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Utilization of Controlled Wastewater Nutrients for Lipid Production from *Botryococcus Braunii*

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Abstract - Diminishing oil reserves, rising oil prices and a significant increase in atmospheric carbon dioxide levels have led to an increasing demand for alternative fuels. Microalgae have been suggested as a means for fuels production because of their advantages related to higher growth rates, higher photosynthetic efficiency and higher biomass production, compared to other terrestrial energy crops. During photosynthesis, microalgae can fix carbon dioxide from different sources, including the atmosphere, industrial exhaust gases and soluble carbonate salts. To determine the most optimal conduction for the growth of Botryococcus braunii in order in order to produce lipids that can be transformed into biodiesel fuel, different nutritional conduction were investigated. For this purpose sewage water is used as a media; and effect of nutrients viz., carbon (C), nitrogen (N), phosphorus (P) and potassium (K) etc.. on biomass growth and lipid accumulation was elucidated and the results achieved are most effective and further analyzed for biomass production chlorophyll content and lipid content. The best growth resulted in an enriched solution from the N+P media condition and the best results were obtained when medium was maintained at 16 hrs light/8 hrs darkness cycles and mechanical stirring at 120 rpm (only during the lighting hrs) with higher lipid content of 70% was observed with (C) condition.

Keywords-- Biofuels, microalgae, Botryococcus braunii, sewage water, batch culture.

I. INTRODUCTION

The colonial alga Botryococcus braunii is widespread in freshwater environments and can be also found in brackish lakes, reservoirs, ponds in subtropical or tropical zones [1-7]. This alga is famous for a notable ability to synthesize a variety of lipids. It produces a high amount of hydrocarbons, excretes them outside the cells, and accumulates them in its extracellular matrix [8, 9]. Some strains of Botryococcus braunii can also produce several certain ether lipids [6, 10]. The hydrocarbon content in Botryococcus braunii is significantly higher than that in other oil-producing algae and it has already been proposed as a future renewable source of fuel

Biotechnological value of Botryococcus braunii: It is known that hydrocarbons from Botryococcus braunii can be used as direct substitute of petroleum-based fuel products without the complexity of tranesterification and low temperature gelling, which showed an inherent superiority of producing hydrocarbons over biodiesel. Botryococcus braunii has a thicker cell wall than most strains, and usually the wet biomass contains a lot of water which cause the efficiency of hydrocarbon recovery to be rather low. Enzymes regulates the unique pathways of triterpene biosynthesis in Botryococcus braunii have attracted many interests.

Biodiesel fuel is becoming more promising as it is produed from non toxic, biodegradable and renewable resources and its use leads to a decrease in the emission of harmful air pollutants [11].

Microalgae are a group of fast growing unicellular or simple multicellular micro organisms, which have the ability to fix CO2 while capturing solar energy with efficiency 10 to 50 times greater than that of terrestrial plants and higher biomass production compared to energy crops [12]. Microalgae have several advantages, including higher photosynthetic efficiency as well as higher growth rates and higher biomass production compared to other energy crops. Several microalgae strains have been reported to have the ability to accumulate large quantities of lipids.

Microalgae Botryococcus braunii was chosen as a subject for this research due to its easy growth in a sewage water as a media without the necessity of utilizing very specific compounds and its significant lipid content, as lipids are the most desirable component from the energy point of view.

The purpose of this work was to establish the growth of B. braunii in a medium where the nutrients and the other organic compounds existing in the wastewater can serve as a source of nutrient medium for the growth of algae. The process also provides economic mode of lipid production from wastewater. Therefore an attempt was made in this study to evaluate the influence of sewage waste water in association with nutrients supplementation on growth and lipid accumulation using Botryococcus braunii and

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sewage wastewater as substrate. Influence of nutrient conditions was studied individually and in combinations. Biomass growths were estimated while lipids were extracted and analyzed. An investigation was based on batch experiments carried out in a lab in order to optimize the medium composition for the best microalgal biomass and lipid yield, and consequently for the economic efficiency of the process if used in an up-scale for biofuel production.

II. MATERIALS AND METHODS

A. *Microalgae strain and medium*

Botryococcus braunii was isolated from mixed microalgae culture. *B. braunii* was grown on CHU media.

B. *Cultivation*

The cells in exponential period were inoculated (10%, v/v) in a sewage culture medium (sewage water was autoclaved at 121^0 C for 20 min and then twice filtered through whatt man filter paper) to start the culture. In addition to this different nutrients viz., nitrogen (N), phosphorus (P), carbon (C) and NPK. Nine experimental variations were studied by varying the nutrients as described in Table 1. A control condition without the addition of external nutrients was operated parallelly to elucidate the effect of native nutrients present in the sewage water.

All the experiments were carried out at a photo period of 16 h light:08 h dark. Light intensity during the experiment was measured using a light meter (Li- Cor measuring device). In the light phase, flasks were placed in a rotary shaker at 120 rpm for mixing. The cultures were maintained at room temperature (25-27ºC) at pH: 8.2 on a fluorescent light with a light dark photoperiod of 16h: 8h. Sterile-air containing 2% (v/v) CO2 was aerated into the flask through an air sparger at the bottom of the flask. The strains were checked for 25 days growth period. All experiments were conducted in duplicates.

C. *Growth measurements*

The growth of *B. braunii* was measured via spectrophotometry and biomass dry weight. Optical density for biomass factor was determined at wavelength 550 nm. One ml of sample was appropriately diluted with deionized water and the absorbance of the sample was read at 550 nm.

The cultures were determined gravimetrically and growth was expressed in terms of dry weight (mg/L) [13]. The cultures were harvested by centrifugation at 3000g for 10 min and the cells were washed with distilled water. Then the pellet was freeze dried. The dry weight of algal biomass was determined gravimetrically and growth was expressed in terms of dry weight (g/l).

The biomass yield was calculated. Biomass yield (mg ml-1) = $\frac{\text{Final weight (g) – Initial weight (g)}}{\text{Sample taken (ml)}}$

D. *Chlorophyll and carotenoid content*

The isolation of pigments from algae cells included the folowing procedures: harvesting 2 ml of microalgae cells by centrifugation at 10000 rpm, two times for 3 min and discarding the supernatant, suspension of cells in 2 ml methanol/water 90:10 v/v and mixing of Vortex for 1 min., heating of the suspension for half an hour in a water bath at 60ºC, cooling of the samples at room temperature, centrifugating the suspension (10000 rpm for 3 min) and discarding the supernatant with dissolved pigments. The absorbance of the pigments extract (665, 652 nm for chlorophyll content (a+b) and 470, 666 nm for carotenoids content) was recorded by using spectrophotometer. The chlorophyll content was computed (mg/l) according Porra et al. [14] and carotenoid content was computed (mg/l) according Lichtenthaler [15].

Chlorophyll (a) and chlorophyll (b) were estimated as given below.

Chlorophyll a (µg ml-1) = 16.75 x A665.2 - 9.16 x A652.2

Chlorophyll b (µg ml-1) = 34.09 x A652.4 – 15.28 x A665.2

Total Chlorophyll (a+b) content was calculated.

E. *Lipid content*

The total lipids were extracted from microalgae biomass using a modified method of Bligh & Dyer, 1959 [16]. The lipids were extracted using a mixture of chloroform/methanol (1:2 v/v). The quantity of lipid residue was measured gravimeterically and expressed as dry weight percentage.

III. RESULTS AND DISCUSSION

A. *Culture of Botryococcus braunii*

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Much research has been carried out on investigating the optimal culture conditions for Botryococcus braunii. It is recognized that the production of hydrocarbons in Botryococcus braunii is positively correlated to the cell growth, regardless of the particular culture conditions and the media used. According to the algal biochemistry, it is such a substantial commitment for Botryococcus braunii to produce a considerable amount of hydrocarbons, the energetically expensive molecules. Therefore, the observed slow growth rate of this microalgae compared to other strains is apparently not the consequence of nutrient limitation, but the energetic consumption to synthesize and accumulate hydrocarbons.

Botryococcus braunii culture requires water, light, CO2, and inorganic nutrients. Factors that govern the algae productivity include pH, pCO2, light intensity, salinity and temperature. Modified Chu-13 medium is often used as growth medium for Botryococcus braunii. This medium has the following composition (g·L–3): KNO_3 (0.05), K_2HPO_4 (0.01), $MgSO_4 \cdot 7H_2O$ (0.025), $CaCl_2 \cdot 6H_2O$ (0.02), ferric citrate (0.0025), citric acid (0.025), boron (0.125 ppm), manganese (0.125 ppm), copper (0.005 ppm), cobalt (0.005 ppm), and molybdenum (0.005 ppm). The pH in the media is adjusted to 7.5 before sterilization [17].

CO_2 is necessary for Photosynthetic cultures of Botryococcus braunii. CO_2 enrichment favors the formation of lighter botryococcenes (C30–C32), while a substantial portion of heavier botryococcenes (C33–C34) are discovered in the cells sparged with ambient air [18].

Although under autotrophic cultivation, Botryococcus braunii could also utilize exogenous carbon sources for enhanced cell growth, as well as hydrocarbon production. A variety of carbon sources, such as C1-C6, lactose and sucrose, have been demonstrated to achieve a decrease in mass doubling time of the microalgae from 6-7 days to less than 2-3 days [19]. Even though we found that a deficiency of nitrogen supply favors lipid accumulation [20], nitrogen is still essential for growth. Studies on the nitrogen source as NO3 –, NO2–, and NH3 indicate that the critical factor in regulating nitrogen metabolism in Botryococcus braunii is the nitrate uptake system. Thereby nitrogen is generally supplied as nitrate salts. An initial NO3- concentration of ≥0.2 g•L-3 favors hydrocarbon production [21].

Brenckman et al. found that with 1 g•L-3 KNO3 the hydrocarbon production after 30 days was 4.8 g•L-3, while about the same level of hydrocarbon production (4.5 g•L–3) was obtained after 30 days if the initial concentration of KNO3 in the culture media was 3 g•L–3. This is because high concentration of nitrate interferes with hydrocarbon production of Botryococcus braunii [22]. Phosphorus is also essential for Botryococcus braunii growth, and usually supplied in the form of K_2HPO_4. However, active growth persists after complete depletion of phosphate in the medium. In the early exponential phase, phosphate levels in the medium can drop to below the detection limit (0.5 mg•L–3) [23].

Botryococcus braunii can rapidly assimilate phosphate even over the cellular metabolic requirement. Cells store the excessive phosphate as intracellular granules. The excessive phosphate is stored as intracellular granules. When the extracellular phosphate is depleted, the cells will begin to use this phosphate reserve. As discussed before, high light intensity may increase the carotenoid/chlorophyll ratio, and thus induce the color change in cell colony [24]. Experiments showed that when Botryococcus braunii are exposed constantly to a light intensity within the range of 25 to 72 μE•m-2s–1, carbohydrate concentration, intracellular nitrogen, and phosphorus content drop [25].

Botryococcus braunii that is exposed to a high light intensity during early stage of cell culture could reach a higher biomass density (7 g•L–3) and hydrocarbon content (approximately 50% of cell dry weight) compared to cells that had been exposed to low light intensity (3 klx, or ~42 μE•m–2s–1) [26]. However, it is showed that high intensity would induce accumulation of carotenoids over chlorophyll, where photoinhibition took place during the exponential phase of cell growth. In controlled close system, There are two approaches to overcome the overexposure of Botryococcus braunii;

1). Partial shading of the bioreactor [26]. 2). Adopting a diurnal light cycle (16 h light/8 h dark).

The pH of culture medium is usually adjusted to between pH 7 and 7.5 before cultivation. A rise in pH is observed during active growth of Botryococcus braunii, and then a slight drop will be seen later [23]. Similar trends in pH are commonly observed in CO2 enriched cultures during exponential growth. Experiments showed that the optimal temperature for cell growth is 25°C [27].

B. Influence of nutrients on micro algal growth

The influence of the media constituent's potassium nitrate, magnesium sulphate, dihydrogen potassium phosphate and ferric citrate on growth and lipid production in *B. braunii* was investigated using response surface methodology (RSM) [31]. In this study nutrients in combination showed positive effect on the biomass growth compared to the individual nutrients operation [Tab:1].

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Table 1: effect of variation in nutrient sources on biomass and lipid production.

#	Experimental deviation	Nutrient source	Chemical Concentration
1	Control	Sewage Water (SW)	138 mg nitrates/L, 58 mg phosphates/L
2	Carbon	SW +glucose	600 mg glucose/L
3	Nitrogen	SW +Potassium nitrate	600 mg KNO3/L
4	Phosphorus	SW +inorganic phosphate	600 mg K_2PO_4/L
5	Carbon + nitrogen	SW + glucose +potassium nitrate	300 mg $C_6H_{12}O_6$ + 300 mg KNO_3
6	Nitrogen +phosphorus	SW + pot. nitrate + inorg. phosphate	300 mg/L KNO_3 + 300 mg/L K_2PO_4
7	Carbon + phosphorus	SW + glu + inorganic phosphate	300 mg/L $C_6H_{12}O_6$ +300 mg K_2PO_4/L
8	Carbon + nitrogen + phosphorus	SW +glu +potassium nitrate+ inorganic phosphate	200 mg/L $C_6H_{12}O_6$ + 200 mg/L KNO_3 + 200 mg/L K_2PO_4

Among the individual conditions, presence of phosphorus (P) showed good biomass growth (1.2 g/l) followed by carbon and nitrogen (C+N) conditions (0.9 g/l). While among the nutrients in combination, N +P condition (1.4g/l) showed high biomass growth followed by C+P (1.2 g/l), C + N+ P (1.0 g/l) & C+N (0.7 g/l) as shown in [Fig:1]. Control condition with sewage water showed comparatively less biomass growth (0.5g/l). Highest biomass concentration observed in P as a individual nutrient condition might be due to the biomass enriching ability of P in its inorganic form. [28,30]. The next best conditions noticed were with N and C. Where carbon is considered as a key factor governing the growth pattern of microalgae. While nitrogen in the form of nitrates get assimilated in the microalgal cells and are used as a nutrient source for enhancing algal growth. Higher biomass growth resulting in N + P as seen in [Fig:2] condition might be due to the involvement of both the nutrients in the growth enhancement. Good algal biomass growth observed with C + P combination might be due to the individual function of both the nutrients facilitating cell division of algae cells. Likewise, C + N combination showed its influence on biomass increment due to the involvement of both the components in the synthesis of amino acids, chlorophyll and other nitrogen containing compounds. C + N + P combinations comprises of all the major nutrients viz., C, N, P and K essential for growth.

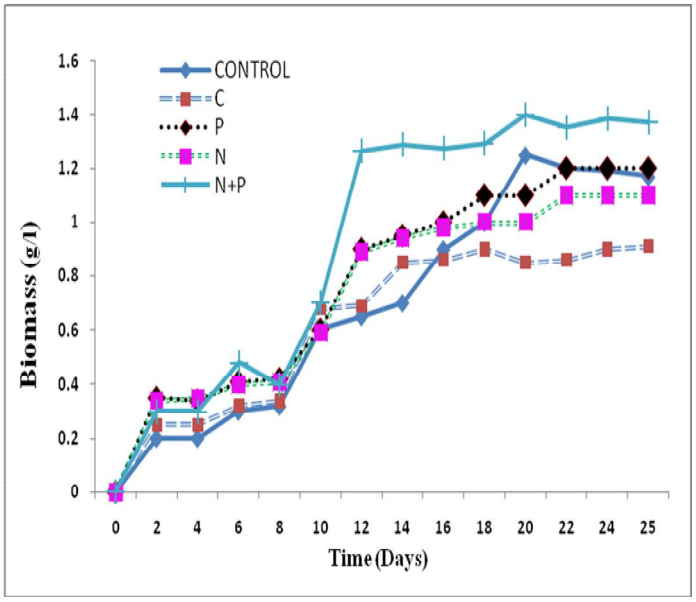

Fig: 1 Biomass yield of B.braunii culture grown in waste waster under the influence of different nutrients.

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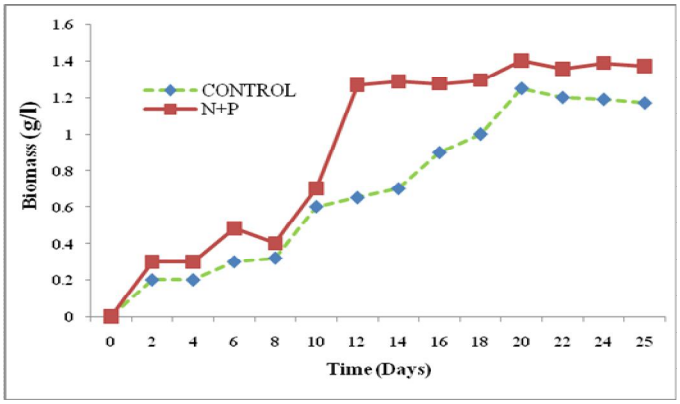

Fig: 2 Comparision of Biomass yield of B.braunii culture grown in waste waster under the influence of N+P with that of control.

Dayananda et al.,[32]; reported the biomass yield of 2.0 and 2.8 g/l in B. braunii culture (SAG 30.81 and LB-572) treaded with different levels of BG11 media. The increase in the biomass yield of B. braunii under light and dark conditions was reported by [33]. According to [34] the maximum biomass of B. braunii is 2.3 g/l. Shen et al.[35] reported dry biomass concentration of up to 2.543 g L-1 for B. braunii. Nitrates & phosphates have positive effects on algal growth [36]. These variables affect algae growth independent of each other without any interaction among them.

Lipid productivities at the end of growth were high compared to initial values which might be due to initialization of reserve food material conversion to lipid granules along with the synthesis of carbohydrates as a part of algal metabolism. While at the end of growth, all the available nutrients initiated towards deprivation and stimulated the lipid synthetic pathways enhancing lipid accumulation. The lipid productivities were noticed to be maximum with C condition (70.0%) followed by N (55%), N+P (50%), control (45%), NPK (42%), C+ N +P (40%), P (31%), C +N (30%), and C +P (30%) as seen in [Fig:3]

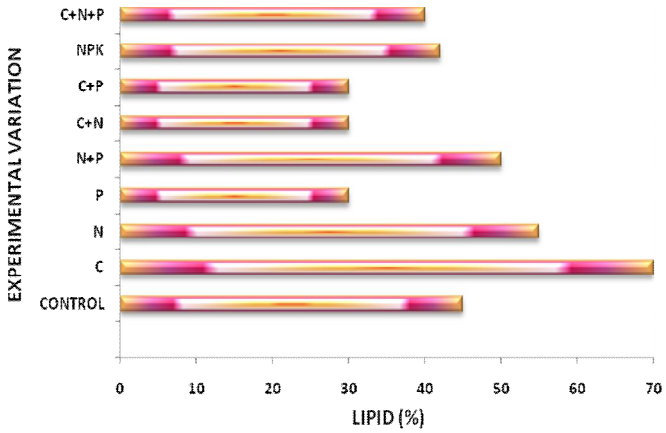

Fig: 3 Lipid yield of B.braunii culture grown under the influence of different experiemtal vaiation.

Individual nutrients showed relatively higher lipid accumulation compared to nutrients operated in combinations. However, both individual nutrients and nutrients in combination showed uniformity in the order of lipid productivity. When each individual nutrient condition is considered, C condition evidenced higher lipid productivity which might be due to the storage of total lipids as neutral lipids instead utilizing for the formation of photosynthetic apparatus.

Dayananda et al.[37] researching Botryococcus in Chu 13 media and 2% CO2 receives similar results in terms of chlorophyll (9.8) and carotenoids (1.8). Anitha et al. [38] reveals that at decreasing concentration of nitrogen sources there was a decreased growth, chlorophyll and biomass. Nitrogen starvation also triggered a rapid decline in nitrogen containing compound such as photosynthetic pigments, causing complete loss of photosynthetic efficiency. Rao et al. [39] also receive close to our values of chlorophyll (11.6) and carotenoids (2.5) as shown in [Fig: 4 & 5]. A high intensity of light increases the carotenoid-to-chlorophyll ratio, and this affects the color of algal colonies [40].

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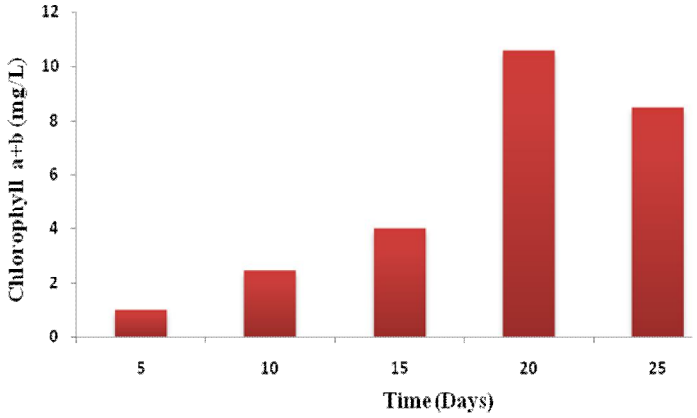

Fig: 4 Chlorophyll content of *B.*braunii culture grown in waste water.

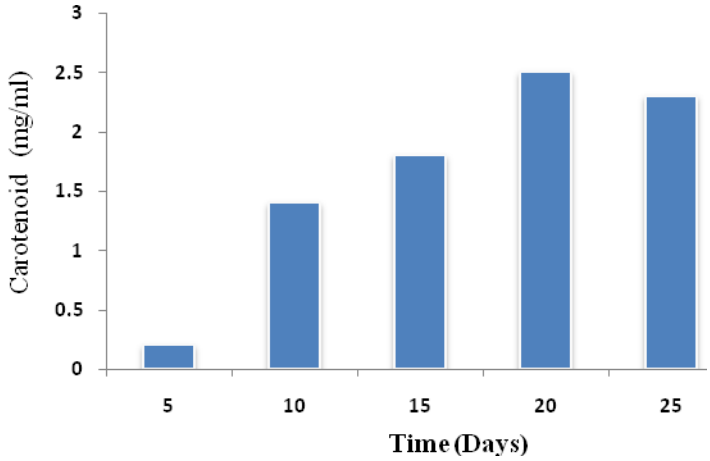

Fig: 5 Carotenoids content of B.braunii culture grown in waste water.

Taking into account the carotenoids of B. braunii again higher values (2.5) occurred in cultures grown in enriched with nitrates medium [Fig:5]. Lutein is the major carotenoid among the total carotenoids from B. braunii as reported by [41].
The green algae B. braunii has received much attention because it contained unusually high levels of hydrocarbons ranging from 15-75% of dry wt [42].

IV. CONCLUSION

Microalgae are termed as ideal candidates for biofuel production due to their ability to utilize sewage water, nutrients and CO2 to make the process economically viable and carbon neutral. Experiments performed with variation in nutrient supplements showed positive influence on both biomass growth and lipid accumulation. The obtained results show that the *B. braunii* develops better in sewage water, as larger values are observed in the biomass and in the percentage of lipids. Chlorophyll content in all cultures follows the dynamics of variation of the curves of growth.

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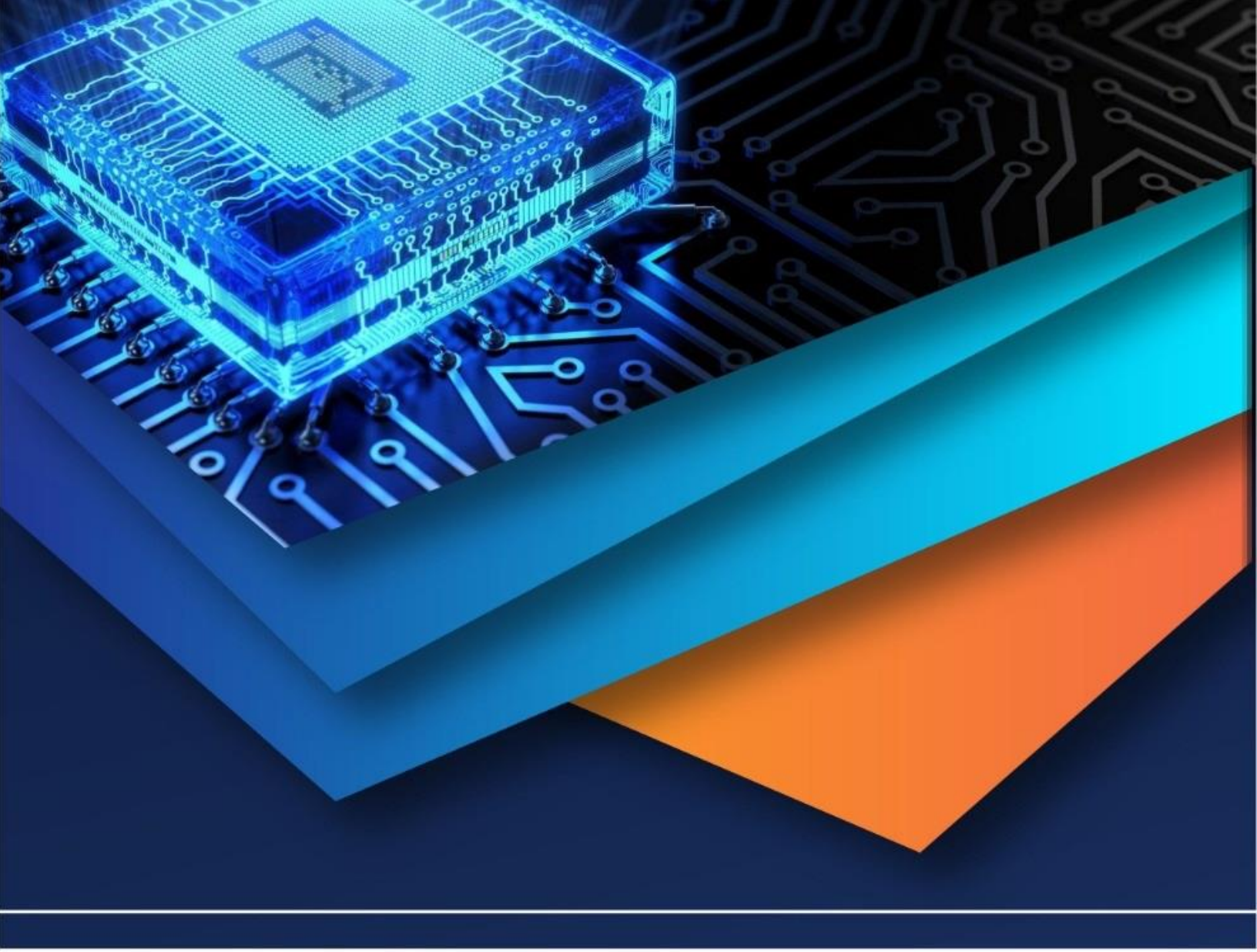

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