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Feruloyl Esterase Producing Hypocrea Lixii SS1: Isolation and Identification

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Abstract: ***Feruloyl esterases represent a diverse group of hydrolases catalyzing the cleavage and the formation of ester bonds between plant cell wall polysaccharide and phenolic acid. It is widely distributed in plants and microorganisms. It has a potentially broad range of applications in the pharmaceutical and Agri-food industries. Fifty fungal species from the fouled soil around an oil refinery were isolated and screened for the production of feruloyl esterase enzyme by enrichment culture techniques. The isolated fungal strain is identified as Hypocrea lixii SS1 based on the results of biochemical tests and 18s rRNA sequencing. Further it was tested for the ability to utilize the sunflower sludge (waste from the oil industry) as the sole carbon source. The growth kinetics and enzyme production of Hypocrea lixii SS1 were also studied and maximum growth was found on the 7th day of incubation. The fungus showed a remarkable feruloyl esterase production of 18.9 U/g. Further the enzyme produced was affirmed as feruloyl esterase with their molecular weight (29 KDa) using SDS-PAGE.***
Keywords : Fungal isolate, Growth kinetics, Hypocrea lixii SS1, Fermentation, Feruloyl esterase.

I. INTRODUCTION

Feruloyl esterase (E.C. 3.1.1.73) also known as ferulic acid esterases (FAEs), cinnamoyl esterases, cinnamic acid hydrolases is a subclass of the carboxylic acid esterases (EC 3.1.1). It plays a key physiological role in the degradation of the plant cell wall (intricate structure) by hydrolyzing ferulate ester groups involved in the cross-linking between hemicelluloses and lignin [1]. Initially, the classification of FAEs was based on the primary amino acid sequence and specificity for aromatic moieties [2-4] and now extended to fungal genome sequences [5]. Recently, there are many research activities on FAE production as it has a considerable role in biotechnological processes for various industrial and medicinal applications.

Feruloyl esterase enzymes play a significant role in biological applications. FAE production have also been reported in various micro-organisms such as Micrococcus sp. [6], Humicola sp. [7], Ophistoma sp.[8], Pencillium sp. [9], Lactobacillus sp.[10], Aspergillus sp.[11], Streptomyces sp.[12]. Since FAEs are produced by many different methods, there is no need of high purity. So different purification strategies are followed [13]. FAE applications include (i) Release of ferulic acid from Agro by-products, which are transformed into other valuable molecules such as styrenes, polymers, epoxides, alkylbenzenes, vanillic acid derivatives, protocatechuric acid-related catechols, guaiacol, catechol, and vanillin [14] and also used as antioxidants [15], [16]; (ii) Recovery of phenolic compounds from nonwood fibers, such as rice straw, wheat straw and sugarcane bagasse by FAE digestion [17]; (iii) FAE has synergistic effects on cellulose and xylanase activities for the release of glucose and xylose from cellulose and hemicellulose for ethanol production [15], [18]; (iv) FAE acts as a biosynthetic tool for the formation of lipophilic antioxidant derivatives [15]; (v) FAE has been used to improve a) the in vitro bioaccessibility and colonic metabolism of phenolic compounds in humans b) digestion of complex plant cell walls in animals [15], [19].

Sunflower (Helianthus annuus) is cultivated worldwide mainly for oil extraction. Sunflower sludge (a by-product rendered by the oil industry) has been generated in large quantities and remains as waste. Fractionation of Sunflower sludge results in a lignocellulosic fraction (LCF) - 23.2–25.3%, a proteinaceous fraction (PF) 55.4–57.6% and a soluble fraction (SF) 17.1–21.4% of the dry weight. As evidenced by the growth of different fungi, Sunflower sludge -LCF (obtained after removal of PF) was found to be a suitable fermentation source for solid-state fermentation (SSF) [20].

Hurried industrialization for protracting economic stability leads to the pollution of the environment. Industrialization and urbanization are responsible for air, soil and water pollution. To remove pigments, objectionable odors and non-triglyceride material from crude oil, various methods such as bleaching, deacidification, neutralization and odorization were used [21]. An enormous amount of by-products are produced from crude oil refining processes, such as deodorizer distillates, soap stocks, and acidic water which are potentially harmful to the environment. If these residues are not disposed properly, it could lead to environmental damage representing a danger to all forms of life.

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There are many inquire on the biodegradation of various aromatic hydrocarbons, using micro-organisms isolated from the oil residues. Researchers have proved the potential of isolated microbes that has the ability to bio remediate oil polluted environments.
The present research is to analyze the potential of a fungal species isolated from fouled soil around an oil refinery in a different endeavor. The fungus isolated is being used for the enzyme production. The principal aim of this study was to isolate, identify and characterize the fungal strain capable of producing feruloyl esterase enzyme from fouled soil around oil refinery. This filamentous fungal strain will be used as potential esterase enzyme producers from the industrial waste as sunflower sludge.

II. EXPERIMENTAL METHODS

A. Isolation and Identification of Fungal Strain

The discrete colonies were picked on agar plates surface and placed into 1 mL of sterilized water and frozen in liquid nitrogen. Cell disruption was done in an ultra-turrax T25 apparatus. The extraction of DNA and its purification was performed as follows: Precipitation of proteins and polysaccharides was done by the addition of Potassium acetate (5M). The supernatant was filtered on Whatman filter paper and 1 mL of isopropanol was added to the tube and mixed. Centrifugation at 9600 x g at 4 °C for 30 s was performed. The supernatant was discarded and the pellet (DNA) was dissolved in 70 mL Tris–HCl 50 mM pH 8, EDTA 100 mM pH 8. The sample was purified with the mixture of phenol, chloroform and isoamyl alcohol (25:24:1), the upper phase was transferred in a separate tube and added 0.10 mL of sodium acetate 3 M and 0.7 mL of isopropanol. After a centrifugation at 9600 x g for 30 s, the supernatant was carefully removed and the pellet was dissolved in 40 mL of Tris–HCl 50 mM, EDTA 100 mM, pH 8.

B. Classic and Molecular Identification of Fungal Strain

The 581-bp fragment within the gene coding for the small ribosomal subunit (18S rRNA) of fungi was amplified in a Thermal Cycler Gene Amp PCR System 9700 (PE Applied Biosystems, Norwalk, USA) using the fungus specific primers TR1 5'-GTTTCTAGGACCGCCGTA-3' and TR2 5'-CTCAAACTTCCATCGACTTG-3'[22]. Two thermal amplification cycles were used to amplify the fungal 18S rDNA. The first cycling parameters were as follows: an initial denaturation step of 96 °C for 10 min, followed by 30 cycles of 96 °C for 1 min, annealing at 46 °C for 1 min and extension at 72 °C for 2 min, with a final extension step of 72 °C for 5 min. Cycle 2 parameters were as follows: denaturation step of 95 °C for 3 min followed by 35 cycles of 95 °C for 1 min (denaturation), 53 °C for 1 min (annealing), 72 °C for 2 min (extension) and final extension of 72 °C for 3 min. The amplification products were verified by electrophoresis in 0.8% w/v agarose gel and DNA stained with ethidium bromide.
The sequences of 18S rDNA were obtained using the primer TR2 and ABI PRISM^{TM} dye terminator cycle sequencing kit (PE Biosystem). The amplified products were analyzed using an Applied Biosystem 373A automated DNA sequencer (Perkin Elmer).

C. Sequence and Phylogenetic Analysis

The sequences were aligned using CLUSTAL W and CLUSTAL X, which is a graphical user interface program and then the sequences were compared with those from GenBank using BLAST [23]. The Biochemical tests were also performed to identify the genus. The 18SrRNA sequence analysis of the isolate was also performed. The sequence was analyzed using MEGA 5 and the corresponding phylogenetic tree was constructed [24].

D. Enzyme Assay and Protein Determination

Enzyme activity was determined according to the method described by [25]. The substrate solution was prepared by mixing 1 vol of 10.5mM 4NPF in Dimethyl sulfoxide (DMSO) with 9 vol of 0.1Mpotassium phosphate buffer solution, pH 6.5, containing 2.5% Triton X-100 followed by immediate vortexing. This buffer–4NPF solution was prepared freshly before analysis; the DMSO solution of 4NPF was prepared within 24 h and kept at room temperature. Decreased concentrations of Triton X-100 were effective at solubilizing the substrate, but solutions were not stable. For periods up to 1 day, the minimum effective Triton X-100 concentration should be 2.5 %. Reagent blanks are strongly recommended, as the emulsions occasionally exhibit variations in absorbance. The reaction mixture comprised 0.1 mL enzyme and 2 mL substrate solutions. The change of absorbance was read at 410 nm. In a control sample, the enzyme was replaced by appropriate buffer. One unit of enzyme activity is defined as the amount of enzyme releasing1 lmol of 4NP from 4NPF in 1 min.
The protein content was determined by the method of Bradford method [26]. The protein content in the chromatographic fractions was estimated by measuring the absorbance at 280 nm.

E. Purification of FAE using Gel Filtration

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The crude enzyme extract was loaded to a Sephadex G-200 column (65 x 1.5 cm). Enzyme elution was done with 0.05 M Na-Phosphate buffer (pH 9.0) with a flow rate of 2 mL/min. Each collected fraction was analyzed for protein and FAE activity. The active fractions were pooled and dialyzed against the same buffer and concentrated using a rotary vacuum evaporator at 40 ºC.

F. Molecular Weight Determination of FAE by SDS-PAGE

The molecular mass of the crude enzyme extract and partially purified FAE was estimated by SDS-PAGE electrophoresis. SDS-PAGE (12%) was performed as using medium range (10 to 100 kDa) molecular weight markers (Banglore Genei Pvt., India). FAE and other proteins were visualized by staining the gel with Coomassie brilliant blue.

G. Growth Pattern of Fungal Strain

Hypocrea lixii SS1 was grown in the growth medium. The temperature and pH of the medium were maintained at 35 °C and 5.5 respectively. Absorbance values of cell suspensions were read at 540 nm at regular intervals of 3 h, over a 192 h period. Cell cultures were shaken well for 60sec before each measurement. The control flask contained only the culture medium. The experiments were carried out as triplicates and their average values were taken into consideration.

H. Determination of Growth Kinetics

Samples were collected at every 3 h interval from the culture flask and subjected for centrifugation at 1118 x g separately. The initial weight of the aluminium foil was taken. The pellet obtained was placed in the foil and kept at 55 ◦C for 10 min until got dried. The weight of the foil with the dried pellet was measured [24].

From the biomass data obtained at various time intervals, the specific growth rate, μ_m and lag time were calculated using modified Gompertz model.

$$ln\left(\frac{X}{X_0}\right) = ln\left(\frac{X_{max}}{X_0}\right) exp\left(-exp\left(\frac{\mu_m . e}{ln\left(\frac{X_{max}}{X_0}\right)}(\lambda - t) + 1\right)\right) \quad (1)$$

Where X_0, X_{max}, X, t, μ_m and λ denote initial biomass concentration (mg/mL), maximum biomass concentration (mg/mL) and biomass concentration (mg/mL), incubation time (h), maximum specific growth rate (h^{-1}) and lag time (h), respectively.

III.RESULTS AND DISCUSSION

A. Screening of the Strain

A total of 50 strains was screened from the soil around an oil refinery on PDA for the production of feruloyl esterase enzyme. Top ten isolates producing the maximum amount of feruloyl esterase is listed in the Table 1.

Table 1
Different isolates of fae production

Isolate No	Dry weight (mg)	Protein (mg/ml)	Feruloyl esterase activity (U/g)
SS1	10.23	4.54	18.90
SS2	9.98	2.09	8.71
SS5	8.90	2.73	6.92
SS7	9.00	1.921	1.08
SS10	5.89	1.93	10.09
SS14	4.07	1.54	5.46
SS20	3.06	1.043	1.23
SS23	5.64	1.0098	0.43
SS33	7.98	1.3956	4.32
SS40	5.54	3.00	6.90
SS42	2.80	1.005	3.04

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Above, all the strains which produced highest enzyme activity were selected and subjected for further studies. The newly isolated strain produces the maximum feruloyl esterase enzyme and its activity was found to be 18.90 U/g. This is found to be higher than the enzyme activity obtained from Aspergillus niger (7.68 mU/g) [27], Streptomyces S10 (2 mU/mL) [28]. This is the first report showing the highest yield of ferulolyl esterase by the isolated new strain to the best of our knowledge.

B. *Identification of New Fungal Strain*

1) *Morphological Characteristics:* The strain isolated from the soil has the following morphological characetistics: Isolated strain grows at an optimum temperature of 35 °C. The colonies were first found to be transparent and later changed into yellow color. The colonies color is change mainly due to the pigment production by fungal strain, which is a characteristic feature of fungal species. The fungal strain under microscope was characterized by the effuse conidiation after 3-5 days of growth, whereas the conidiophores aggregations and pustles developed later. The microscopical observations were found to be the similar with [29]. This confirms that the new species identified belongs to Hypocrea sp. However, these methods are problematic as there are different biotypes within a species and time consuming. Hence 18s rRNA sequencing was carried out for getting reproducible and accurate results.
2) *Identification of Fungal Genome:* Identification of fungal genome was carried out by 18s rRNA sequencing, and the sequence was obtained. This was blasted against the entire microbial genome database in National Center for Biotechnology Information (NCBI). The BLAST result showed only 93% similarity with other existing fungal species and thus was found to be a new strain (named SS1). Thus, the sequence was deposited in the GenBank and was provided with a new accession number (JN687587).
3) *Phylogenetic Tree:* Phylogenetic tree was constructed from neighbour-joining program, using bootstrap consensus test with 500 in MEGA 5 and the branch lengths are in the same as those of the evolutionary distances used to infer the phylogenetic tree. The newly isolated strain was closely related to *Hypocrea lixii* with 96% similarity. Based on this similarity the isolated new fungal strain was identified as a *Hypocrea lixii* strain SS1 shown in Fig 1.

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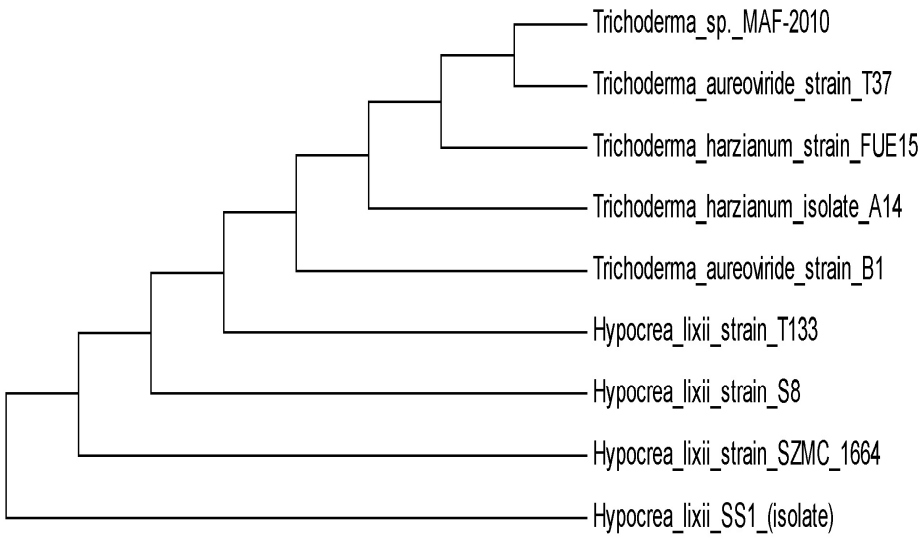


Fig. 1 Neighbour-joining tree based on nearly complete 18S rDNA gene sequences showing relationships between *Hypocrea lixii SS1* (isolate) and the related Hypocrea species strain.

C. Growth Pattern of Fungal Strain

The growth kinetics of the *Hypocrea lixii* SS1 obtained by their cultivation from 0 to 192 h was shown in Fig. 2.

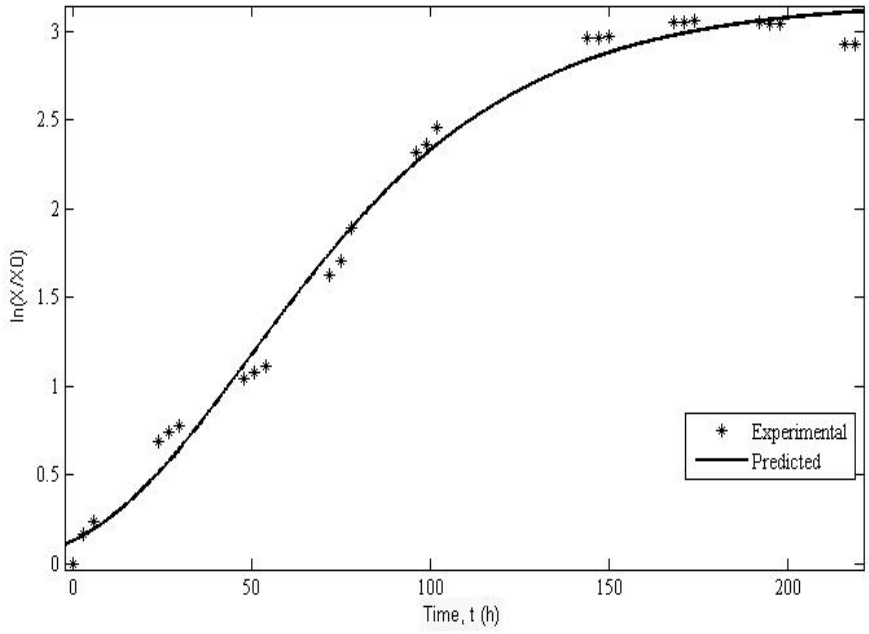


Fig. 2 Growth Kinetics of fungal *Hypocrea lixii SS1*

The biomass data obtained from the growth analysis was modeled using modified Gompertz model which was denoted by Eq. (1). Modified Gompertz model was found to represent accurately with experimental data of fungal isolate using Matlab 7.0. The growth kinetic parameters such as specific growth rate,lag time and

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Coefficient were determined for the fungal isolate were listed in Table 2. The exponential growth was observed from 96th h to 168 h after which it attains a steady state around 192 h. This indicate that the maximum growth observed on the 7th day of incubation. No colonies were resembling in the uninoculated control which indicates that the biomass obtained on the inoculated medium was only due to the fungal growth.

TABLE 2
DETERMINATION OF GROWTH KINETIC PARAMETERS

Fungal isolate	Specific growth rate, μ_m (h^{-1})	Lag time, λ (h)	Coefficient of determination, R^2
Hypocrea lixii SS1	0.036	6.532	0.999

D. Molecular weight determination of FAE by SDS-PAGE

The extracellular enzyme produced by the isolated Hypocrea lixii SS1 was found to be feruloyl esterase. This was further confirmed and characterized by SDS-PAGE. The supernatant (crude enzyme) and gel filtration active fraction of supernatant (representing partially purified enzyme) from Hypocrea lixii SS1 culture were used to perform the zymogram analysis. Fig. 3 shows the protein content on the SDS-PAGE (Lanes 1, 2, 3). Lane1 & Lane2 contain the crude enzyme extract and Lane 3 contains the product after the gel filtration procedure. The gel-filtration procedure is proved to be effective for feruloyl esterase enzyme purification since Lane 3 shows the predominant band. The results indicate that *Hypocrea lixii* strain SS1 has the ability to produce feruloyl esterase enzyme and its molecular weight was found to be 29 kDa. The results obtained were similar to [30].

IV. CONCLUSIONS

The present study showed that soil near oil refineries could be a rich source of feruloyl esterase producing fungi using sunflower sludge as the waste from the oil industry. The following conclusions were made from this study:

A. Out of various microorganisms isolated from the fouled soil, fungal strain *SS1* was found to be high potential for the production of feruloyl esterase enzyme.

B. The genus of the fungal isolate was identified as *Hypocrea* by performing the morphological and biochemical tests. Furthermore, the 18srRNA and the phylogenetic analysis reasserted the isolate as *Hypocrea lixii SS1*.

C. The enzyme was further affirmed by SDS-PAGE with their standard molecular weight (29 KDa).

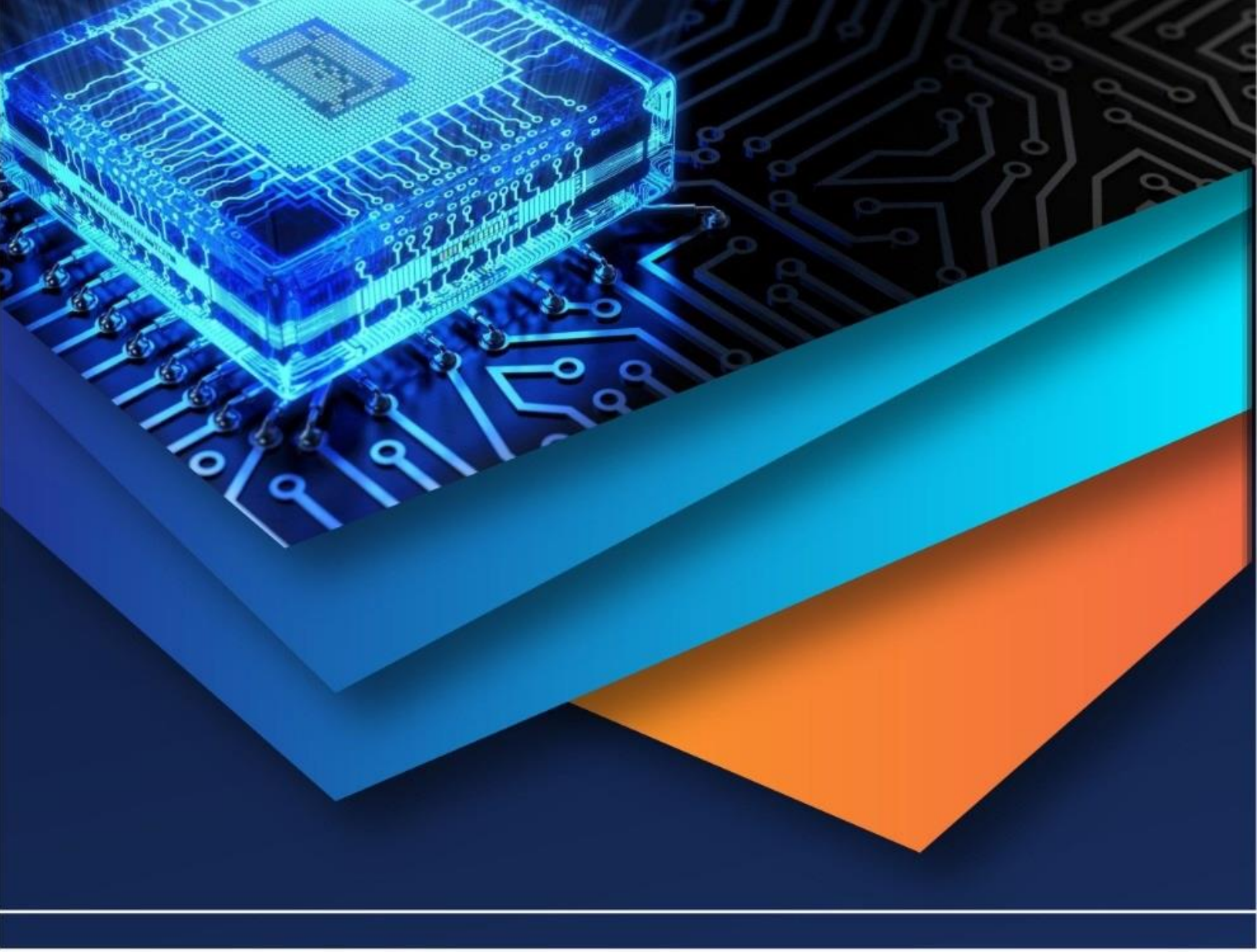
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