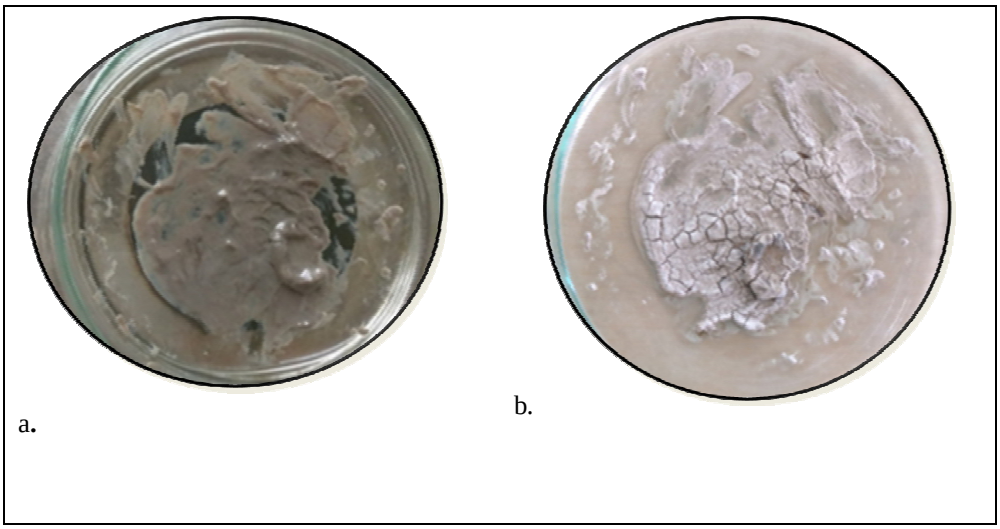

Fig3: Extraction of PHB granules using sodium hypochlorite-chloroforma. before evaporationb. after evaporation

The PHB accumulation was calculated forall the five isolates and is shown in Table 1. The table indicates two promising bacterial isolates which showed increased production of PHB of 35.72% by sample 1 of 0.8g/L CDW from Sau and 24.03% of 0.498g/L CDW by sample 2 from Pen.

Table 1: Residual biomass and PHB accumulation of the five isolates after extraction

SAMPLE NO.	CDW (g/L)	PHB (g/L)	PHB (%)
1	0.800	1.440	35.72
2	0.498	1.575	24.03
3	0.591	2.020	22.64
4	0.550	1.902	22.43
5	0.401	1.472	21.41

C. Quantification of PHB after extraction

The quantification method involves the breakdown of the PHB polymer into its monomers. These monomers are then converted into a compound that was measured using crotonic acid as the standard on a calibration curve. The amount of UV radiation absorbed by crotonic acid directly corresponds to the amount of PHB present in the sample.

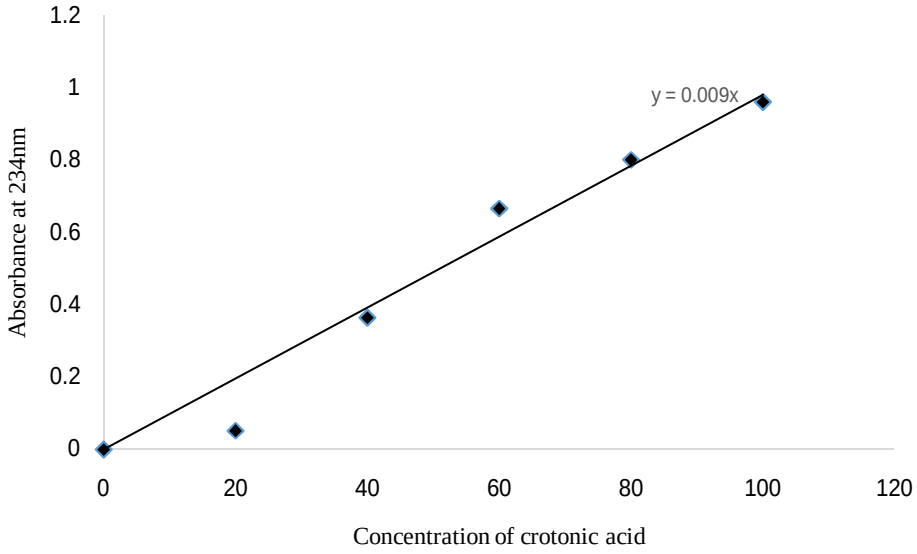

Fig 3: Standard calibration curve of crotonic acid

The equation from the standard curve was made use of to quantify the amount of unknown PHB extracted from the five isolates, as shown in Figure 3 and the values are mentioned in Table 2. The concentration of PHB is indicated graphically in Fig. 4.

Table 2: Determination of Concentration of PHB from the standard calibration curve using crotonic acid as the standard

SAMPLE NO.	CONCENTRATION OF PHB (mg/L)
1	108.57
2	59.60
3	58.88
4	42.55
5	39.39

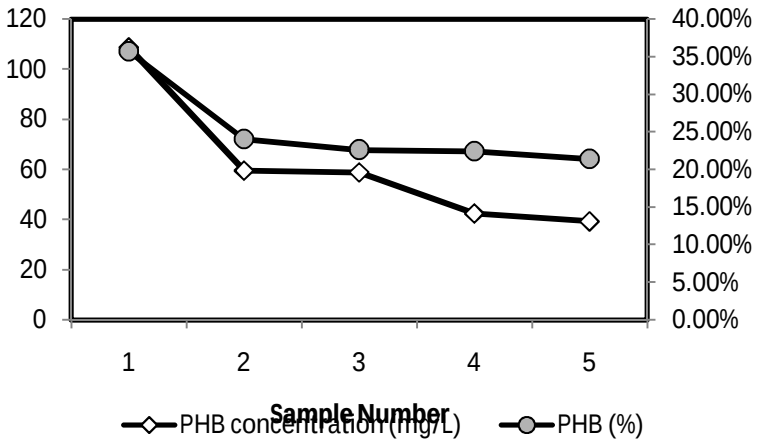

Fig. 4 Comparative graph of concentration of PHB obtained on extraction and after quantification using crotonic acid as the standard

Ceyhan and Ozdemir, 2011 reported polyhydroxy butyrate production from domestic waste water using Enterobacteraerogenes 12Bi strain with good yield ranging from 16.66 to 96.25%[12]. Hassan et al., 2016 reported PHB of 0.17 g/L and calculated in relation to cell dry weight as 20% CDW for Bacillus sp. obtained from the soil sample of Egypt[13]. Dhangdhariya et al., 2015 carried out the maximum PHB production of 21.7% from Bacillus magaterium JK4h using novel dry sea mix as the growth media[14]. Ghate et al., 2011 obtained the highest production of PHB of 0.284 g/L using Neera as the agro-industrial waste material with Bacillus subtilis[15].

D. Characterization of PHB Producers

The isolates were found to be gram positive rods. Sample 1 colonies were off-white in colour with irregular surface with 3mm diameter whereas; sample 2 showed orange yellow colour with rough surface with pinpoint colonies. The biochemical characteristics of the samples were investigated according to Bergey's manual of Bacteriology. Sample1 and 2 were found positive for catalase, citrate and Voges -Proskauer and both could ferment glucose. Positive results for Methyl red and sucrose and mannitol fermentation were observed for sample 1unlike sample 2. Based on the morphological and biochemical characterization the samples were identified as Bacillus sp. The additional characterization by 16S rRNA sequencing helped us to identify the genome of the isolate along with its phenotypic relation with other species and the results revealed that sample 1 and 2 contains 1256bp and 1302bp. Blast analysis of the sequence had a high scoring similarity of 99% and were identified as Bacillus sp. and Enterobacter sp.The sequence of 16S rRNA was submitted to the GenBank database and can be accessed under GenBank accession numbers KY801697 (Bacillus sp. strain YXA3-50) and KY801696 (Enterobacter sp. strain tv1)

IV. CONCLUSION

Although there are many sources which are capable of producing PHB, the bacterial source is supposed to be the best of all due to its rapid growth and multiplication in very short time and higher yield of polymer.The carbon rich medium and nitrogen deficient medium served as the best source for PHB production. The PHB yield of the hot spring water isolate Sau showed the best result with highest concentration of 108.57mg/L. Studies need to be carried out for large scale production of PHB utilizing simple and cheaper nutrient sources which would replace the petroleum derived polymers. The biopolymers from bacteria will help to produce biodegradable products in large quantities at a very low cost. So, focusing research in these areas will help to completely avoid the usage of non-biodegradable products in future.

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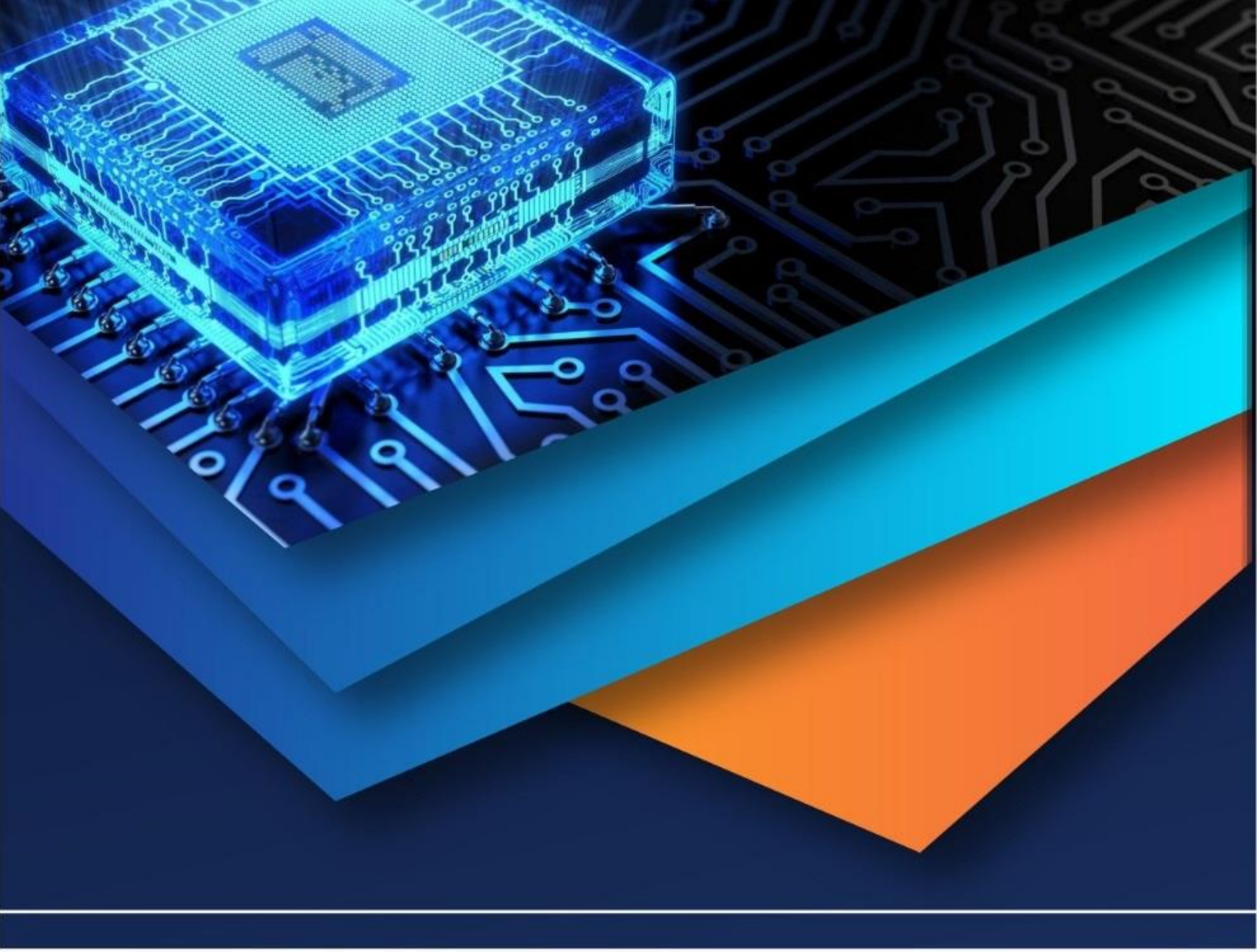

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