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Identification of Potential Marine Filamentous Heterocyst Cyanobacterium Producing Higher EPS Coupled with Higher Viscosity

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Abstract: ***Marine cyanobacterial exopolysaccharides are natural biopolymers well known for their potentials in various fields. As there is an increase in demand for a new polymer, fifteen (15) strains of different morphotypes unicellular, filamentous and filamentous heterocystous belonging to varied habitat were screened for EPS production. EPS synthesis was found to be coupled with incubation duration, growth phase, which reflected on the rheology of the medium. Filamentous heterocystous strain Nostoc sp BDU 80591 of salt pan habitat yielded (2.8 gL^{-1}) EPS exhibited (4.64 cP) as viscosity.***

Keywords: Exopolysaccharide, Cyanobacteria, Viscosity, Nostoc sp, Growth phase

I. INTRODUCTION

Exopolysaccharides are high molecular weight polymers rich in sugar residues coupled with lipids and proteins secreted to the environment by most of the prokaryotic and eukaryotic organisms [1], [2]. Increase in demand for natural polysaccharides for the industrial applications has created interest in the search for an alternative new source microorganism for replacing existing natural and synthetic polysaccharides [3], [4].

Cyanobacteria are photosynthetic prokaryotic organisms coupled with oxygenating, diazotic potential which is also involved in regulating biogeochemical cycle [5]. It is well known for producing valuable biological compound like carbohydrates, lipids, proteins and pigments [6], and found in diverse habitats including freshwater, marine environment, hot springs, hypersaline localities, cold and arid deserts [7].

Both the types of EPS are composed of carbohydrate and non-carbohydrate constituents like protein, lipid, sulfate, uronic acid and pyruvate [8].

Presence of sulfate and uronic acid in EPS contribute negative charge and provide sticky behavior to the organisms [9], [10].Basically, EPS is involved in protecting the cell from drastic environmental changes [11]. Due to its wide range of composition and unique properties it has wide applications in various industrial sectors such as textiles, enhancement of oil recovery, wastewater treatment, cosmetology, pharmacology and food additives as an emulsifier, thickener and suspending agent [12]. The present work focuses on identifying a potential strain that is capable of producing the maximum EPS from the cyanobacterial repository among the EPS producers.

II. MATERIALS AND METHODS

A. Growth and Maintenance

EPS producing marine cyanobacteria were obtained from the repository of National Facility for Marine Cyanobacteria (NFMC), Bharathidasan University, Tiruchirappalli. Heterocystous forms were maintained in ASN III Nitrogen free medium and non-heterocystous forms in ASN III N^+ complete medium at 27 ± 2ºC under fluorescent light of about 200 µmol photon m^{-2} s^{-1} light intensity [13].

B. Morphological Identification and Staining of Exopolysaccharide

The selected organisms were identified under an inverted trinocular microscope (Leica DMI 3000b) fitted with a camera set up (DFC 425C) following the manual Cyanophyta [14]. To identify the strain possessing EPS, one drop of culture was mixed with four drops of nigrosin dye (Conc.10 mg/ml) [15]. Wet mounts were observed and the microphotograph exhibiting the zone of EPS production was photographed under bright field illumination using Leica light microscopy.

C. Precipitation and Extraction of EPS

EPS was extracted by ethanolic precipitation method with slight modification [16]. In 30 days old cultures from the milieu, cells were separated from the growth medium by centrifugation at 10,000 g at 4°C for 20 mins. The polysaccharide was extracted by adding three folds of cold ethanol to the supernatant. The precipitated EPS was purified by dialysis using 12,000 Da dialysis membrane to get rid of ions then freeze-dried and stored at 4°C and expressed in gL^{-1}.

D. Viscosity Analysis

Supernatant of cyanobacterial strains were obtained on the 15th and 30th day of incubation by centrifugation at 10,000 rpm for 20 mins. Viscosity of cell-free supernatant was measured using LVD VII + PRO (Brookfield) and expressed as cP (centipoise).

E. Analytical methods

The dialyzed 1mg EPS dry powder was dissolved in 1ml distilled water and was chemically analyzed.

1) Total sugars following by phenol-sulphuric acid method. Formation of yellow-orange colored product with phenol was measured at 490 nm with glucose as a reference [17].

2) The alkaline reagent along with Folins ciocalteau reagent in dark incubation makes the amino acids in the EPS to be expressed by the occurrence of blue color and absorbance was taken at 750nm. The amount of protein present was calculated using standard Bovine serum albumin based on Lowry's method [18].

3) The amount of sulfate present was estimated by the turbidimetric method. The precipitate in colloidal form was measured at 420nm [19]

4) The uronic acid content was analyzed by carbazole method. Uronic acid gives pink color with carbazole at acidic condition measured at 525nm, and galacturonic acid serves as standard [20].

III. RESULTS AND DISCUSSION

A. Morphological Identification And Negative Staining

Fifteen EPS producing cyanobacterial strains selected for the present study from different habitats to identify the potential EPS-producing strains. The selected strains were observed under a microscope, and the marine cyanobacterial strains represented unicellular, filamentous and filamentous heterocystous forms (Table. 1). The EPS producing capacity of the organisms was identified by the negative staining as EPS is not visible directly under the microscope (Fig.1), The slimy layer remains unstained and is found to be around the organism denoting the production of negatively charged EPS in *Nostoc sp*. BDU 80591. Like EPS producing organisms, namely *M. flos-aquae* C3-40, *Anabaena sp* and *Nostoc sp* [15], [21] has been identified by this technique. The organisms identified based on morphology are tabulated below.

Table. 1 Selected strains for screening EPS Production.

S.no	Organism	Order	Morphology	Habitat and area of collection
1	*Aphanocapsa species* BDU130052	*Chroococcales*	Unicellular	Stagnant sea water, Bheemunipatnam, Bay of Bengal region, Andhra Pradesh.
2	*Aphanocapsa species* BDU 50261			On submerged rocks, Palkulam, Idinthakarai, Bay of Bengal region, Tamil Nadu.
3	*Chroococcus minor* BDU 40402			Stagnant seawater, Athangarai, Palk Bay, Ramnad, Tamil Nadu.
4	*Synechococcus elongatus* BDU 141741	*Synechococcales*		Decaying wood, Rangat Bay, Middle Andaman Island.
5	*Synechococcus elongatus* BDU 10144			Brackish water, Vettaikaranirupu, Tamil Nadu.
6	*Synechococcus elongatus* BDU 130192			Abandoned saltpan, Kakinada, Andhra Pradesh.
7	*Leptolyngbya fragile* BDU 141754		Filamentous	On submerged stones, Mayabunder Jetty area, Middle Andaman.

8	*Leptolyngbya valderianum* BDU 20041			Ephiphyte on wood in sea, Point Calimere, Bay of Bengal, Tamil Nadu.
9	*Oscillatoria salina* BDU 91691	*Oscillatoriales*		On the muddy soil, Cheituvai backwaters, Arabian sea, Kerela.
10	*Oscillatoria willei* BDU 130511			Under the boat, Visakhapatnam Port, Andhra Pradesh.
11	*Oscillatoria laetevirens* BDU 10143			On muddy soil, Marakkanam Salt pan, Bay of Bengal region, Tamil Nadu.
12	*Spirulina subsalsa* BDU 141021	*Spirulinales*		Attached on submerged rock, Rose Island, near South Andaman Islands.
13	*Nostoc calcicola* BDU 40302	*Nostocales*	Filamentous heterocystous	Stagnant sea water, North mandapam Gulf of mannar, Tamil Nadu.
14	*Nostoc species* BDU 80591			Marakkanam Salt pan, Bay of Bengal region, Tamil Nadu.
15	*Nostoc species* BDU 80701			Marakkanam Salt pan, Bay of Bengal region, Tamil Nadu.

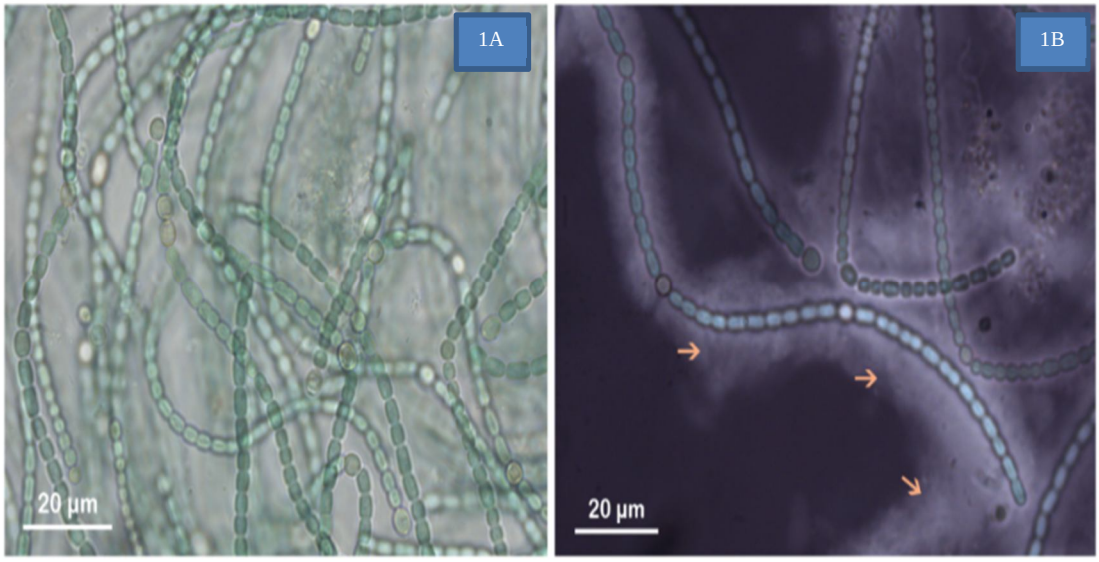

Fig. 1 Light microscopic images of *Nostoc sp.* BDU 80591 15th day old culture
a. Unstained, b. Arrow marks showing EPS with negative stain nigrosin

B. EPS Extraction

EPS was extracted by ethanol precipitation, and the results are depicted in (Fig.2), as ethanol precipitation method is found to be fast and easy to isolate and purify EPS [22]. Production of EPS is highly species and strain dependent. Among the fifteen strains of different morphotypes from varied habitats screened, filamentous heterocystous *Nostoc* forms were found to possess highest EPS (1.5 gL^{-1}, 2.8 gL^{-1}) BDU 80591> (1 gL^{-1}, 2 gL^{-1}) BDU 40302 > (0.9 gL^{-1}, 1.8 gL^{-1}) BDU 80701 on the 15th day and 30th day respectively. Production of EPS though started at early growth phase and time-dependent indicating that at stationary phase on the 30th day of incubation showed the maximum. In *Anabaena flos-aquae* A37 [23] also similar findings have been reported. In the earlier studies stated in *Anabaena sp*. BTA992 was found to produce 1.7 gL^{-1} on the 30th day of all the tested cyanobacterial strains [24], *Nostoc calcicola* RDU-3 was found to produce 105 mg/L on 44th day [25]. In the present investigation, exopolysaccharide productivity by salt pan isolates, a halophilic cyanobacteria.

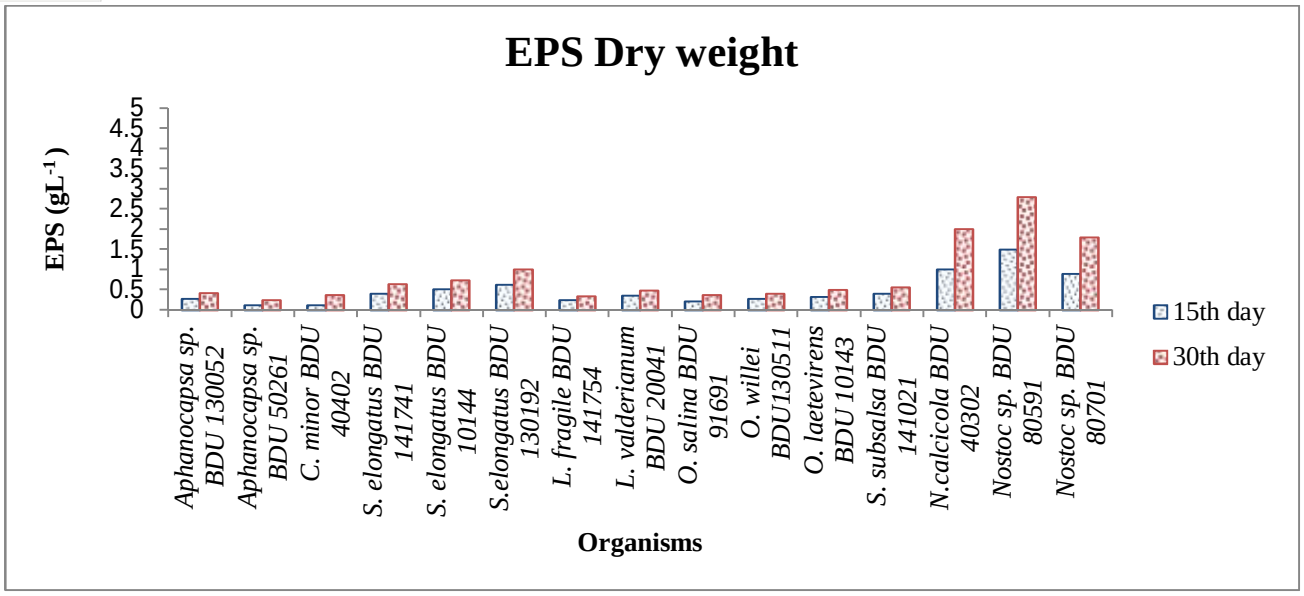

Fig.2 EPS production for the selected strains on 15th and 30th day

C. Viscosity Analysis

EPS accumulation has its effect on culture medium so assessing viscosity can be used for monitoring EPS production [22]. During growth, increase in the concentration of EPS linearly enhances viscosity to the medium [26], [27], [28]. From the result, (Fig. 3) All the chosen 15 organisms were measured for viscosity their viscosity nature varied. The heterocyst filamentous were the ones which showed high viscosity compared to others. The rate of production among *Nostoc* were 80591>40302>80701 and the viscosity rates were 4.64cP> 3.87cP> 3.37cP, The order next to exhibit higher viscosity was *S. elongatus* BDU 130192 which belong to unicellular orders. The continuous release of EPS to the surrounding environment leads to progressive increase in viscosity of the medium on the 30th day. However previous studies with culture medium viscosity have shown highest of (6.55cP) occurred on 12th week (i.e.) 90 days in *Microcystis aeruginosa f. flos-aquae* [29]. Whereas in our study maximum viscosity recorded at the end of the 4th week was found to be three times higher than *Anabaena sp*· whose viscosity was 1.75cP at the 14th week of incubation.

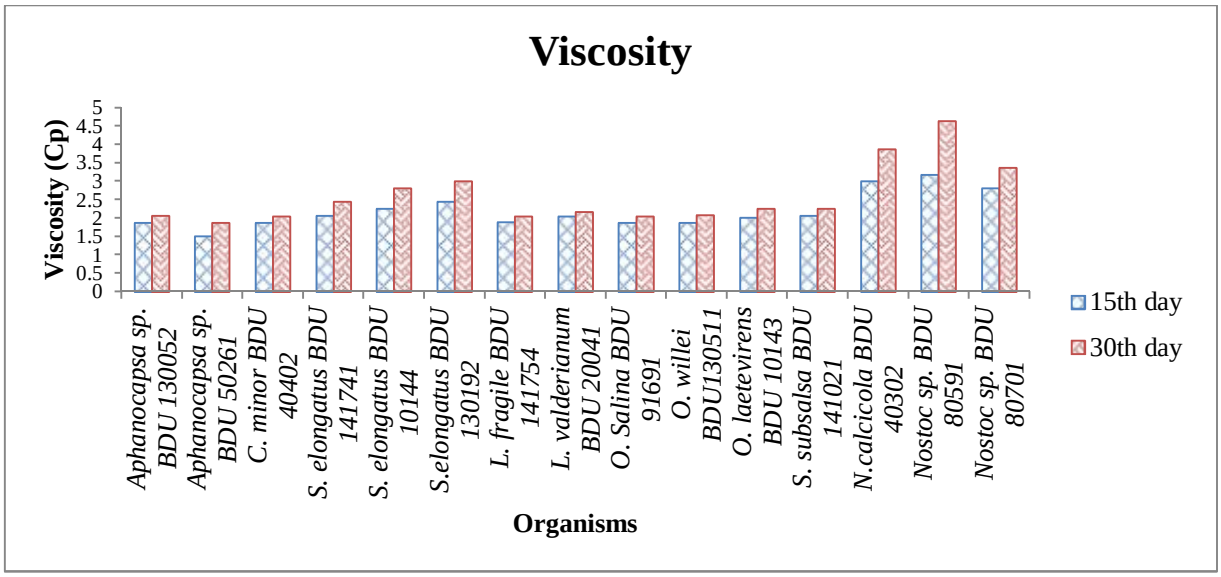

Fig. 3 Viscosity for the selected strains on 15th and 30th day

D. Biochemical Analysis

Three strains that exhibited highest EPS production were analyzed further for its biochemical properties and is presented in (Table. 2). All the three organisms had a predominant composition of total sugars indicating polymer is mainly of the polysaccharide. Presence of sulfate and uronic acid plays an essential role in providing anionic nature [9, 10]. Moreover, this anionic EPS has its application in wastewater treatment, sequestration of heavy metal. It is worth stressing that among three *Nostoc* strains, highest sulfated polysaccharides producing strains of BDU 80591 and BDU 80701 found to share same habitat (salt pan). Sulfated EPS has special significances as the pharmaceutical value [30]. From all the above results *Nostoc sp* BDU 80591 the sulfated polysaccharide from salt pan habitat can act as novel promising candidates which can be further exploited for its biotechnological potentials. Of the screened organisms, *Nostoc* being a diazotroph is of immense significance as it reduces nitrogen input to the medium making it economically viable.

Table. 2 Biochemical analysis of EPS in the three strains with highest EPS productivity presented as $\mu g\ ml^{-1}$ of EPS dry weight

Organisms	Total carbohydrate	Protein	Sulphate	Uronic acid
N. calcicola BDU 40302 (Sea water)	311.82±1.9	72.44±2.1	132.12±1.9	114.98±2.1
Nostoc sp. BDU 80591 (Salt pan)	326.45±1.7	79.21±1.3	159.31±1.2	134.87±1.3
Nostoc sp. BDU 80701 (Salt pan)	298.64±1.8	69.31±2.2	149.42±1.5	129.82±2.4

IV. CONCLUSION

Thus, we conclude that filamentous heterocystous forms are the best candidate for EPS production. Although many species of cyanobacteria were reported for EPS production only very few, have been extensively exploited till now. For successful commercialization, further media optimization and characterization is required.

V. ACKNOWLEDGEMENT

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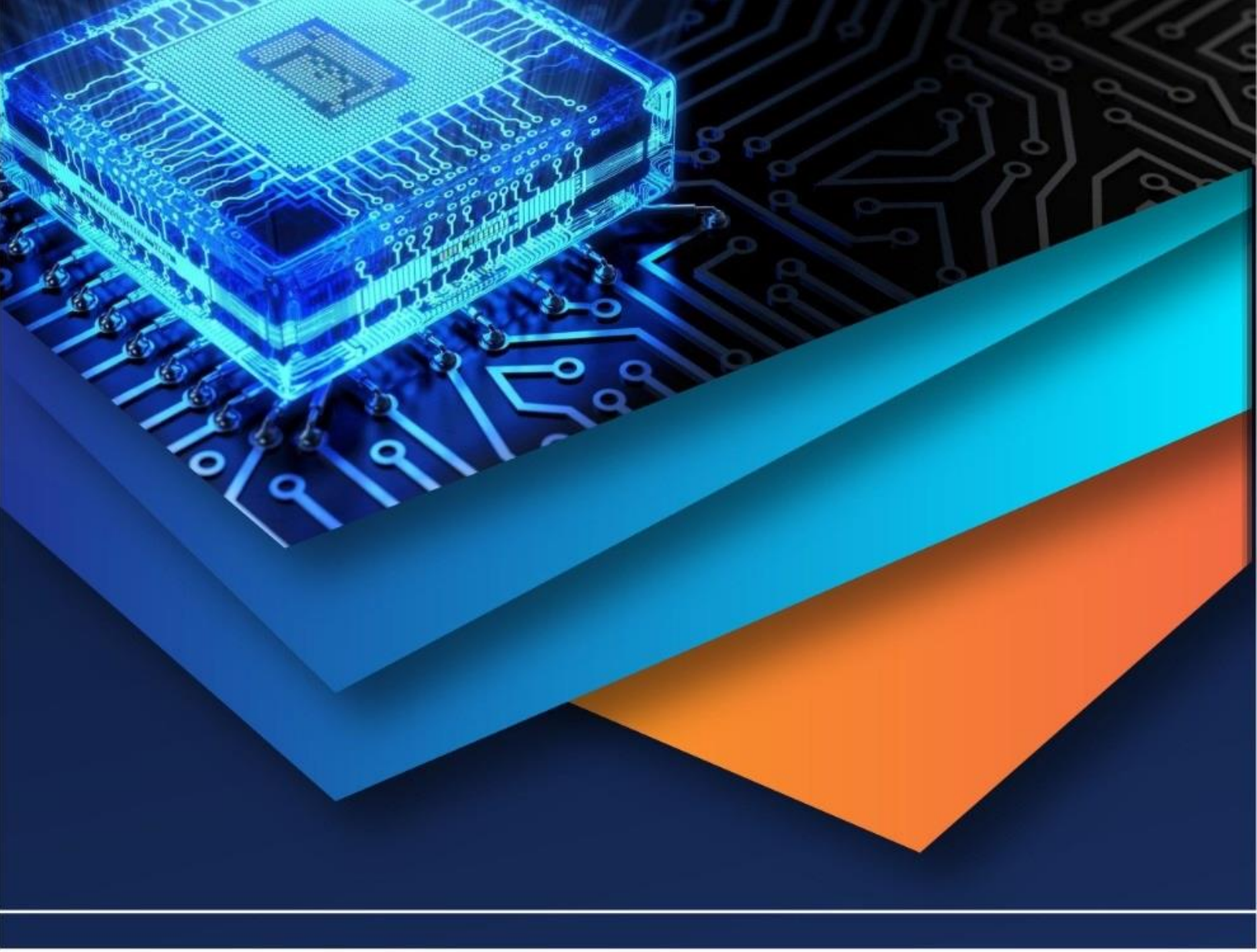

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