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Isolation and Characterization of Carotenoids of Halobacillus sp. SC8 Isolated from Camalti Saltern Izmir, Turkey

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Abstract: ***Carotenoids considered as yellow-orange, red fat-soluble pigments and are usually composed of 40 carbons, found dominantly in algae, prokaryotes, and plants. Carotenoids have a variety of uses in the fields of biotechnology, cosmetic industry, and food technology.***
Çamaltı saltern is Turkey's largest salt lake and has great importance in terms of economic and ecological. It has the conditions for the production of halophilic bacteria which is important in biotechnology.
In this study, halophilic and carotenoid-producing bacteria SC8 strain was isolated from marine salt environment using marine agar medium. 16S rDNA gene sequence analysis revealed that the SC8 strain belongs to the genus Halobacillus and has 100% similarity to Halobacillus locisalis. Carotenoids were isolated by methanol/acetone method, and were detected by thin layer chromatography and spectrophotometric and identified by HPLC. Nine peaks were determined on HPLC (High Performance Liquid Chromatography) separation. After the spectra, absorbance maxima and retention times are obtained, these peaks can be divided into several groups according to the λmax and UV spectra defined as isomeric forms of lutein, zeaxanthin, lycopene, a-carotene, and p-carotene. The total carotenoid amount is obtained as 2.4550 µg / ml extract.
Keywords: Carotenoids, halophilic, bacteria, HPLC

I. INTRODUCTION

Carotenoids are lipid-soluble pigments and generally referred to the composition of 40 carbons called tetraterpenoids which are classified into two groups. In the literature [1,2]. Carotenoids which are not containing oxygen atoms are called carotenes such as lycopene, α-Carotene, β-carotene. Carotenoids like astaxanthin, lutein which contain oxygen atom are called xanthophylls.
According to Giovannucci, E. et al. [3] higher plants and certain prokaryotes produce yellow-orange and red colored carotenoids. Carotenoids were reported [4,5] to have various usages in biotechnology, cosmetic industries, and food technologies.
Asker D. And collegues [6] showed that e xtreme environments such as low temperature, hypersalinity, intensive light, acidic or alcoholic host microorganisms that could produce carotenoids.
Carotenoids play a crucial role in microorganisms and found in all photosynthetic organisms. They provide light harvesting, protection against intensive light by free radical detoxification which in return it minimizes possible damage to the cell membrane as reported by Namitha and Negi [7].
Carotenoids according to Foy et al. [8] are believed to be effective against many diseases. They have significant effects on cardiovascular diseases, Alzheimer, Parkinson, and hypertension. Antioxidant activities against free radicals.
Antioxidant properties are believed to be the preventive role of carotenoids against disease. In literature it's [9, 10] reported that they can capture oxygen free radicals (atoms or groups of atoms with an unshared electron) because of their conjugated double bonds system. Carotenoids with nine or more double bonds provide the most protection.
Furthermore, It's reported that the mechanism by which cancer prevention could be achieved by enhance celliure communication by increasing connexin-43 expression and eventually they upregulate gap junction.
Bacteria and specifically those ones that are able to survive in extreme conditions are valuable sources of carotenoid.
Considering the importance of carotenoids in our daily life as well as discovering novel and productive sources we aimed to discover carotenoids from halophilic bacteria from camalti saltry which is the one of biggest saltern lake in Turkey.

II. MATERIAL AND METHOD

A. Isolation and Growth Conditions

Water samples were obtained from camalti saltry and were handed to the laboratory in sterilized containers and were cultured immediately on marine medium with 10%, 15% and 20% NaCl concentrations. Marine medium contained (per liter); MgCl2.7H2O, 8.8 g; Na2SO4, 3.24 g; CaCl2, 1.8 g; KCl, 0.55 g; NaHCO3, 0.16 g; KBr, 0.08 g; SrCl2, 34.0 mg; H3BO3, 22.0 mg; Na2O3Si, 4.0 mg; NaF, 2.4 mg; (NH4)(NO3),1.6 mg; Na2HPO4, 8.0 mg; peptone 5 g; yeast extract, 1 g.

Colony growth was first observed after about 7 days. To obtain pure cultures, single red colonies were picked from the plates and were used for Gram-staining and stock preparation, by growing in marine broth in an orbital shaker.

Cultures were stored at −70◦C in marine broth supplemented with 30% glycerol.

B. Extraction of Genomic DNA and Sequencing of 16S rRNA

DNA was extracted using “Genomic DNA isolation” kit (Intron) .

The 16S rRNA gene was amplified using polymerase chain reaction (PCR) using fD1 5’-AGAGTTTGATGGCTCA-3’ and rD15’CGGCTACCTTGTTACGACTTC-3’. (Weisburg WG 1991). PCR was performed using a thermal cycler (Eppendorf) with a 50-µl reaction containing 1.5 µl MgCl2, 1 µl of each dNTP, 0.5 µl of each primer, 5 µl PCR buffer, 36.5 µl H2O and 1 U of Taq DNA polymerase (Geneaid). Initial denaturation was carried out for 2 min at 95 °C. In literature [15,16] it was followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 42 °C for 30 sec and extension at 72 °C for 4 min with a further 10 min extension at 72 °C. The amplified DNA fragments were separated using 1% agarose gel electrophoresis. The sequence was compared with reference 16S rRNA gene sequences available in NCBI GenBank database BLAST using blastn and megablast and the phylogenetic tree constructed using Mega 6.0 software.

C. Pigments Extraction and Analysis

To extract carotenoids, 300 ml of marine broth cultures were centrifuged at 4500 rpm for 20 min at 4 °C. Jehlička and Oren reported [5] that, the supernatant was separated and a mixture of acetone-methanol (1:2 v/v) containing butylhydroxytoluene (BHT) (0.1%; as antioxidant) was added to the pellet.

The pelleted cells were sonicated for 5 minutes to facilitate extraction and followed by centrifugation at 4000 rpm for 15 min at 4°C as stated by Gupta et al. [11].

Samples were wrapped with aluminum foil to protect them from light. The extracts were stored under nitrogen at -70 °C. Asker reported [12] that extraction procedures and analysis tests were conducted in dark conditions.

D. UV-Visible Spectroscopy

Extraction solution UV spectra were recorded [13,14] at 300-800 nm using a spectrophotomete. The approximate content of total carotenoids was determined by measuring the optical density of the sample in 495 (λmax of our extraction solution).

HPLC analysis

III. EXTRACTION AND DETERMINATION OF CAROTENOIDS

Determination of carotenoids was performed using the LC Agilent Technologies 1200 Rapid Resolution (Waldbronn, Germany) system equipped with a UV-Vis detector (DAD 1260,Waldbronn, Germany) and a Poroshell 120, SB-C18 column (4.6 x 150 mm, 2.7 µm) (Agilent Technology InC, USA). The mobile phase was composed of acetonitrile contained 0.5 g/L of triethylamine (solvent A) and acetonitrile:methanol:ethyl acetate (solvent B) (Sigma Aldrich Chemie GmbH, Germany) in a gradient from 95:5:0 to 60:20:20 in 20 min, the latter proportion being maintained until the end of the run (45 mi). Flow rate was set at 0.5 mL/min according to Álvarez R et al. [19]. The eluate was detected with a DAD detector (Agilent Technology, Germany) set at 454 nm. Quantification of carotenoids was performed by the external standard method. Carotenoids were quantified, related to β-carotene. The peaks were identified on the base of spectrum data and by comparison with data obtained from literature.

IV. RESULTS

A. Morphologic and Physiologic Characteristics

Cells were Gram-positive, motile, endospore-forming and rod-shaped halophilic bacteria. This organism grows at the temperature of 10.0-42.0 degrees ˚C with an optimum of 30 degrees ˚C. Strain SC8 formed round and brownish-red colonies on marine agar.

Carotenoids according to their order of elution in the column, chromatographic behavior (e.g., retention time (Rt) and spectral characteristics), UV–visible absorption spectrum (absorption maximum wavelength, λmax) were identified by the HPLC system's software tentatively.

B. 16S rRNA Gene Sequence Analysis

16S rRNA gene sequence analysis revealed that strain SC8 belongs to Halobacillus genus and has 100% similarity with Halobacillus locisalis.

Table 1 presents the characteristics of peaks identified, they were identified as carotenoids having peak purities of more than 95%, according to tR, the shape of UV-absorption spectra (from 300 to 800 nm) and the fine-structure spectrum at 450 nm.

Upon separation by HPLC (Table 1,2) 9 peaks appeared. The spectra are shown in Fig.1. The absorbance maxima were shown in tab2. These peaks could be categorized into several groups according to their □max and UV spectra on which isomer types were identified as lutein, zeaxanthin, lycopene , α-carotene and β-carotene. Peak1. Eluting between 5 to10 with □max of 470 is characterized as lutein. The total amount of carotenoid is obtained as 2.4550 □g/ml of extract.

V. DISCUSSION

The presence of carotenoids is widespread in many cells of extremophile bacteria, living in various conditions and environment, such as: strong light, low temperature, high salinity, thermophilic conditions. One of the reasons for their production, i.a. by halophilic bacteria might be oxidative stress caused by high salinity. The synthesis of carotenoids, as compounds with a strong antioxidant potential, can reduce the effects of stress. Among the carotenoids synthesized by halophilic bacteria: phytoene, lycopene, β-carotene, apo-carotenoids are often found. Authors [5,14] also identify the presence of bacterioruberin and its derivatives, this carotenoids are a yelow-orange or orange-red in color with absorbtion maximum above 510 nm.

The total content of carotenoids in the obtained sample of the bacterial biomass of Halobacillus sp. SC8 isolated from Camalti Saltern Izmir (Turkey) was 2.66 µg / ml of extract. Extraction was performed using a mixture of acetone and methanol in proportion 1:2 v/v. The absorption maxima of carotenoids exstract in spectrophotometric measurement were: 428, 455, 483 nm (Fiure 1) and the obteined biomas and extract was a light yellow in color. As a result of the chromatographic separation of the carotenoids extract 9 peaks were obtained (Figure 2) with the retention time between 5,4 – 26,2. In the HPLC separation one prominent compound, peak 2, was obtained with a retention time of 5.8 min, which constituted 40% of the total carotenoids. Maxima of absorption for this peak were: 429, 455 and 485 nm (Table 1). The same absorption maxima also had compounds no. 5, 7-9 (Figure 3). Compounds 2, 5, 7-9 represented 79.2% of the total carotenoids content. In literature (6,11,20), an additional parameter of the spectral characteristics may be the ratio of the longest wavelength absorption peak III, and the peak of highest absorption II, taking the minimum between those two peaks as the baseline, multiplied by 100 . For compounds 2, 5, 7-9 obtained different values calculated according to the above description and they were respectively 63.5, 66, 62, 68.5 and 69.5. The diferences betwen the value are smal but this fact may indicate diferences in chemical structure of those compounds, despite the same λmax. As stated by Perez-Fons et al. (18) on the base of retention time, DAD-spectrum and literature data this compounds are probably apo-8-carotenoids and cis-apo-8-carotenoids. On the base of absorpion maxima and III/II% ratio value is big similarity of this compounds to apo-8-lycopene, which was yelow or light yelow in colour. The peak 2, 7 and 8 have the small absorbtion maximum characteristic for cis isomers (344 nm) with intensity (AB/AII%) 14, 11 i 4 respectively were reported [20, 10].

The second group detected in in the HPLC separation were three compounds, peak 1, 4, 6 with the same absorbance maxima of 441, 467 and 495 nm, constituting a 19.9% of total carotenoids. This peaks characterised by III/II%, 21, 15 respectively for peak 1 and 4, but for peak 6 is not to posible to calculate this paremeter. Peak 1 had absorption maximum charakteristic for cis-isomers (344 nm), with intensity (AB/AII%). Examining moderately halophilic bacterium Halobacillus halophilus received similar spectral characteristics of carotenoids as the yellow color of bacterial biomass, however the proportions of compounds were different. Thos authors obteined 3 compounds with absorbace maxima 430, 453, 485 nm with similar III/II% value like in presented study and 3 carotenoid compounds with absorption maxima 442, 468 and 495 nm, which were the bigest peaks obteined after HPLC separatin.

Camalti slterin is the biggest salt harvesting site in western turkey nearby the Agean sea. Few studies have been carried out using FISH and real time PCR to discover prokaryotic diversity in the saltern. Erdogmus et al [21] reported that the archaeal diversity is limited to holoferay, halorubrom, halobacterium and haloarchea. The pattern of bacterial diversity in camlati saltern shows resemblance with order saltern in the world and the most dominant genera are *halpbacillus sp. And halomonas sp.* Mutlu et al also reported similar findings applying metageomics studies. Some studies also analyzed the bioremediation potential of prokaryotes. Erdogmus et al [21] studied the phenotypic characteristics and exopolysaccharides producing capability in camalti saltern.

As shown in literature [21, 19] carotenoid production has significant importance due to their commercial interests, which are used as coloring agents in neutraceuticals, pharmaceuticals, cosmetics, and foods. This very fact led to extensive investigation of carotenoids from biological sources in the last decade.

In the present study, we aimed to find and characterize carotenoids from camalti saltern as intact sources of bacteria in order to produce carotenoids.

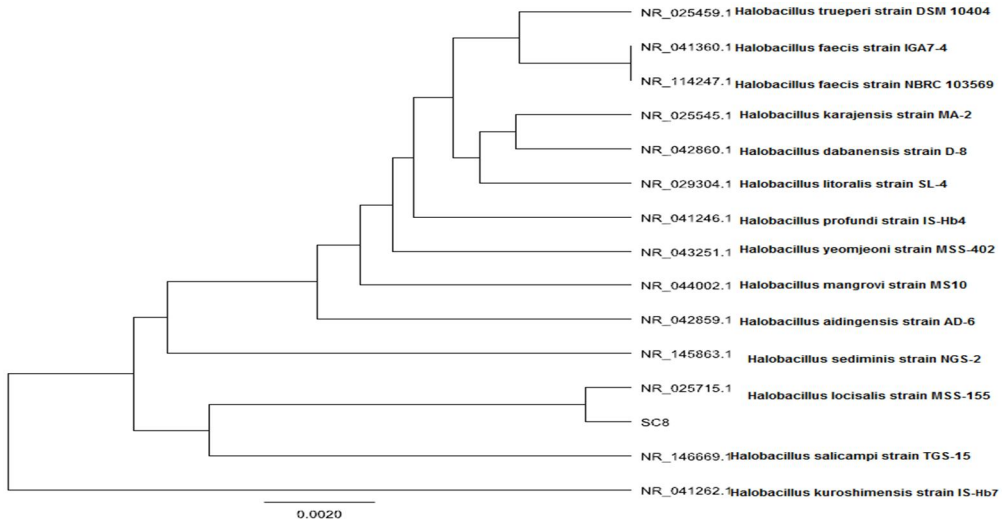
To the best of our knowledge this study is the first one carotenoid producing halophilic bacteria from camalti saltern.

Carotenoid contents.

The total carotenoids obtained from halobacillus sp SC8 was 2.46 µg/ml of extract.

VI. CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.



A. Physiologic tree

Retention time of carotenoids	No of peaks	Carotenoids content [µg/ml of extract]	Percentage share [%]
5,49	1	0.082	3.1
5,907	2	1.065	40.0
6,904	3	0.023	0.9
13,552	4	0.345	12.9
17,63	5	0.210	7.9
18,668	6	0.105	3.9
20,16	7	0.568	21.3
23,623	8	0.230	8.6
26.196	9	0.036	1.4
Total carotenoids content		2.66	100

Table 1 Identification of carotenoids of SC8 obtained by HPLC-DAD.

No of peaks	Retention time	□max 1	□max 2	□max 3	III/II%
1	5,49	441	467	495	21,4
2	5,907	429	455	485	63,5
3	6,904	-	462	-	-
4	13,552	441	465	495	15
5	17,63	429	455	485	66
6	18,668	441	467	495	-
7	20,16	429	455	485	62
8	23,623	429	455	485	68,5
9	26.196	429	455	485	69,5

Table 2 Spectrofotometric measurement of absorbtion

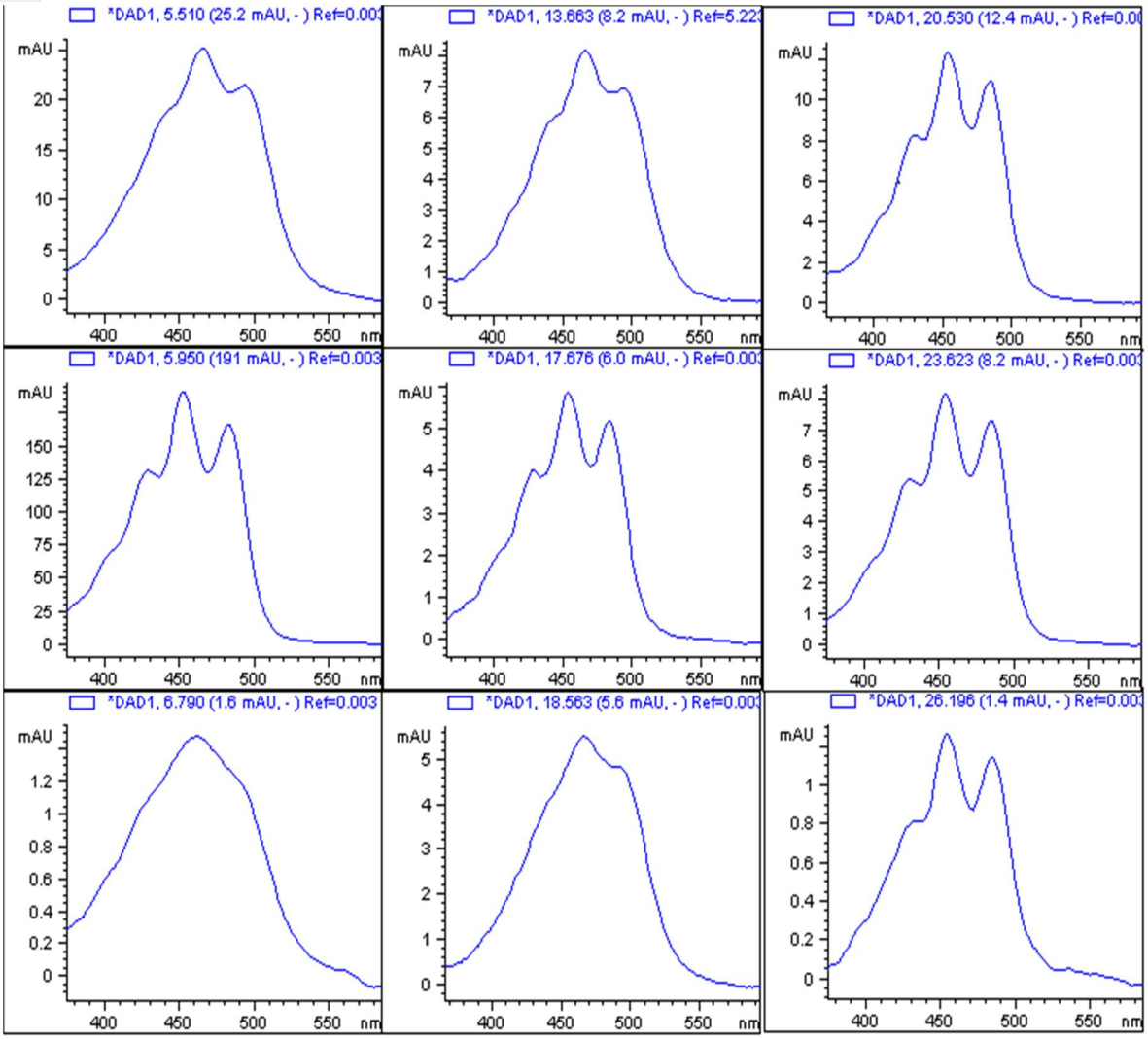


Fig. 2 Figures of absorption chart for compounds

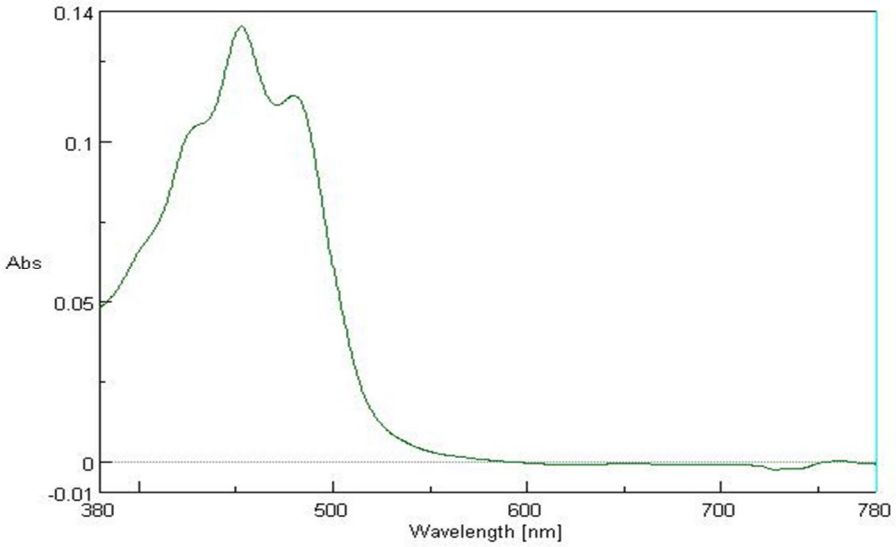


Fig. 1 Total Caratonoids absorption of Sc8

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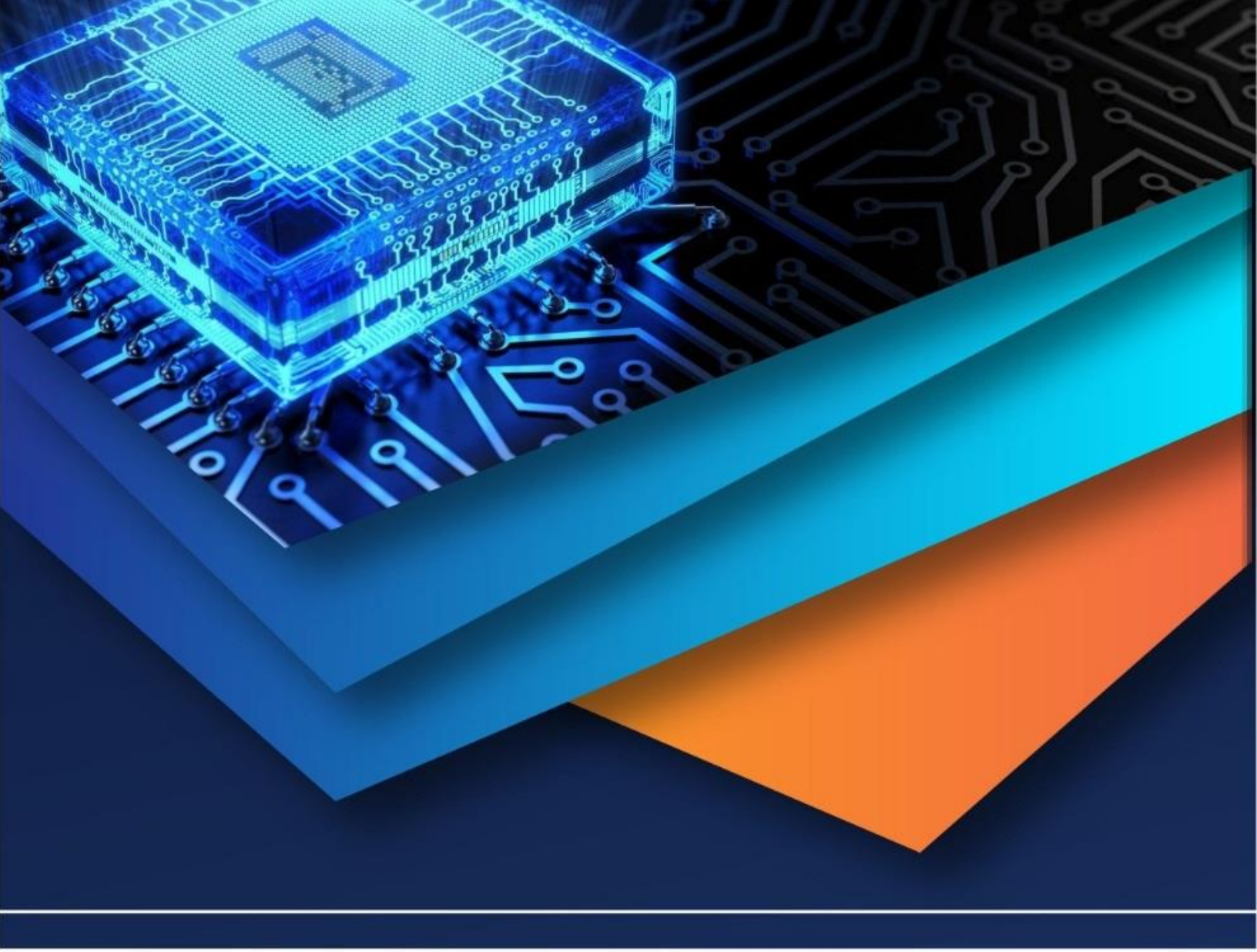

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