INNOVATIVE THEORIES IN SCIENCE AND ENVIRONMENT

EDITED BY
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PREFACE

It is an honor for us to present the book named **Innovative Theories in Science and Environment**. In this process, where we heal the wounds caused by the Covid 19 epidemic in our country and in the world, the importance of science and technology has been understood once again. For this reason, we are happy to contribute to the topics mentioned in this book, both scientifically and technologically, at national and international level.

This book was published for the first time and prepared in chapters. The book consists of eight chapters that describe new and current issues, after a brief introduction at first. It is a professional theory book in the field of Science and Environment. It includes very effective views and the latest determinations of scientists in the fields of Basic Sciences and Environment.

The work has been prepared with the thought of partially filling the gaps felt in the aforementioned fields in our country and in the world and to make use of undergraduate / graduate students and our colleagues working in the field of basic and applied sciences.

We would like to thank İksad Publishing's managers and employees for their meticulous and patient work in conducting the typesetting, editing and printing of the book named **Innovative Theories in Science and Environment**. Yours truly…

Editor of Innovative Theories in Science and Environment

Assist. Prof. Dr. Tülay GÜRSOY

CHAPTER 1

LOCATION AND GENETICS OF MEMORY

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INTRODUCTION

Parallel with the advancement of technology-dependent techniques, memory, which is an important part of cognition, has been studied intensively. Findings of memory studies are expected to contribute to the knowledge of all the biology (Kandel, 2001). Nevertheless, studying memory is not easy since it has multi-layers. It comprises behavioral level on one side and molecular level on the other side. Due to its multilayered structure, memory, as a subject, is in the conjunction of a wide variety of disciplines from philosophy to physiology. Each of the disciplines approaches the subject of memory from their own perspectives. One of the perspectives is studying memory at a molecular level. This approach sometimes is criticized for being reductionist as the Nobel Prize winner neuroscientist Eric Kandel who had preferred this approach stated (even, he called it as radically reductionist). To him, this way is the most experimental one. Dubnau and Tully (1998) state that the genetic approach can uncover cellular, anatomical, biochemical and behavioral aspects of memory and learning. Along the similar line, Thompson (2005) defines memory as neuronal memory circuits. Beyond the multilayered structure of memory, one should consider the miscellaneous processes that constitute memory, namely, encoding, storage, retrieval, etc. when studying it.

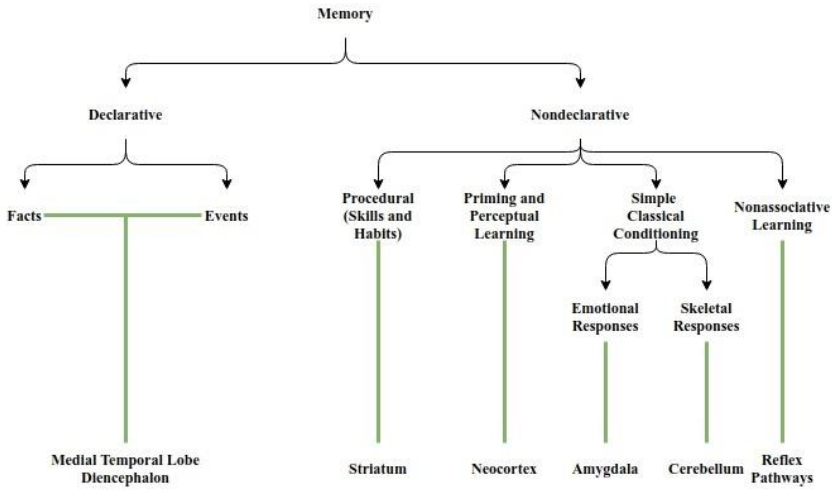
Eric Kandel decided to study complex memory on simplest cases, especially on animals, to relate behavior to molecular mechanisms in the brain. He justified his decision by stating that even though human

brain is different from brains of simpler animals, they have some commonalities. Thus, he assumed that biochemical mechanisms between human and animals are similar or same. He concluded that the molecular mechanism of memory is conserved from more basic creatures such as *Aplysia* to mammals for both short and long term memories, along with implicit and explicit memories.

Nevertheless, Kandel (2001) claims that the approach of molecular biology, which studies in a bottom-up manner, is not sufficient to understand memory. Top-down processes studied by psychology, cognitive sciences, etc. also should also be considered to solve the mystery of memory.

Kandel selected *Aplysia* as the model organism for his reductionist memory studies. He and his colleagues determined the "gill withdrawal reflex" as a simple behavior and "sensitization" as a form of learning. When *Aplysia* is given an aversive shock to its tail, it withdraws its gill as a defensive mechanism. Then, non-aversive stimuli given to its siphon causes it to withdraw its gill. This occurs because of learning. One aversive stimulus to the tail leads to short-term memory, whereas for long-term memory at least four stimuli is needed. It is not necessary to synthesize new proteins for short-term memory. This memory is rather needed for long-term memory. However, both memories are required to strengthen communications through covalent alterations of existent proteins (Kandel, 2001). Both of the memory types are synapse-specific which means that stimulus effects only the synapse is applied. Transcription of proteins caused

by long-term memory process leads all synapses of the neuron to be marked for utilizing the proteins for a potential long-term process. However, during short-term process, stimulated synapse is marked for using proteins activated by CREB, which is activated by the long-term process. Thus, these processes show a difference concerning the number of synapses they mark. The short-term mechanism marks synapses locally, while the long-term does systemically (Kandel, 2001). Learning and long-term memory development call for inducement of the CREB/CRE pathway (Impey et al., 1998). CREB functions as a "molecular switch" or modulator on long-term memory (LTM). Repression of CREB causes LTM blocking, while activation of CREB leads to enhanced LTM in *Drosophila melanogaster* (Yin et



al., 1995).

Figure 1: Long-term memory system taxonomy (Squire, 2004)

According to a single-gene mutant *Drosophila melanogaster* model, memory formation has five sequential processes, which are learning, short-term memory, middle-term memory (MTM), anesthesia-resistant memory (ARM) and long-term memory (LTM). After MTM, either LTM or ARM occur. Both of them are long lasting. LTM requires protein synthesis and spaced training. ARM is independent of protein synthesis and constructed by mass training. MTM is also protein synthesis-independent (Dubnau & Tully, 1998).

Activity-dependent synaptic plasticity is a mechanism for memory storage (Tanaka & McHugh, 2018). In another approach, plasticity and memory include non-synaptic mechanisms in addition to synaptic ones (Thompson, 2005).

Multiple memory systems theory (MMS) suggests that memory is a modular system instead of unitary. The parts of the system process specific kinds of information independently and produce different representations while occupying different locations in the brain in other words, having different neural networks (Ferbinteanu, 2019). The basic question is how these separate systems form only one behavior. To answer this questions other theories and models such as attribute model of memory have been developed. A new approach, dynamic brain network system, suggests that memory networks are not static, but dynamic. Thus, the dynamic brain network system can reconfigure and work together, if the situation requires it. One system acquires information and later, the other system can use this information. This is beyond the memory system, it is memory meta-

system. Memory is more than neural circuits, which is only one of the blocks that comprise it. Each type of memory is controlled by more than one brain part and each of the brain parts affects more than one type of memory (Ferbinteanu, 2019).

Engram theory of memory suggests that memory is the changes in cells called “engram cells” that occurs while learning. Stimulation of these cells causes recall. According to this theory, memory is not held by engram cells on only one place, rather, cells in different areas form an engram cell pathway in accordance with the memory components such as spatial, fear etc. A pathway for memory is not compulsory. When the default memory pathway is not available, a compensatory pathway is employed (Tonegawa et al., 2015). In an interesting study, Liu et al. (2014) incepted false fear memory by optogenetically activating dentate gyrus (DG) cells that carry contextual memory engrams.

1. LOCATION OF MEMORY

There are two widely used strategies to study memory. First one is neural plasticity, especially long-term potentiation (LTP). The second one is determining a form of learning and memory, and then attempting to find loci on the brain and a memory trace. Neuronal records yield a form of memory sits on certain parts of the brain. This does not mean that these loci are the centers for this type of memory. A claimed memory locus of the brain can be necessary for memory, but not sufficient. Thompson (2005) suggests the methods of reversible inactivation in which they inactivated the suspected regions

and observed whether it prevents memory formation and retrieval or not. If memory functions do not work, then it can be concluded that the regions are essential for memory. Another problem occurs by lesion methods in which parts of the brain are damaged or a subject with a lesion on the area of interest is studied. Nevertheless, the problem is that given lesions are scarcely limited to the aimed sites.

It is generally assumed that learning and memory is distributed to different parts of the brain. For instance, contextual conditioning and passive avoidance are hippocampus-dependent learnings, in which CRE-dependent gene expression increases in CA1 and CA3 regions of the hippocampus. Passive avoidance leads to increased gene expression on the dentate gyrus of the hippocampus as well. Whereas, auditory signal fear-conditioning is an amygdala-dependent learning and is linked to elevated CRE-mediated gene expression in the amygdala (Impey et al., 1998). In a study done by Santangelo et al. (2020) people with highly superior autobiographical memory (HSAM) showed a stronger neuronal activation on their ventromedial prefrontal cortex (vmPFC) than the control group. Nevertheless, two of the brain regions are more closely related to memory; medial temporal area and prefrontal cortex (Schacter et al., 1998). Moreover, the most addressed region in the brain, for memory, is the hippocampus. Synaptic connections on this structure are strengthened by synaptic plasticity on encoding when neocortex is activated. In this way, hippocampus had stored the activity pattern of the neocortex. Later, a stimulation can activate this activity pattern and neurons fire in the hippocampus, which is called “retrieval” (Tanaka & McHugh, 2018). Learning, in

addition to the strengthening of synaptic connections, involves an elevated number of synapses (Bailey & Chen, 1989). However, researchers do not agree on the central role of hippocampus on memory. For instance, Thompson (2005) claims that hippocampus is not essential for a specific kind of learning, standard-delay classical eye-blink conditioning, rather it is formed and stored at interpositus nucleus which is one of the four cerebellar nuclei. Hippocampus is the locus for higher-order memory mechanism, not for, as he called it, "essential memory circuit".

One of the memory theories, temporal lobe memory system, claims that memory is a result of the function of brain's medial temporal area including hippocampus and parahippocampal cortex (Squire & Zola-Morgan, 1991). Another memory system theory suggests that there are six separate kinds of memory; each of them has its own central neural networks. These memory systems work parallel and independently (Squire, 2004). Later, non-unitary memory system theories with the functional principle of "independent parallelism" have changed to theories propose that neural networks of memory systems are not separate determinately rather; they can be shared between memory systems in some situations (Ferbinteanu, 2019). Similarly, Thompson (2005) claims that the two terms, memory trace and memory trace circuits, point to two different things. Generally, memory trace circuits comprise memory trace, but not always. The memory trace circuit refers to loci of the memory process, whereas the memory trace is the processes, which can happen in different loci of the brain.

In addition to the above discussions, the location problem has a deeper level. Consolidation theory claims that memory formation takes place in soma with the processes of transcription and protein synthesis. In contrast to that, post-translational modification (PTM) theory suggests that proteins for memory are locally synthesized and modified in dendrites. The proper protein level is determined by synaptic dialogue from postsynaptic to presynaptic neurons. This raises the question of whether memory process including storage of it is located in nucleus or synapses (Hernandez & Abel, 2008).

2. NEURAL AND GENETIC COMPONENTS OF MEMORY

Two distinct categories of neural circuits in behavior and learning are mediating and modulating. The mediating circuits are used in reflex and habituation. Whereas, the modulatory circuits involve utilization of modulatory interneurons such as serotonergic ones in intricate modes of learning, such as sensitization and classical conditioning. In *Aplysia*, the modulating circuit alters the mediating circuitry to coordinate the stability of its connections (Figure 2). In mediating circuits, neurotransmitters can act by ionotropic receptors and metabotropic receptors that activate second messengers. Activation of second messengers provides long-lasting synaptic action. In modulatory circuits, neurotransmitters cause second-messengers to move to the nucleus and stimulate genes. Further, a mechanism causes synapse-specific local protein synthesis to stabilize long-term facilitation and structural change. This last one is a part of long-term memory process (Kandel, 2001).

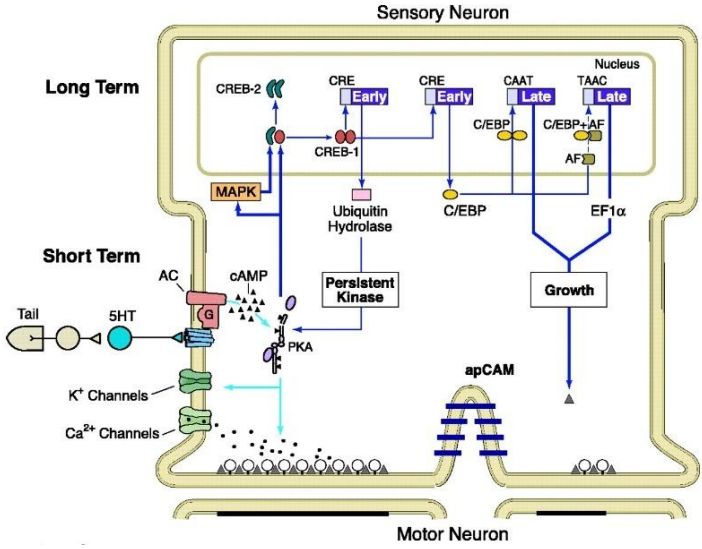


Figure 2: Molecular mechanism of short- and long-term sensitization on *Aplysia during* gill-withdrawal reflex (Kandel, 2001)

Short-term sensitization generates a single time discharge of serotonin that promotes covalent alteration of existent proteins. Whereas, long-term sensitization promotes persistent activity of protein kinase A as well as the growth of new synaptic connections. The stimulus leads serotonin to release and acts upon a transmembrane receptor to stimulate adenylyl cyclase (AC) and cAMP activation. cAMP then stimulates the cyclic AMP-dependent protein kinase A (PKA). PKA phosphorylates and covalently alters a group of target proteins. The activity of PKA increases Ca+ influx that in turn give rise to neurotransmitter release.

In long-term sensitization, overstimulation causes repeated release of serotonin that activate cAMP to translocate to the nucleus. cAMP phosphorylates the cyclic AMP response element-binding (CREB) protein and prompts discharge of the restraining activity of CREB-2

and engages the mitogen-activated protein kinase (MAPK) there (CREB-2 may inhibit CREB-1 via MAP kinase, which is also activated by cAMP.). Transcription factor CREB-1 binds to a cAMP response element (CRE) in the promoters of target genes and activates a cluster of instant response genes to synaptic strengthening and developing of new synaptic connections. One of the targets is the gene that encodes a ubiquitin hydrolase, a protease that leads to proteolysis of the inhibitor subunit of PKA. This gap generates constant activity of PKA, leading to persistent phosphorylation of the substrate proteins of PKA. Besides, CREB-1 activates C/EBP, which is a gene that induces the generation of new synaptic connections. C/EBP can act together with activating factor (AF). They activate some of the genes which cause new synapses to grow. cAMP, PKA and CREB mechanisms are involved in both learning and memory (Kandel, 2001). Memory-related regulators such as CREB has been conserved among organisms including *Homo sapiens*. One can assume that genes that play role in memory can be shared between organisms, too. CREB expressed in non-neuronal tissues regulates development and growth, whereas CREB functions in neurons regulates long-term memory (Lakhina et al., 2015). Weng et al. (2018) found a molecular pathway for encoding long term contextual memory which induces synaptic plasticity at synapses between mossy fibres (MF) and cornu ammonis 3 (CA3) pyramidal neurons of the hippocampus, mediated by transcription factor Neuronal PAS domain-containing protein 4 (Npas4) and polo-like kinase 2 (Plk2).

Mammalian memory-related genes and long term memory-related genes on worms are orthologs suggest that memory pathway has been conserved so that organisms such as worms can be studied as model organisms for mammalian memory (Lakhina et al., 2015). Berto et al. (2018) identified more than 100 genes correlated with episodic memory encoding in human. They claimed that their data showed for the first time that specific genes related to cognitive tasks like memory encoding may be determined in humans. They recorded intracranial EEG where participants performed an episodic memory task. Then, they correlated these signals for concerned regions with gene expressions of the same regions. Tan et al. (2019) utilized the spatial correlation method in which they conducted correlation for whole human brain transcriptome and memory relevance of regions of the brain via neuroimaging map for cortical and subcortical areas in an effort to identify memory-associated genes. They found 8383 positively (refers to higher gene expression) and 7243 negatively (refers to lower gene expression) correlated genes for the cortical areas, and 7642 positively and 7984 negatively correlated genes for the subcortical areas. According to their analysis, genes mostly expressed in cortical and subcortical regions are distinct. Cortical genes are linked to immune and epigenetic regulation, whereas subcortical genes are related to neurogenesis and glial cell differentiation.

CONCLUSION

Finding a place and related genetic components of memory are necessary for revealing the memory mechanism. However, this is not easy. Genes can be pleiotropic, meaning that an individual gene can affect more than one phenotype. Thus, identifying genes that have effects on memory and learning does not mean that the functions influenced by a gene are related to memory or learning. This is a limitation for genetic studies of memory and learning (Dubnau & Tully, 1998). Additionally, memory is not a monolithic, but a multifaceted structure. Studies show different regions for each kind of memory. Further approaches and studies should be developed to overcome these obstacles and disclose memory.

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CHAPTER 2

CHARACTERIZATION AND ANTIMICROBIAL EFFICACY OF SILVER NANOPARTICLES OBTAINED FROM THE FUNGUS EXTRACT BY GREEN METHOD

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INTRODUCTION

Metal nanoparticles have a wide range of applications in pharmaceutical, agricultural, diagnostic and bioengineering studies. In particular, the potential of silver nanoparticles in the food industry, packaging, cosmetics, medicine, environment or the automotive sector is noteworthy (Thombre, Shinde, Thaiparambil, Zende, & Mehta, 2016). These nanoparticles are studied extensively with good conductivity, chemical stability, catalytic and UV filtering properties (Chauhan, Reddy, & Abraham, 2015). However, the production of metal nanoparticles by physical and chemical methods is both costly and toxic by means of harmful and environmentally harmful methods (Forough & Fahadi, 2011). Green synthesis is an environmentally friendly and economic approach, which eliminates or reduces the formation of harmful substances during production. In addition, the production of controlled and narrow-sized, well-defined, stable nanoparticles with green synthesis may also enable the development of novel pharmaceutical drugs. Metal nanoparticles are also known to have a strong antimicrobial effect, especially silver has toxic effect on microorganisms. So, silver-based components are therefore used to treat burns or infections in medicine (Poor, Khatami, Azizi, & Abazari, 2017). That's why, it is desirable to synthesize silver nanoparticle economically recently with a green approach. Plant, algae, fungi and bacteria have used as organic material (Betül Yilmaz Öztürk, 2019; Sanguiñedo, Estevez, Faccio, & Alborés, 2019). It contains a wide variety of organic materials in fungus. For instance, *Pichia membranifaciens*, which is an effective antagonistic yeast, can

display a wide antibacterial spectrum, and it has also been reported that post-harvest various diseases occurring on some fruits can be controlled (Zhang et al., 2017).

Today, it is known that pathogen or opportunistic microorganisms develop an increased resistance against various antimicrobial agents. This resistance can be developed against a single drug, as well as multiple drug resistance. Resistance mechanisms are still not fully elucidated, and biofilm development of microorganisms is one of the most important mechanisms of resistance. Biofilm is a coordinated microbial community that colonizes live or non-living surfaces and is embedded in their own EPS. When microorganisms form biofilms, they show higher resistance to antimicrobials than their planktonics (Singh et al., 2018). Most of the available antibiotics are ineffective against microorganisms that are associated with biofilm-associated and multidrug resistance. Many studies focus on the development of new, effective, cost-effective and non-resistant antimicrobials. The effects of organic and inorganic nanoparticles on biofilm inhibition continue to be investigated, but their mechanisms of action have not yet been fully elucidated. In the present study, we studied extracellular biosynthesis of silver (Ag) nanoparticles from *Pichia membranifaciens* NRLL-Y 2026 (*P. membranifaciens*) extract. Obtained nanoparticles were characterized by analytical instruments and their antimicrobial and antibiofilm activities were evaluated against bacterial and fungal isolates. In addition, ultrastructural effects on Gram negative bacteria *Escherichia coli* ATCC 25922 (*E. coli*) and *Candida albicans* ATCC 14053 (*C. albicans*) yeast of biosynthesized

silver nanoparticles were investigated by transmission electron microscopy.

1. MATERIAL AND METHODS

1.1. Materials

Analytical grade silver nitrate ($AgNO_3$ Solution), XTT [2,3-bis(2-methoxy-4-nitro-5- sulfophenyl)-2H-tetrazolium-5-carboxanilide], were purchased from Sigma-Aldrich. The medium used for antimicrobial studies were Nutrient Broth (NB), Yeast Extract Peptone Dextrose (YPD) and Roswell Park Memorial Institute (RPMI) 1640 Medium. All chemicals were also purchased from Sigma-Aldrich.

1.2. Organisms

In our study, *P. membranifaciens* NRLL-Y 2026, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 14053 standart strains were used. *P. membranifaciens* NRLL-Y 2026 was maintained on YPD agar at 4°C and subcultured on same medium at 30°C for 24 h prior to use. *C. albicans* isolate was activated in RPMI 1640 broth medium and E. coli isolate in nutrient broth medium by incubation at 37 ° C for 24 h. In our study, the method was used proposed by Ronavari et al. with some modifications for AgNP biosynthesis (Rónavári et al., 2017). *P. membranifaciens* was harvested and washed three times with distilled water. 5 g of biomasss was ground and boiled for 40 min with distilled water, maintaining a total volume of 100 ml. Particulates were removed from the environment by filtration. The aqueous extract was further centrifugated at 4500 rpm

for 5 min to remove any particulates. The obtained extract was diluted in water at different ratios of extract to water. Thus, the amount of extract to be used during the synthesis has been optimized. Firstly, the color change in the reaction mixture was observed and then results were supported by UV-Vis analysis (Singh et al., 2018). After the metal salts are completely reduced to nanoparticles, they were centrifuged first at 3000 rpm 5 min and so large particulates were removed, then second centrifugation was made at 18.000 rpm for 15 min to collect the nanoparticles. Synthesis products were centrifuged three times with distilled water to remove non-reduced metal ions. The nanoparticles collected after centrifugation were brought to powder form by freeze drying (-76 ° C).

1.3. Characterization techniques of synthesized nanoparticles

Optimization studies have been carried out for the biological reduction of AgNPs. For this process, 3 ml samples were taken and UV-Vis measurements were analysed. Absorbtion measurements were performed by a UV-Vis (AE-S90-2D UV-Vis Spectrophotometer, China) at 190-1100 nm. In our study, optimization process has been realized for the factors that will affect the synthesis. The first optimization was to determine the amount of extract. The synthesis was carried out by diluting the extract in different proportions. Other factors affecting the synthesis, pH, temperature, time and salt concentrations ($AgNO_3$) were also optimized. The results were evaluated primarily by visual color change in the reaction mixture and then by spectral analysis data (Betül Yılmaz Öztürk, Gürsu, & Dağ,

2020). In our study, optimization process was performed for the factors that affect the synthesis. The first optimization was the determination of the amount of extract. The synthesis was carried out by diluting the extract in different ratios. Other factors affecting the synthesis, pH, temperature, time and salt concentrations ($AgNO_3$) were also optimized. The results were evaluated by visual color change in the reaction mixture and then by spectral analysis.

To visualize the synthesized AgNPs, TEM (Hitachi HT 7800) at 100 kV acceleration voltage was used. For the preparation of the sample, the nanoparticle solution dropped on the carbon coated grid was examined after drying. Energy-dispersive X-ray (EDX) analysis connected to TEM device was performed to determine the amount of elemental silver in the sample (Oxford Instruments X-MaxN). In addition, TEM was used to investigate the effects of nanoparticles on yeast and bacterial cells in detail. For this purpose, samples were fixed, dehydrated, clarificated and embedded in resin. After polymerization, obtained TEM blocks were cut into 60 nm thick ultrathin sections and taken into 300 mesh sized copper grids.

X'pert PRO PAN analytical device was used to get information about the crystal structure of powdered AgNPs. For the X-ray diffraction model, the device was operated at 30 mA and 40 kV (Cu K$\propto$ radiation (λ = 1.5406 Å)) scanning mode. Diffraction densities were recorded at 2 theta angles and from 10 ° to 80 °. Diffraction densities were compared with standard JCPDS files. The program of X'pert PRO PAN gave information about the crystal structure of the AgNPs.

For FTIR analysis, Diamond ATR technique was used with PerkinElmer Spectrum Two device. In order to remove organic substances that do not play a role in the reduction of silver ions during green synthesis, samples were washed three times with pure water. In this analysis, in order to determine the biomolecular bond changes in the extract content during the synthesis, spectra were obtained by scanning in the range of 4000 to 650 cm^{-1}.

The ICP-MS device (Thermo iCAP RQ) was used to quantitatively determine the amount of synthesized silver nanoparticles. Firstly, the organic components in the extract were burned using a microwave ash oven. During this process, pure water dilutions were performed and a standard calibration curve (Redox-423A) was obtained. The amount of silver nanoparticles was determined by software, and the primary concentration to be used in the subsequent antimicrobial studies was determined.

In our study, CLSI (Clinical Laboratory Standards Institute) criteria were used to determine the MIC values of AgNPs synthesized. Ampicillin for bacteria and amphotericin B for yeasts standard drugs are used. Antimicrobial susceptibility studies were carried out considering the initial concentration amount (68.5 µg / ml) determined in the ICP-MS analysis.

1.4. Antimicrobial Activity

Broth microdilution test was performed according to the CLSI (CLSI M7- A8) criteria for E. coli isolate used as the test organism (Wikler, 2006). For this purpose, 96-well microplates were used. After the bacterial culture was grown one-night in MHB, the suspension turbidity was adjusted to McFarland 0.5 (1- 2x105). Serial dilutions of the AgNPs ranging from 34.25 µg/ml to 0.07 µg/ml were made and microorganism inoculations were performed. Plates were incubated 24 hours at 37° and optical density values were determined at 545 nm. The lowest nanoparticle concentration that prevents the growth of microorganisms is determined as MIC.

CLSI M27-A2 criteria were based for determination of antifungal activity of AgNPs on *C. albicans* isolate (Standard). This study was carried out with 96-well microplates and using RPMI-1640 (Sigma, Germany) medium. The turbidity of the cells after overnight grown in RPMI-1640 medium was adjusted to McFarland 0.5 (0.5-2.5 × 10^3). The final concentration ranges of AgNPs are 34.25 µg/ml - 0.07 µg/ml. After inoculation of yeast cells, plates were incubated for 24 h at 37 ° C. Absorbance values were measured with a microplate reader (Chromate Microplate Reader 4300) and the MIC detection was determined as the lowest NP concentration that prevents yeast growth.

According to the results of the MIC test, ten microliters samples were taken from the wells that did not show any growth and inoculated to the medium. RPMI agar were used for C. albicans and MHA were used for E. coli. The lowest AgNPs concentrations that did not show

visible growth in plates for both bacteria and yeast after 24 hours incubation at 37 ° C were defined as MBC and MFC values, respectively.

To examine cell-nanoparricle interactions in ultrastructural level, TEM device was used. For this purpose, the samples were grown for 24 h in the medium containing AgNP at Sub-MIC concentration (1/2MIC). After fixation, dehydration, clarification, embedding and polymerization steps, ultra thin sections (60 nm thickness) taken with an ultramicrotome (Leica Ultracut R). Samples were stained with uranyl acetate and lead citrate and TEM grids were analysed under the Hitachi HT 7800 model TEM (Li et al., 2012).

1.5. Antibiofilm Activity

The method proposed by Serrano-Fujarte et al. was used to determine the antibiofilm activities of AgNPs (Serrano-Fujarte et al., 2015). For this purpose, the effects of AgNPs on cells both before and after biofilm formation were evaluated. To investigate the pre-biofilm effect, the active substance was applied to the cells at the inception of the experiment and used to be at the MIC value. TSB media for bacterial isolate and RPMI media for yeast isolate were used. Ten different concentrations were selected and mean values were taken. The study was repeated three times. To investigate the effect after biofilm, the active substance was applied to the grown cells in plates after 24 hours and the results were evaluated. For XTT assays, 100 ml of XTT saline (1 mg / ml) and menadione solution (1 μM; prepared in acetone) was added into wells of 96 well microtiter plates including

biofilm. The XTT assay was added to wells containing 100 ml of XTT saline (1 mg / ml) and menadione solution (1 µM) (Molecular Probes; prepared in acetone) biofilm. Colorimetric changes due to the reduction of XTT at 492 nm were interpret with a microplate reader (Chromate Microplate Reader 4300) after 37 ° C for 2 hours incubation in the dark (Baillie & Douglas, 1999; Ramage, Martínez, & López-Ribot, 2006).

3. RESULTS

3.1. AgNPs biosynthesis and Characterization

In our study, silver nanoparticles (P-AgNPs) were successfully synthesized by *P. membranifaciens*. Optimization studies for reaction parameters such as extract amount, temperature, pH, salt were carried out by wavelength scanning (190-1100 nm). In current studies, AgNP synthesis in the UV-Vis experiments has been reported to be in the range of 350-500 nm (Haider, Mohammed, Al-Mulla, & Ahmed, 2014; Betül Yılmaz Öztürk et al., 2020). AgNPs showed surface plasmon resonance (SPR) response at 420-430 nm which meant the stimulation of free electrons of nanoparticles. The symmetrical shape of the band indicates that the spherical shaped nanoparticles are uniformly distributed (Suriya, Raja, Sekar, & Rajasekaran, 2012). The effect of extraction mixture ratio, temperature, time and metal ion concentration factors were examined to determine the optimum reaction parameters in nanoparticle synthesis. After determining the most suitable parameter conditions, characterization studies were carried out.

In our results, the ratio of extract to water (2: 8) and this indicates that synthesis can occur at a very low cost (Fig. 1a). Especially the determination of the optimum pH plays an important role in nanoparticle formation and reaction rate (Badawy et al., 2010). Because the ph of the mixture prepared for synthesis prepares the ground that allows the silver nitrate to react with the organic material. For pH optimization, extract pH was based and a change between pH 5-9 was observed. A change in wavelength is a measure of nanoparticle size, shape, and properties between particles (Prasad, Kambala, & Naidu, 2013). According to the absorbance value and the shape of the peak, the best result was determined as pH 7 (Fig. 1b).

Temperature and time optimization studies revealed that 80 ° C and the best time for P-AgNPs synthesis was determined as 15 minutes (Fig. 1 c-d). Since increasing the temperature caused disruption and precipitation the reaction mixture, this condition showed that the nanoparticles were unstable at temperatures of 90 ° C and above, and the reaction may not take place. Many proteins degrade before they reach 100 ° C. In literature, proteins have been reported to play a role in the biosynthesis and coating of AgNPs (Barwal, Ranjan, Kateriya, & Yadav, 2011). In the synthesis of P-AgNPs, the concentration of salt tested for silver salt optimization was determined as (1-20mM), and 15mM silver nitrate was determined as the optimum concentration. When the salt concentration was increased further, significant shift was observed in the absorbance peak (Fig. 1e). The color of the reaction mixture began to change from a yellowish color to dark brown from the 10th minute. This demonstrated the reduction

of silver metal ions and the formation of silver nanoparticles through active molecules. After 15 minutes, the color did not change anymore and the best absorbance was obtained at this time (Fig. 1f). The optimal reaction parameters in nanoparticle synthesis are 2: 8 for extract ratio; 7 for pH; 80 ° C for temperature, 15mM for salt concentration and 15 minutes for time.

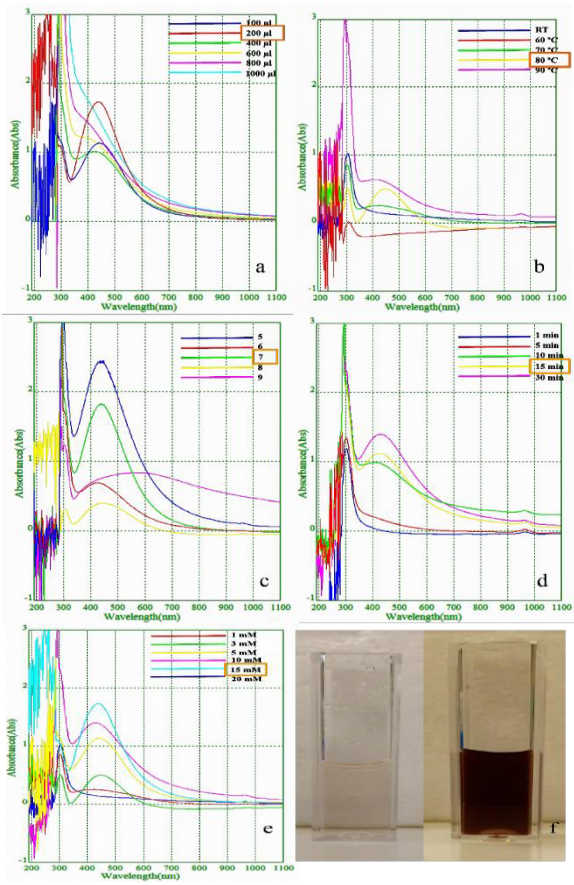

Figure 1: Optimization studies based on the UV-Vis spectral analysis for AgNP production by the *P. membranifaciens* The optimized parameters for P-AgNPs were as follows: (a), the reaction mixture ratio (extract:water) (b), pH (c), temperature (d), time (e), silver salt concertation and (f), The photograph of test tubes with optimizing concentrations.

Synthesized P-AgNPs was examined in TEM analysis for the morphological character and homogenous distribution (Fig. 2). As a result of 10 different grid scans, P-AgNPs were found to be spherical and their average size was between 20-40 nm. Besides, according to the TEM-EDX analysis result, 93.65% silver elements were found.

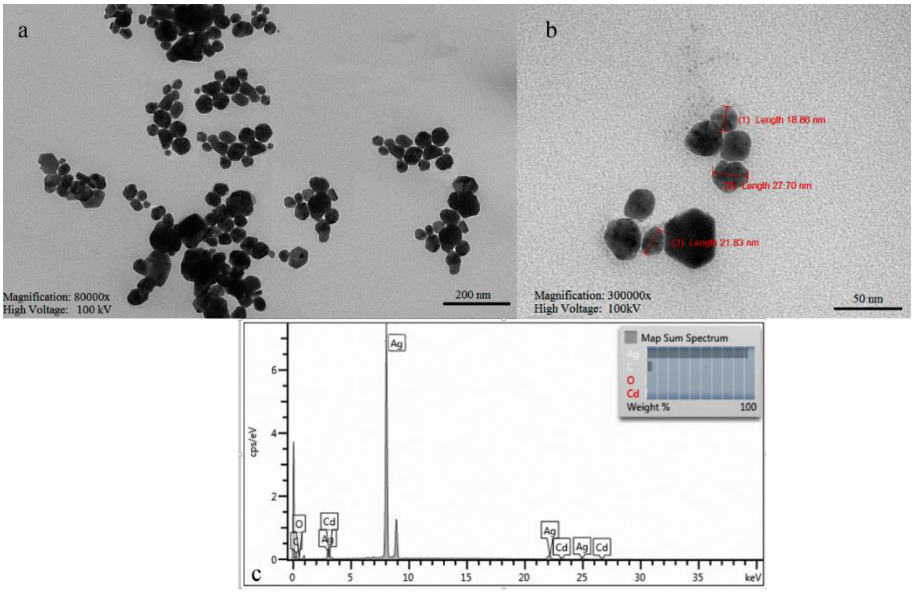

Figure 2: Transmission electron microscopy (TEM) images of nanoparticles showing the particle shape of P-AgNPs (a,b) and TEM-EDX spectrum of nanoparticules (c).

XRD is a widely used technique that shows the crystal structure of nanoparticles due to the wavelength of the X-ray. XRD patterns of silver nanoparticles synthesized using *P. membranifaciens* are shown in Fig. 3. The X-ray diffractogram of the biosynthesized nanosilver exhibited the Bragg reflection corresponding to the face center cubic (fcc) silver. The spectra obtained confirmed that the silver

nanoparticles were in pure crystalline form according to the 2θ values. Three additional broad bands at 25.447º (2θ), 35.453º (2θ) and 41.580º (2θ) respectively showed Bragg reflection corresponding to the silver planes (2 0 0), (2 2 0) and (3 1 1) respectively (Fig. 3). Fracture peaks were expanded along their bases in spectra showing that the silver nanoparticle was in nanoscale. Other false diffraction results from impurities of organic materials. Evaluation of the results, Bragg peak location and intensities were taken into account and compared to standard JCPDS files (Reference code: 98-002-8103). As a result, it has been shown that Ag^{+} is reduced to Ag^{0} by *P. membranifaciens* in crystal structure.

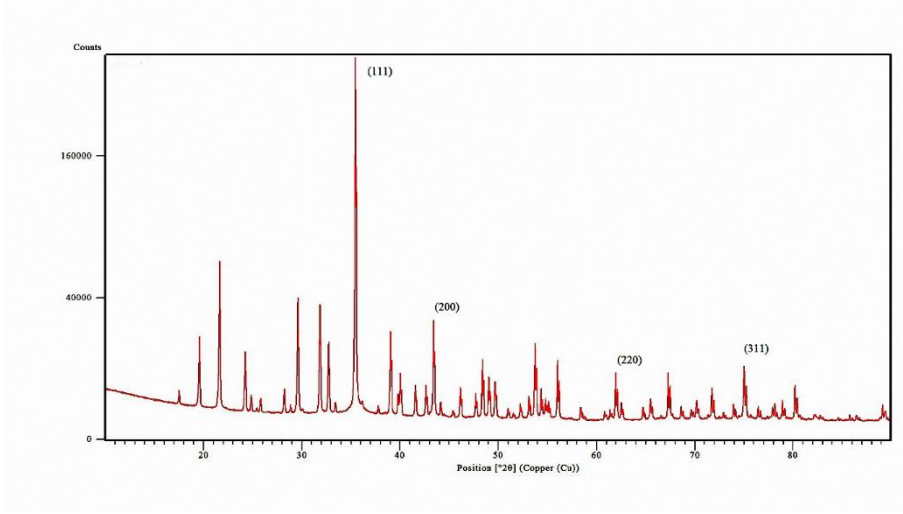

Figure 3: X-ray diffraction pattern of AgNPs synthesized using *P. membranifaciens* extract

In our study, FTIR analysis was performed using *P. membranifaciens* extract to determine the components that reduce nanoparticles, especially the bioactive components, which we consider to be a

capping agents. As a result of analysis, many peaks 3266, 2931, 1631, 1524, 1455, 1401, 1303, 1216, 1127, 1027, 974, 899, 811 cm^{-1} were observed (Fig. 4). The FTIR spectrum obtained from the fungus extract and the analysis of the silver nanoparticles obtained from this extract showed extensive similarities between the samples. In particular, *P. membranifaciens* extract and the spectrum of AgNPs obtained from this extract were characterized by the OH band at 3266.03 and 3298.7 cm^{-1}. This band contains essentially the alcoholic, phenolic and carboxylic groups indicated by an intense broadband around 3400 cm^{-1}, corresponding to the O-H stretching of the hydroxyl groups and the N-H stretching and the primary and secondary amines and amides (Ronavari et al., 2017). The C-H band was observed between 2931.20 and 2971.27 cm^{-1}. The C = C voltage was observed between 1631.12 and 1731.12 cm^{-1}. The new peak at 1731.12 cm^{-1} is most likely due to the aldehyde oxidation of the alcoholic group, while the silver nitrate metallic salt decreases to form silver nanoparticles. The peak at about 1,630 cm^{-1} shows the C = C vibration of the aromatic structures. The peaks of 1524.9, 1455 and 1401.17 cm^{-1} appear in the extract removed, and these peaks correspond to azo compounds. The elimination of these peaks suggests that these compounds are effective in the formation of nanoparticles. In addition, in our example, C = O, ranging from 1027.40 to 974.79 cm^{-1} was also determined. Aromaticity can be mentioned between 900-690 cm^{-1}. Aromatic azo compounds are important intermediate compounds. These intermediate compounds have wide application as pioneers for chemical stabilizers,

polymerization inhibitors, dyes and materials used in pharmaceuticals (Teng et al., 2019). In our study, these azo compounds in *P. membranifaciens* extract are thought to act as capping agents in our green synthesis.

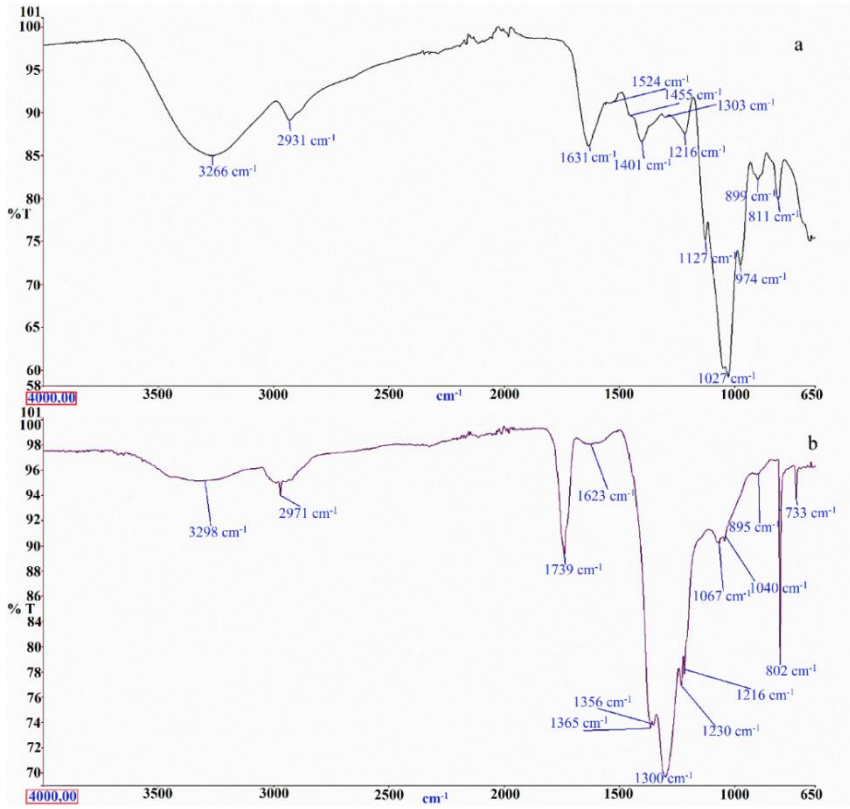

Figure 4: Fourier transform infrared spectroscopy (FTIR) of (a) *P. membranifaciens* extract; (b) *P. membranifaciens*-synthesized Ag nanoparticles

3.2. Antimicrobial Activity of AgNPs

The Antimicrobial studies of biosynthesized P-AgNPs toward isolates were sum up in Table 1. Our data showed that the synthesized AgNPs have strong antimicrobial efficacy compared to traditional drugs. While the MIC values we obtained were 0.54 µg / ml for *C. albicans*,

which is a yeast isolate, it was found 0.27 µg / ml for E. coli isolate, which is a gram negative bacterium, so, it was observed that the antibacterial effect of P-AgNPs was higher than the antifungal effect. While MBC value was 2 times higher than MIC, MFC values were found 4 times higher.

Table 1. Minumum inhibitory concentration (MIC, µg/mL), minimum fungicidal concentration (MFC, µg/mL), and minimum bactericidal concentration (MBC, µg/mL) values of biosynthesized AgNPs against *E.coli* and *C. albicans* isolates

Parameter	microorganisms	
	C. albicans	*E.coli*
MIC ($\mu g\ mL^{-1}$)[a]	0.535	0.268
MFC/MBC ($\mu g\ mL^{-11}$)[a]	2.14	0.535

[a] Values for $n = 3$; CV ≤ %5

3.3. Antibiofilm activity studies of AgNPs

The amount of AgNPs (0.54 µg / ml) on prebiofilm was found to be 79% reducing. On postbiofilm, it decreased 73.5% with 2.14 µg / ml (Table 2). According to these results, it has been found that the effect on postbiofilm is as significant as the effect on prebiofilm.

Table 2. Percentage inhibition rates (%) of biosynthesized AgNPs on C. albicans biofilm before and after exposure

Test compound	%Biofilm inhibition (concentartion)	
	C. albicans -pre biofilm	*C. albicans -post biofilm*
Silver nanaoparticle	%79±3.2 (0.54 $\mu g\ mL^{-1}$)	%73.5±1.5 (2.14 $\mu g\ mL^{-1}$)

[a] Values are mean ± SD for n=3.

3.4. Ultrastructural evaluation in planktonic bacteria and yeast cells used with AgNPs

Planktonic *C. albicans* cells after exposure to P-AgNPs are shown in Fig. 5. When examining the control group of *C. albicans*, typical Candida morphology was found and the nucleus was located centrally and was quite evident. While the cell wall and the stoplasmic membrane were observed as a whole, the stoplasma was also seen regularly (Fig. 5a).

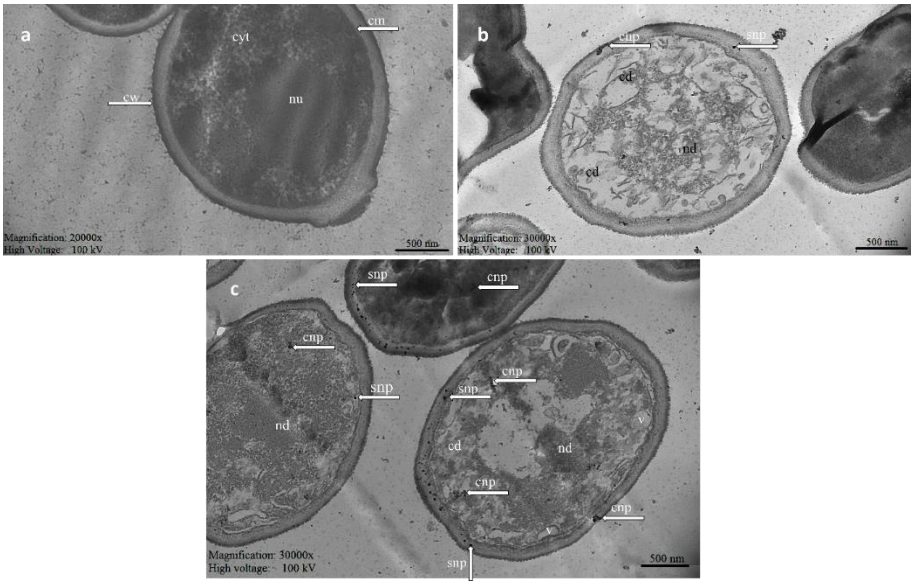

Figure 5:TEM micrographs obtained from *C. albicans* ATCC 14053 cells grown in the absence and presence of P-AgNPs at a concentration of 1/2 MIC. (a) Untreated *C. albicans* cells show preserved ultrastructure: Characteristic ovoid morphology, intact cell wall (cw), continous cytoplasm membrane (cm), central located nucleus (nu) and electron dense cytoplasm (cyt). (b, c) In cells treated with AgNPs; cytoplasmic (cd) and nuclear (nd) dissolutions, vacuoles (v), localized . clustered nanoparticles (cnp) both in the inner side of cell walls and cytoplasm were observed. Nanoparticles were shown as both single and clusters (Scale bar, 500 nm).

When we examine the cells exposed to 1/2 MIC P-AgNPs, the nanoparticles were found to be more aggregated. As shown in the Fig. 5 b and c, NPs are mostly located between the cell wall and the membrane and they are also found to be in the cytoplasm. Cytoplasma dissolution was seen in some cells.

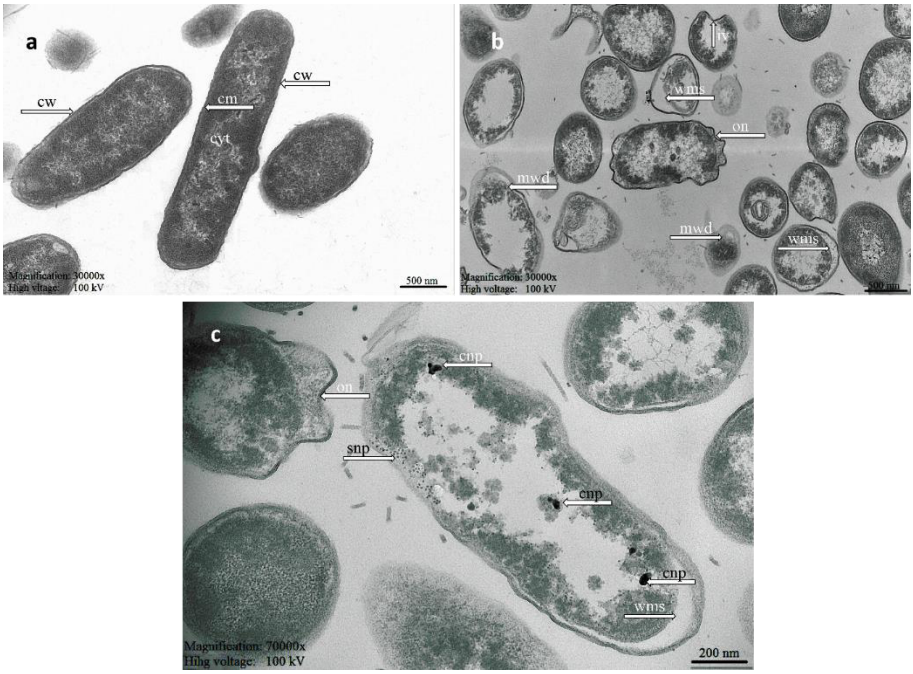

Figure: 6 TEM micrographs obtained from *E.coli* ATCC 25922 cells grown in the absence and presence of P-AgNPs at a concentration of 1/2 MIC. (a) Untreated control cells showed well preserved morphology: Characteristic cylindrical morphology, intact cell wall (cw) and cytoplasmic membrane structure (cm) and homogeneous cytoplasm (cyt). (b,c) In cells treated with AgNPs; Cell wall and membrane separations (wms), cell wall ondulations (on) and invaginations (iv), membrane-wall dissolutions (mwd), in general, single nanoparticles (snp) were distrubuted at the peripheral region of the cells. Clustered nanoparticles (cnp) were also shown mostly in the inner side of cells (Scale bar, A-B=500nm, C=200 nm).

Control *E. coli* cells showed a normal morphology, well preserved cell membrane and wall structure (Fig. 6 a). In the cells exposed to AgNPs, NPs were found to be densely localized within the cell, and they appeared to be more contrasted in some regions. Cell wall and membrane separations are a prominent feature. In many cells, ondulation, swelling and invaginations were determined in the cell wall structure. Some cells showed membrane and wall dissolutions. Generally, nanoparticles localized in the cell wall and the cytoplasm content was highly damaged in some cells (Fig. 6 b, c).

5. DISCUSSION

One of the critical steps in the field of nanotechnology is the development of reliable and environmentally friendly processes in the metal nanoparticle synthesis. These nanoparticles are extensively studied for their catalytic activity, magnetic, electronic and optical properties or antimicrobial properties. Metal nanoparticles such as gold, silver, platinum or palladium are synthesized in a variety of ways, including UV irradiation, aerosol technologies, lithography or photochemical reduction techniques. However, these techniques usually include toxic chemicals, expensive and environmentally harmful practices. Toxicity problems can be caused by various organic solvents, reducing agents and stabilizers. Some nanoparticles may also be toxic due to their surface chemistry, size, shape and composition and therefore are not used in clinical and biomedical applications. All these factors are becoming controllable through biologically mediated production such as green synthesis (Shah, Fawcett, Sharma, Tripathy,

& Poinern, 2015; Velusamy, Kumar, Jeyanthi, Das, & Pachaiappan, 2016). Microorganisms are also widely used in this area and the ability of different microorganisms to synthesize metal nanoparticles is different and detailed studies are needed (Babu, Sridhar, & Gunasekaran, 2011; Qayyum, Oves, & Khan, 2017).

In our study, the formation of stable silver nanoparticles by reducing silver nitrate solution was carried out with a green synthesis method and a low cost approach. Characterization of the obtained nanoparticles was performed by UV-Vis, TEM, TEM-EDX, XRD, FTIR, ICP-MS techniques. Our results showed that the reduction of silver nitrate to silver nanoparticles was achieved with high stability and no impurities. The size of these particles is about 30-40 nm in average. In previous studies, silver nanoparticles have been synthesized using organisms such as plant extract, algae and fungi, and their results support our study data (Azizi, Namvar, Mahdavi, Ahmad, & Mohamad, 2013; Govindaraju, Kiruthiga, Kumar, & Singaravelu, 2009; Tippayawat, Phromviyo, Boueroy, & Chompoosor, 2016).

Niknejad et al. studied silver nanoparticle synthesis using *Saccharomyces cerevisiae* yeast and they reported that the average size of nanoparticles was below 50 nm (Niknejad, Nabili, Ghazvini, & Moazeni, 2015). The results of the researchers are similar to our results. In the same study, MIC values of silver nanoparticles were determined as 2-4 µg / ml against fluconazole resistant and susceptible *C. albicans* isolates, while MIC values obtained in our study were

found to be lower. The antimicrobial effects of silver nanoparticles vary depending on their particle morphology and surface characteristics. Therefore, in the synthesis of nanoparticles suitable for biological applications, the size and shape of the surface chemistry is of great importance.

The metal nanoparticles obtained with biosynthesis are shown to be ideal candidates for medical applications and antimicrobial / antibiofilm studies since their properties of biocompatible and naturally stable. Several studies have reported strong antimicrobial activity of silver nanoparticles against drug-resistant clinical isolates. Although the mechanism of action of silver on cells is not yet fully understood, damage on membrane is frequently reported. In particular, it has been reported that silver interacts with proteins in the cell wall and disrupts membrane permeability. So porous formation occur on membrane and cell may die. On the other hand, the reduction in particle size facilitates the passage through the membrane and increases the inhibitory effect (Souza et al., 2018). The thickness of the cell wall structure of the microorganism also affects the passage of the active substance. Several studies showed that smaller size particles have a larger surface area and thus a better interaction with the microorganism, thereby increasing the bactericidal effect. Morones et al. reported that silver nanoparticles were present not only in the cell membrane but also inside bacteri (Morones et al., 2005). As reported by Suriya et al., silver nanoparticles can interact with phosphorous and sulfur-containing components such as DNA and penetrate into the bacterium or fungal cell (Suriya et al., 2012). In also our study,

nanoparticles were observed both on the cell surface and in the cytoplasm and the cell damage was noted.

AgNPs have been reported to be highly effective against most drug-resistant clinical isolates. In also our study, P-AgNPs were found to be very effective with low MIC values. (0.54 µg / ml for *C.albicans*) and (0.27 µg / ml for *E. coli*). When the effects of P-AgNPs on planktonic cells are examined ultrastructurally, although damaging effects on both microorganisms are observed, some differences are also observed. Nanoparticles in *Candida* cells were mostly localized on walls and membranes, whereas in *E. coli* cells, nanoparticles were observed both in the wall structure and in the cytoplasm. Cell wall damage in *E. coli* was higher and further deterioration was observed in membrane and cytoplasm contents. In contrast *Candida* cell wall and membrane structure was integrate, generally. These difference may be caused by different cell wall and membrane structures of *C. albicans* and *E.coli.* Considering both prebiofilm and postbiofilm effect studies, P-AgNPs showed a high biofilm reducing effect. In prebiofilm effect studies, AgNPs was used at lower concentrations (0.54 µg / ml) and a strong reducing effect was observed (about 79%). In the postbiofilm effect study, biofilm reduction (approximately 73.5%) was achieved with a higher active substance concentration (2.14 µg / ml), but this rate can still be considered as an effective reduction.

In our study, *P. membranifaciens* extract was successfully applied in silver nanoparticle synthesis by eco-friendly, inexpensive and easily. The natural extracts contain many biological active ingredients which

may be responsible for both the reduction of silver ions and the stabilization of the obtained nanoparticles. *P. membranifaciens* produces some chemical compounds such as organic acids, acetaldehyde, ethyl acetate or isoamyl acetate during its oxidative metabolism. In the chemical synthesis of nanoparticles, organic acids are used as reducing agents (Dağlıoğlu & Öztürk, 2019; Iravani, Korbekandi, Mirmohammadi, & Zolfaghari, 2014). It is difficult to fully define the chemical components of *Pichia*, however, active substances secreted by fungi play an important role in reaction as reducing or capping agents (Li et al., 2012). According to another report, it is emphasized that this reduction can be due to enzymatic activities and may be due to the transfer of NADH and NADH-dependent enzymes that act as electron carriers (Ahmad, Mukherjee, et al., 2003; Ahmad, Senapati, Khan, Kumar, & Sastry, 2003; Ingle, Gade, Pierrat, Sonnichsen, & Rai, 2008).

CONCLUSION

With the increase of antibiotic resistant strains, failures in the treatment of infectious diseases are increased and this situation considerable threats public health. Antibiotics are insufficient against multi-resistant microorganisms and biofilm associated infections. Microorganisms can be resistance to antimicrobials with a single or multiple mechanisms. Therefore, it is important to investigate new approaches and new components in the management against resistance. In this study, P-AgNPs were successfully synthesized with the green synthesis method and using *P. membranifaciens* extract. Our

results revealed that AgNPs biosynthesized from *P. membranifaciens* extract have strong antimicrobial and antibiofilm effects against Gram negative bacteria *E. coli* and *C. albicans* yeast. P-AgNPs were very effective even at very low concentrations. Such low concentrations may not lead to cellular and genomic toxicity for advanced organisms, but this should be investigated in detail. Our TEM analyses showed that this effect is differentiated depend on microorganism type. The strong antimicrobial effect of synthesized silver nanoparticles on bacteria and yeasts suggests that these nanoparticles may have potential for use in surgery rooms or medical waste sterilization. P-AgNPs obtained by green synthesis can be a promising candidate in the production of various pharmaceutical and biomedical products and medical applications however detailed studies are needed to confirm our findings.

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CHAPTER 3

FREE RADICALS AND THE MECHANISMS CAUSING THEIR FORMATION

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INTRODUCTION

Although oxygen is essential for the life of all aerobic organisms, its toxicity is a significant paradox. Oxygen-centered free radicals are based on oxygen and their metabolites are called reactive oxygen species (ROS). Certain ROSs produced during normal metabolism have the potential to cause significant damage to the human body. ROSs, which are mostly made up of free radicals, are forms of oxygen with higher chemical reactivity compared to normal oxygen molecules. In short, free radicals are molecules containing unpaired electrons in their outer atomic orbitals. Especially when they exceed the antioxidant capacity, these molecules can cause significant damage to DNA, proteins, carbohydrates and lipids in tissues. Particularly the oxidative destruction of poly unsaturated fatty acids, also known as lipid peroxidation, has considerably harmful effects. Considering that free radicals are oxygen-centered, an increase occurs in various reactive oxygen species such as superoxide, hydrogen peroxide and hydroxyl radicals due to the increase in both oxygen use and electron leak from the mitochondrial electron transport chain. While free radicals can come from outside of the body, they may also form as a natural result of the human metabolism. There are many studies showing that free radicals cause various health problems including cancer and aging due to their detrimental effects.

1. FREE RADICALS AND MECHANISM

Atoms or molecules containing unpaired electrons in their outer atomic orbitals are categorized as radicals. The dot shown above the atom represents the unshared electron. These unstable structures aim to become stable as soon as possible. These compounds may form during the functioning of normal metabolic pathways, or with the effect of various external factors. These structures, which are very short-lived but also very active due to the imbalance in their composition, demonstrate an ability to interact with all cell compounds (Kehre and Smith, 1994; Uysal, 1998). Free radicals are high-energy, unstable molecules containing one or more unpaired electrons in their outer atomic orbitals. These unpaired electrons provide great reactivity to free radicals and cause them to damage many biological materials including proteins, lipids, DNA and nucleotide coenzymes. Although oxygen is essential for human life, certain reactive oxygen species (ROSs) produced during normal metabolism have the potential to cause significant damage to the human body (Diplock, 1998). ROSs, which are mostly made up of free radicals, are forms of oxygen with higher chemical reactivity compared to normal oxygen molecules (Nawar, 1996).

Primarily, free radicals possess different chemical compositions including hydroxyl, superoxide, nitric oxide and lipid peroxide radicals that are released with the reduction of molecular oxygen in normal metabolism steps (Cetin, 2011). Nitric oxide radical ($NO^{\cdot}$), superoxide ion radical ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), alkoxyl radical

($RO^{\cdot}$), nitrogen dioxide ($NO_2^{\cdot}$), peroxyl ($ROO^{\cdot}$), lipid peroxyl ($LOO^{\cdot}$), ozone (O_3), hypochlorous acid (HOCl), nitrous acid (HNO_2), peroxynitrite ($ONOO^-$), dinitro trioxide (N_2O_3), lipid peroxide (LOOH), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) can be listed as some examples of free radicals (Kurtdede, 2018). Among these, the highly reactive HO- attacks lipids, proteins, polysaccharides, DNA and other macromolecules. Oxidized molecules isolate electrons from other molecules, causing a chain reaction. If this reaction is not brought under control, it can cause significant tissue damage that can affect membrane permeability, enzyme function, and even muscle contraction (Miller, 1993).

1.1. Main Mechanisms Causing Free Radical

1.1.1. Autooxidation

Autooxidation is a typical free radical chain reaction catalyzed by atmospheric oxygen (Nawar, 1996). The reaction of free radicals with oxygen occurs very fast, and many mechanisms have been specified for the initiation of these reactions. PUFAs and phospholipids are particularly prone to autooxidation. Hydroperoxides (ROOH) are considered to be the first-formed products during autooxidation (Porter, 1984). Three main mechanisms are proposed for hydroperoxides to initiate a chain reaction (Foote, 1985).

I. Hydroperoxide may react with an initiator radical ($X^{\cdot}$) originating from certain sources to form a peroxy radical ($ROO^{\cdot}$) that can get involved in the chain reaction.

$$ROOH + X\cdot \longrightarrow ROO^{\cdot} + XH$$

II. Hydroperoxide may be reduced by a metal ion or a different reducing agent to form the alkoxy ($RO\cdot$) radical (or, less likely, the hydroxy ($^{\cdot}OH$) radical).

$$ROOH \xrightarrow{[H]} RO\cdot + OH^{-} \text{ (veya } RO^{-} + {}^{\cdot}OH \text{)}$$

III. Albeit less significant compared to the other mechanisms, the O-O bond in hydroperoxide may dissociate and convert into alkoxy and hydroxy radicals at ambient temperature rather than higher temperatures.

$$ROOH \longrightarrow RO^{\cdot} + {}^{\cdot}OH$$

Lipid oxidation consists of three phases: initiation, propagation and termination. In the initiation phase, a lipid radical ($L^{\cdot}$) is produced through the transfer of H-atom as a result of the reaction between an initiator radical ($X\cdot$) and a fatty acid (LH) substrate. In the propagation phase, a peroxy radical ($LOO\cdot$) is formed with the addition of oxygen to the $L^{\cdot}$ radical produced in the first phase. This peroxy radical can combine with a hydrogen atom from another fatty acid (L'H) molecule to form fatty acid hydroperoxides and a new lipid radical. In the termination phase, the radicals produced react with each other and turn into stable decay products such as non-radical ester, ether, aldehyde, ketone and alcohol (Porter, 1984).

1.1.2. Impact of Transition Metal Ions

Transition metal ions such as iron and copper also serve as strong oxidative catalysts that form free radicals in the living system. Iron is a more effective metal in encouraging oxidative reactions (Halliwell and Gutteridge, 1984).

Free forms of iron, which play an important role in oxygen transport, ATP production, and DNA and chlorophyll synthesis in biological systems, can cause toxicity in living cells. The active oxygen species formed as a result of this toxicity are able to encourage lipid oxidation or attack DNA molecules (Miller, 1996). The "Haber-Weiss reaction", which forms harmful hydroxyl (·OH) radicals, occurs when the superoxide anion ($^{\cdot}O_2^-$) reacts with H_2O catalyzed by Fe^{+2} (Duthie et al., 1989).

$$\cdot O_2^- + H_2O \xrightarrow{Fe^{+2}} O_2 + OH^- + \cdot OH$$

(Haber-Weiss reaction)

$$\cdot OH + RH \longrightarrow R\cdot + H_2O \text{ } \textit{(damage)}$$

The Fe ion also catalyzes Fenton-type reactions where hydroperoxides are transformed into harmful hydroxyl ($^{\cdot}OH$) radicals. The highly reactive hydroxyl radical rapidly generates lipid radicals and initiates lipid peroxidation chain reactions (Miller, 1996).

$$Fe^{+2} + H_2O_2 \longrightarrow Fe^{+3} + OH^- + {}^{\cdot}OH$$

(Fenton Reaction)

1.1.3. Photooxidation

Photochemical pathways are of great importance for the formation of peroxides, which initiate oxidations. Direct absorption of light by a molecule may cause electron transfer processes that can produce superoxide anions. Photosensitized processes are likely more significant than direct photochemical reactions. In these types of direct oxidations, a molecule called sensitizer (Sens) absorbs the light and causes the oxidation of certain other species. In these reactions, the sensitizer itself is usually not consumed, and the molecule absorbing the light turns into an active form (Sens*) (Foote, 1985).

$$\text{Sens} \xrightarrow{h\upsilon} \text{Sens}^*$$

Erythrosine, which is a synthetic dye, and pigments such as chlorophyll-a, pheophytin-a, hematoporphyrin, hemoglobin and myoglobin are among the photosensitizers generating singlet oxygen (Nawar, 1996). Photooxidation reactions are categorized into two as Type 1 and Type 2. In Type 1 reactions, the activated sensitizer produces radicals by reacting with the substrate to transfer hydrogen atoms or donate electrons. These radicals react with oxygen and generate oxygenated products.

$$\text{Sens}^* + \text{Subs} \longrightarrow \text{Radicals} \xrightarrow{O_2} \text{Products (Type 1)}$$

In Type 2 reactions, the active sensitizer generates singlet oxygen by directly reacting with O_2 This singlet reacts with the substrate to generate oxygenated products.

$$\text{Sens}^* + O_2 \longrightarrow \text{Sens} + {}^1O_2 \xrightarrow{Subs} \text{Subs-}O_2\text{(Type 2)}$$

1.1.4. Enzymatic Oxidations

Reactive oxygen species are also generated as a result of the activity of enzymes such as lipoxygenase, cyclooxygenase, xanthine oxidase, myeloperoxidase and cytochrome P_{450} in the body (Meydani, 2001).

Xanthine Oxidase (XOD): It is among the main enzymatic sources producing ROS in the living system. Although XOD is a dehydrogenase enzyme that executes electron transfer to NAD^+ while oxidizing hypoxanthine (an intermediate compound in the purine catabolism) first to xanthine and then to uric acid, it transforms into an oxidase enzyme that oxidizes thiol groups and causes proteolysis under certain stress conditions. As a result of the activity of XOD, the superoxide anion and hydroperoxide radicals are formed (Lavelli, 2000).

NADPH oxidase: NADPH oxidase, which is another enzyme that creates free radicals, is also found in the plasma membrane of neutrophils. Approximately 1-4% of the oxygen uptake of the mitochondria is used for superoxide anion production, and approximately 20% of the superoxide anion produced is given to cells. NADPH oxidase, which gains activity with the increase in O_2 uptake in the phagocyte cells containing macrophages and monocytes,

converts this oxygen into the superoxide anion and increases its amount in extracellular fluids (Duthie et al., 1989).

Neutrophil myeloperoxidase (MPO): The neutrophilic myeloperoxidase enzyme, which catalyzes the production of hypochlorous acid through the oxidation of chloride ions by hydrogen peroxide, is another significant source of oxidants in the living system. The toxicity of this reaction contributes to the killing of bacteria in the defense system. On the other hand, the emerging hypochlorous acid also inactivates α1-antiproteinase and causes inflammation by damaging healthy human tissue (Lavelli, 2000).

1.1.5. Halogenated Hydrocarbons

Other elements that lead to the formation of free radicals are: the nitrous oxides known as air pollutants and toxic halogenated hydrocarbons found in contaminated drinking water. It is reported that hydrocarbons such as carbon tetrachloride (CCl_4) and bromotrichloromethane ($CBrCl_3$) are effective in initiating oxidative damage in biological systems. Highly reactive species such as trichloromethyl and trichloromethyl peroxyl radicals are produced during the metabolism of CCl_4 as a result of the rapid reaction of the cytochrome P_{450} monooxygenase enzyme system with various aminoacids and unsaturated fats. Protein denaturation and lipid peroxidation occur as a result of this (Chen and Tappel, 1996).

1.2. Free Radicals and Lipid Peroxidation

Biomembranes and intracellular organelles are sensitive to the attacks of oxidants due to the poly unsaturated fatty acids (PUFA) in the membrane phospholipids. Malondialdehyde (MDA), which is one of the most significant products of lipid peroxidation, affects the exchange of ions from cell membranes, causes the compounds in the membrane to be cross-linked and leads to negative outcomes such as ion permeability and changed enzyme activity. MDA may react with the nitrogenous bases in DNA and therefore has genotoxic and carcinogenic effects on mutagenic cell cultures (Mercan, 2004).

Free radicals initiate lipid peroxidation by isolating a hydrogen atom from the alpha methylene groups of the PUFA chain within the membrane structure. In biological systems, this free radical is considered to be the superoxide anion and the hydroxyl radical. On the other hand, the hydroxyl radical ($OH^{\bullet}$) is regarded as the main radical in the excitation of lipid peroxidation. This radical is formed with the superoxide radical or H_2O_2 under the catalytic effect of iron (Kehre and Smith, 1994).

The fact that it is very difficult to directly measure free radicals due to their high reactivity and that their primary targets are membrane phospholipids, lipid peroxidation is among the most significant parameters in the determination of oxidative damage. Lipid peroxidation induced by free radicals continues until membrane phospholipids are completely oxidized, and the ionic balance of the

cell is disrupted due to increased membrane permeability. This situation causes the surface receptors attached to the membrane mediating the entry of biochemical molecules and hormones to lose their activation (Avci, 2008).

1.3. The Relationship of Free Radicals with Oxidative Stress

Oxidative stress is triggered by increased free radical formation. In basic terms, oxidative stress is defined as an imbalance between prooxidants and antioxidants in the biological system in favor of prooxidants (Berk et al., 2008). Although cells are able to tolerate mild oxidative stress on their own, they generally activate antioxidant enzyme systems. However, in cases where intracellular defense systems are inadequate, the balance between ROS and antioxidants is disrupted. Therefore, cellular macromolecules such as DNA, proteins, carbohydrates and lipids, which are sensitive to oxidant damage, are damaged (Zadák et al., 2009; Wildburger, 2009; Berger, 2005).

Free radicals are in constant production with normal metabolical processes. However, their production rate increases under certain conditions of inflammation or disease. Ordinarily, the body is protected against ROS and their toxic products by a wide range of defense mechanisms. The imbalance between ROS and their safe elimination may initiate oxidative chain reactions (Altıner, 2018).

1.4. The Effect of Free Radicals on Carcinogenesis

Free radicals constitute the first step in oxidative stress-related carcinogenesis. The $OH^{\cdot}$ radical causes the formation of modified purine and pyrimidine bases through DNA damage. It is estimated that 106 bases of each DNA molecule in normal human cells are subjected to oxidative impact each day. Endogenous DNA damage formed by free radicals causes age-related carcinogenesis (Willcox et al., 2004). If the DNA repair mechanism does not regenerate DNA immediately, the erroneous base pair during replication will result in mutation. This mechanism explains the increased prevalence rate of cancer in individuals exposed to oxidative stress (Nordberg and Arner, 2001).

Radiotherapy and certain chemotherapeutic agents generate free radicals and cause cell death as well (Prasad et al., 2002; Simone et al., 2007). Previous studies have shown that cytostatic agents in various categories caused the formation of free radicals both *in vivo* and *in vitro* (Crohns et al., 2009; Simone et al., 2007; White et al., 2006). In previous studies, it has also been stated that chemotherapy increased the amount of lipid peroxidation products in cancer patients (Weijl et al., 1997, Yildiz et al., 2018).

Free radicals that are generated in excess amounts or cannot be neutralized naturally despite being produced in normal amounts cause lipid peroxidation, loss of enzyme activity in proteins due to catabolism, and mutagenesis and carcinogenesis as a result of DNA

catabolism (Sivanandham, 2011; Ozcan, 2015). Mutation caused by ROSs cause the formation and progression of cancer, and it may also cause the spread and proliferation of oxidative stress mutated cell clones, and cell death. Previous studies have shown that ROSs accelerated cancer progression while also turning benign tumors into malign tumors (Okada, 2002).

1.5. Free Radicals and Antioxidants

Antioxidants are defined as substances that have the ability to capture and stabilize free radicals and prevent the oxidations caused by them (Elliot, 1999). As long as the formation rate and inactivation rate of free radicals are in balance, the organism does not get affected by these compounds. On the other hand, if the defense mechanism decreases or the generation rate of these malign compounds exceeds the defensive force of the system, this balance is disrupted and negative effects related to free radicals may emerge (Halliwel, 1996; Nakazawa, 1996). Various natural defense systems in the body keep free radicals under control against the harmful effects of ROSs. These systems complement each other as they are influential over different cells and different free radicals (Diplock, 1998).

Endogenous [such as Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione S-Transferase (GST), catalase (CAT), melatonin, ceruloplasmin, transferrin and myoglobin] and exogenous [such as α-tocopherol (vitamin E), β-carotene, ascorbic acid (vitamin C) and folic acid (folate)] antioxidants serve to prevent

the catabolic effects of ROS on healthy cells. The antioxidants affect ROSs, they intercept them or turn them into new, weaker molecules. Similarly, they can also interact with ROSs to reduce or inactivate their activity by transferring a hydrogen.

Agents that demonstrate their cytotoxic effects by generating free radicals (such as alkylating agents, radiation) constitute the most common point of discussion on the subject of antioxidant supplementation in combination with chemotherapy. It is thought that antioxidants may scavenge free radicals and reduce their impact. Many publications have reported that antioxidants do not reduce the effectiveness of chemotherapy (D'Andrea, 2005; Block et al. 2007; Simon et al., 2007; Yildiz et al., 2018, 2019). Additionally, it has been argued that as antioxidants reduce chemotherapy-induced toxicity, higher and more effective doses can be used (Christen et al., 2000; Block et al. 2007; Yildiz et al., 2018, 2019).

CONCLUSION

In recent years, the significance of the role played by free radicals in biochemical reactions has been acknowledged, and an increase was observed in the types and amounts of free radicals in the organism during disease states. The significance of this subject is increased by the fact that free radicals were reported to cause various diseases including cancer, aging, diabetes mellitus, ischemia-reperfusion injury and muscular diseases in line with the damage (as severe as cell death) they cause in macromolecules such as DNA, RNA, proteins and

lipids. Particularly the antineoplastic agents used in cancer chemotherapy accelerate the formation of free radicals in the biological system. While free radicals cause cell death, the use of antioxidants against free radicals is supported by the fact that antioxidants neutralize free radicals and oxidative reactions mediated by free radicals. Thus, it is of great importance to support the endogenous defense mechanisms of the body, which is unable to cope with the damage caused by free radicals, with dietary antioxidant nutrients.

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CHAPTER 4

EXAMINING THE DEVELOPMENT OF HEARING IMPAIRED STUDENTS WITH NATURE EDUCATION

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INTRODUCTION

Disabled individuals are defined as the person who lost their physical, mental and / or spiritual characteristics when they were born or as a result of an accident or illness that occurred later. As a result of these congenital or acquired adversities, the quality of life of disabled individuals is affected and disabled individuals face some problems in their daily lives. Together with these problems, the psychological negativities brought about by the problems make the lives of disabled people more difficult.

5.4% of the households in Turkey have a disabled individual. 211,000 of these disabled citizens are those with hearing impairment. The hearing impaired, whose numbers are close to two and a half million according to the United Nations report, are the least striking disability group since they have no visible defects. Decrease in the individual's hearing sensitivity due to hearing loss, congenital or acquired problems; Hearing impairment, on the other hand, is the impairment caused by the decrease in hearing sensitivity in the individual (Tüfekçioğlu, 2003).

As a result of hearing loss, the baby is unable to perceive speech sounds and has difficulties in decoding the codes coming from the receiver. Due to hearing insufficiency, perception of sounds and understanding of stimuli cannot be realized. As a result of this difficulty, verbal communication is interrupted. Therefore, the fact that hearing-impaired children cannot fully acquire the spoken and literacy dimension of their mother tongue has caused hearing-impaired

individuals to be called deaf and mute for many years. " In Turkey, it is estimated that nearly 3 million hearing impaired individuals live (Akmeşe, 2016).

Considering this number, it is obvious that a significant segment of the society has important rights in education, health, work and other legal fields. However, problems have arisen due to the low visibility of the hearing impaired in our country, the inadequacy of studies in the field of hearing impairment and, as a natural consequence of this, the inability to reflect appropriate policies on public spaces. Hearing-impaired individuals have difficulties in perceiving the surrounding sounds due to their hearing loss, and consequently face problems in performing their daily activities. Communication is at the top of these problems. Communication methods used by hearing-impaired individuals are established by signing, listening, by signing and listening, by lip reading, by writing and by means of an interpreter (Gürboğa and Kargın, 2003).

The main factors affecting the communication skills of hearing impaired individuals are the rate of hearing loss, the age at which the hearing loss occurs, the age at which the hearing loss occurs, the age of using hearing aids, the age of starting education, early education (Elfenbein et al., 1998), the language used in the family, and the socio-economic status of the family.

Nature has formed the material of many studies for the physical, mental and social development of people. It was used for the treatment of soldiers who returned home after the war, after being injured both

mentally and physically due to the calming and pain relieving effect of nature on individuals (Söderback et al., 2004). In addition, patients can be rehabilitated with the use of remedial garden designs accessible to disabled individuals in hospital gardens (Akın, 2006). In addition, nature-themed landscape arrangements make people feel better psychologically (Kaplan & Kaplan, 1989).

Due to the positive characteristics of nature on people, nature activities are also preferred in studies to be carried out with disabled individuals. In many studies that are mentioned here and also cannot be written here, the positive effect of nature on humans is revealed. In recent years, nature-themed trainings have emerged by combining this constructive power of nature with education. With these trainings, it is ensured that disabled individuals can better understand the events that take place here by using and applying nature as a laboratory.

They contribute to the physical and sensory development of persons with disabilities, support the psychological sense of trust and achievement, and they are recognized as a productive member of society with the trainings to be held in nature. Therefore, it is aimed to increase the personal, social and psychological development of the disabled people with the trainings to be held in nature.

With these trainings, at the end of an activity week integrated with nature, individuals with disabilities can gain a different perspective from the life they live within four walls. Thus, these individuals who cannot integrate with any natural area due to their disability, especially staying in nursing homes and rehabilitation centers, can be

provided with education in nature, especially in national parks and protected natural areas, with educational activities (such as dramas, games, competitions, basic nature trainings).

Purpose of the study; To be able to develop an interdisciplinary and holistic educational program in environmental education, including social, scientific, technological and cultural aspects, using the real language of nature, and then to reveal the effect of this program on the target audience. For this purpose, primary school 5th, 6th, 7th and 8th grades hearing impaired students are trained by academic staff who are experts in their fields and experienced in nature education for 7 days in Yazılı Canyon Nature Park in Isparta, Kasnak Oak Nature Reserve, Kovada Lake National Park, Eğirdir Lake, Kubad-ı Abad Seljuk Palace and Eşrefoğlu Mosque. In addition, training was provided in the Beyşehir Lake National Park, Zindan and Pınargözü Caves, the Trout Production Farm, Çaydere and İslibucak Forests and Atabey Horse Farm (TUBITAK, 2018). There are scientific, social, artistic and sportive activities in nature education (Figure 1,2,3).

Figure 1. Meeting drama

Figure 2. Horse riding

Figure 3. Artificial climbing

MATERIALS AND METHODS

National parks are among the protected areas in our country due to their natural character; It is one of the suitable places where nature education can be given.

It is observed that the masses in need of nature education learn from encyclopedias or books and movies on Nature in our region as well as in the country. These sources of information are undoubtedly

beneficial for society. For example, nature documentaries that attract people of all ages and all walks of life contribute a lot to the promotion of Nature. However, none of these can replace the place of nature and the introduction of information directly on the living object.

The natural and cultural values offered by the protected area and its environment, the teaching of natural resources, ecosystem, human-nature relations and the language of nature through the activities carried out within the framework of nature education projects started by TÜBİTAK in 1999, with the contributions of university faculty members and other experts. It is seen that it is aimed to spread the language of nature by young academicians, teachers and students who are informed on their subjects and to spread what they learn voluntarily and to be understood by the masses.

Natural habitats are outdoor classrooms where learning happens the fastest. The damage to natural life, its causes and consequences, is an important meeting point where nature education intersects with environmental education, and nature education provides our children with the awareness of the environmental problems they live and will experience, develop strategies to solve these problems, and develop themselves in personal and social criteria.

In Turkey, the newly developed and implemented in all elementary schools in the 4th and 5th grade Science and Technology Curriculum is provided to give ample space to environmental issues. However, in the handling of these issues, it is seen that there are still areas and gaps

open for improvement during the implementation phase. One of these gaps appears in field trips and practical activities to be held on these trips. However, field trips have the feature of accelerating the development of the student in many ways at the same time by creating an active learning environment. Therefore, as in the whole society, there is a need for the hearing impaired students to gain a formation in this direction. Nature education is one of the alternatives to meet this need.

Similar educational studies started in the first half of the 20th century in developed countries such as the United States of America, Canada and Germany and have become very common today. In the beginning, this educational activity style, which contributed to the development of domestic tourism, which cannot be ignored, formed the main style of ecotourism, which started to develop rapidly in the 1980s. The number of those who visit national parks in the USA and document these trips with the "Green Passport" application has become millions.

For this purpose, hearing impaired students in primary school 5, 6, 7 and 8th grades were selected as participants. Participants were choosen an equal number of boys and girls from three different schools in general a total of 48 hearing impaired students and 12 accompanying teachers working in these schools are given the nature of training a total of 60 people. Thanks to the visual mini-tests performed for the participants at the beginning and end of the training period, it was revealed that the participants were able to recognize the changes before and after the training about various plants, animals and

geological formations. In addition, with the questionnaires conducted for the participants at the beginning and at the end of the training period, it was ensured that the change in the participants' perspectives on science and nature were observed, whether their awareness of nature increased, and their sensitivity to environmental problems were measured. In the survey and visual mini-test evaluations, visual mini-tests and questionnaire forms related to the beginning and after the training program were used and these evaluations were analyzed with the help of the statistical package program called SPSS 20.0. Before the beginning of the training, the participant students were asked to draw a picture of nature and bring it with them, and at the end of the training, the participants were asked to draw a picture of nature again by providing the appropriate time and place. By examining the differences between the two pictures, it was determined whether there was an increase in the knowledge and awareness levels of the participants. In addition, video interviews were conducted with the participants.

In order to determine the effect of the training, 33 questions pre-test and post-test were applied to the participants. Test questions in general;

1. Personal information about the students participating in the training program (participants' age, gender, etc.),
2. What are the factors that affect the decision to participate in the education program (such as the idea of seeing new places, the

program's offering of leisure time, or having information about wild animals and fauna, etc.),

3. Participants' opinions about the application area before and after the training program (such as the rate of seeing the activity areas before, the priority of the activity areas, their opinions after the training program about the activity areas),

4. Level of knowledge of the participants measured before and after the training program (To measure the knowledge level of the participants before and after the training program on subjects such as protected area, nature park, national park, forest, wildlife, ecological observation, visual quality, etc.),

5. The level of satisfaction of the participants with the training program (the evaluation of the content of the training program from the participants in the training program, the adequacy of the trainers, whether enough practice has been done, whether enough visual material is presented, whether enough games are included or not).

RESULTS

Wilcoxon Rank Statistics, one of the nonparametric tests used for 2 dependent variables, was used to determine whether there was a statistically significant difference between the pretest and posttest.

These tests were carried out for a total of 18 subjects and these subjects are as follows: Nature, Wetlands, Insects and Fungi, Sky and space, Forests, Natural Protected Areas, Natural Park and National

Parks, Wild animals, Landscape and visual values, Geology, Plants, Ecology, Camping technique, Marbling, Making ornaments, T-shirt painting, Nature photography, Horse riding.

In order to determine the effect of nature education on the participants, tests were conducted for a total of 18 subjects, and according to the test results, the participants improved in 12 subjects at the end of the training. These subjects are Nature, Insects and Fungi, Sky and space, Forests, Wild animals, Landscape and visual values, Camping technique, Marbling, Making ornaments, T-shirt painting, Nature photography, Horse riding (Table 1).

Table 1: Wilcoxon Rank Statistics

	15s - 15	16s - 16	17s - 17	18s - 18	19s - 19	20s - 20	21s - 21	22s - 22	23s - 23	24s - 24	25s - 25	26s - 26
Z	-3,788[a]	-4,441[a]	-4,194[a]	-4,320[a]	-3,290[a]	-3,634[a]	-4,087[a]	-3,857[a]	-2,979[a]	-3,599[a]	-3,764[a]	-4,358[a]
Asymp. Sig. (2-tailed)	,000	,000	,000	,000	,001	,000	,000	,000	,003	,000	,000	,000

a. Based on negative ranks.

b. Wilcoxon Signed Ranks Test

CONCLUSION

Purpose of the study; To be able to develop an interdisciplinary and holistic educational program in environmental education, including social, scientific, technological and cultural aspects, using the real language of nature, and then to reveal the effect of this program on the target audience. For this purpose, primary school 5th, 6th, 7th and 8th grades hearing impaired students are trained by academic staff who

are experts in their fields and experienced in nature education for 7 days in Isparta.

In order to determine the effect of nature education on the participants, tests were conducted for a total of 18 subjects, and according to the test results, the participants improved in 12 subjects at the end of the training. According to the results, it was understood that the participants were more successful in social and sports activities. The desired success was not achieved in some scientific activities. Considering the subjects that the participants did not improve, it is seen that there are subjects such as Natural Protected Areas, Natural Park and National Parks, Wetlands, Geology, Plants, Ecology with technical terms. The main reason for this is that experienced by the hearing impaired in language development are the fact that other people do not know sign language and their vocabulary is limited (Sarıkaya and Börekçi, 2016).

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CHAPTER 5

THE RELATIONSHIP BETWEEN THE PRODUCTIVITY OF RED PINE AND SOME SITE FACTORS: THE EXAMPLE OF DİNAR

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INTRODUCTION

Turkey about 13 million hectares of productive forest is about 10 million hectares of skilled defective (OGM, 2020). These degraded areas can be brought into forestry by determining the appropriate species that can be used in these areas. Turkish red pine rapid growth of industry in Turkey with the use of wood as lumber and attract the beginning of the preferred species. In addition to this, Turkish red pine is preferred in afforestation works because it serves functions such as carbon capture, erosion prevention and rehabilitation, recreation and wildlife.

In afforestation studies, it is necessary to identify potential places with high site efficiency, and prioritize existing areas with high productivity in natural regeneration studies. The determination of potential places where Turkish red pine can be productive can be done by ecological studies on the productivity of the species. In this study, it was aimed to determine some environmental factors affecting the productivity of Turkish red pine stands spread in Dinar region (Afyon) and to investigate the mutual relations of these factors with productivity.

In this study, it is aimed to estimate the efficiency with Artificial Neural Networks (ANN) technique, which is an application based on artificial intelligence, and to compare the results with the results of regression analysis.

When the studies in the field of engineering are examined, it is seen that the 'Regression Analysis' method is one of the most used statistical methods (Yavuz and Deveci, 2012). However, it is predicted that the regression analysis equations will provide reliable and accurate estimates if they meet some conditions (normal distribution of model errors, homogeneous error variance, no autocorrelation, no correlation between independent variables) (Orhunbilge, 2002).

However, ANN, which is an artificial intelligence application that gives successful results in modeling complex relationships, is preferred by many scientists today. ANN finds widespread use in many fields (electronics and communications, industry, mechatronics and aviation, etc.) (Ashraf et al., 2013).

MATERIAL AND METHOD

The study area is Çzab$_2$ stand in section 224 in the Dinar region of Afyon province (Figure 1). It is located in the Innerwest Anatolia part of the Aegean region between 29°35'-31°45' east and 37°15' -39°20' north latitudes. The surrounding mountains and plateaus intertwined and rose by knotting. The annual evaporation amount of the research area is more than the annual average rainfall. According to the climate classification results of Emmanuel De Martonne and Sırrı Erinç, Afyonkarahisar province has a "semi-arid" climate (Günok, 1999).

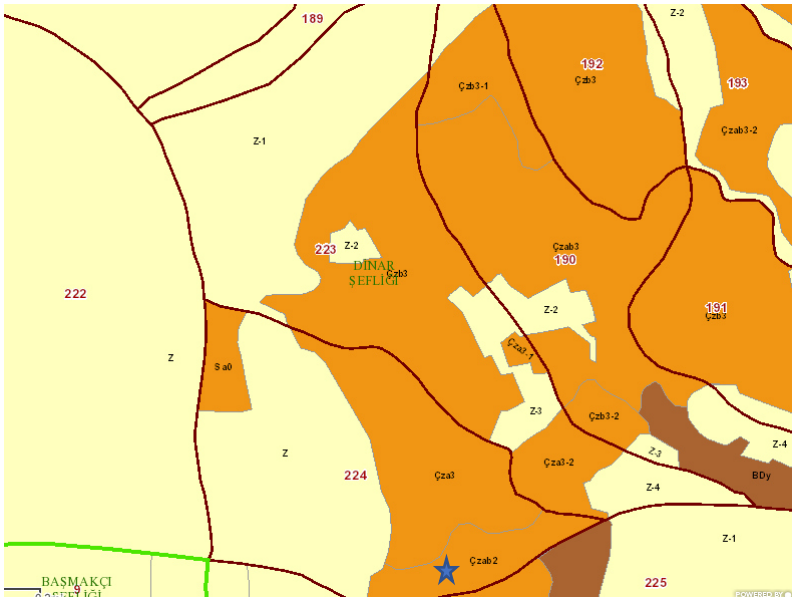

Figure 1. Study area

For the bonitet index representing the dependent variable, 3 trees with the width were determined in the sample area of 400 m^2, the ages of these trees were determined with the help of increment auger and their height was determined with the help of the height gauge and indexed to the age of 100 with the help of the revenue table of Turkish red pine (Alemdağ, 1962).

The locations of the sample areas were determined by GPS, altitude by altimeter with the help of compass and slope by clizimeter. Apart from that, the slope location of the sample area, the land surface roughness and the characteristics of the land shape were determined. Slope position of these features; valley floor, lower slope, middle slope, upper slope and ridge, land surface shape is recorded in the inventory cards as convex, flat, corrugated and concave (Özkan and Kuzugüdenli, 2010).

As numerical variables were obtained, non-numerical variables such as slope location and land shape were digitized and included in the analysis. The slope position is towards the base land from the ridges of the land; ridge (hill): 1, upper slope: 2, middle slope: 3, lower slope: 4, base land (valley): 5, the land shape is flat land: 1, corrugated: 2, concave: 3, convex: 4. It has been digitized and made ready for analytical evaluation.

Before analyzing, it was checked whether these data were normally distributed. Various normality control methods are used to determine the conformity of the data to normal distribution. One of the most common of these methods is the examination of skewness-kurtosis values. The kurtosis-skewness method was used in this study to determine the compliance of variables to normal distribution.

The distribution of variables should also be normal in regression analysis. Like the correlation coefficient, the regression coefficient can be positive or negative. While the correlation coefficient varies between -1 and +1, the regression coefficient can take any value (Pagano and Gauvreau, 1993).

Multiple regression method is one of the most widely used and well-known techniques in scientific studies, and they give a mathematical expression of the relationship between two or more variables. This method provides information about whether there is a relationship between variables and the strength of the relationship (Özdamar, 2002).

Artificial neural networks are a mathematical modeling method developed inspired by the functioning of the human brain. Models are obtained by some computer software that takes the communication principle between neurons, which are human brain cells, as an example (Elmas, 2003). The ANN model includes layers with interrelated nerves (Figure 2). These layers consist of three basic groups as input layer, hidden layer and exit layer (Chandwani et al., 2015).

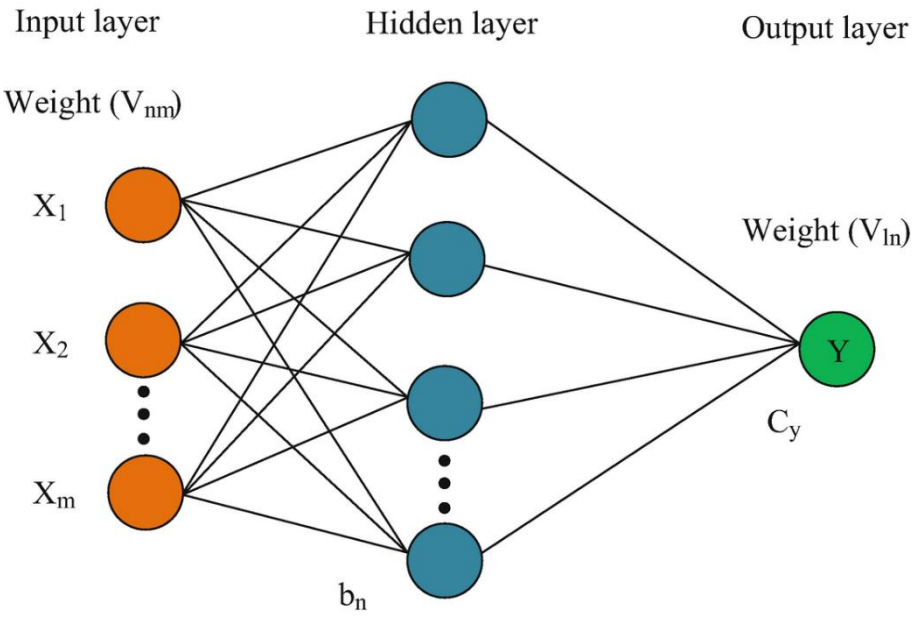

Figure 2. ANN model structure

The way neurons are connected to each other, activation function and learning rules have led to the formation of various ANN structures. Basically, these structures are divided into 3 basic classes as feedback neural networks, feed forward neural networks and radial-based neural networks (Fırat, 2002). Of these structures, feedback neural networks

are more preferred due to their success in prediction and classification processes (Elmas, 2003). In the application of ANN, which includes different neural structures, firstly, input variables and output variables are defined in the system to be predicted, and with these definitions, ANN analyzes the data and estimates the weights in a way to optimize the success in the prediction and minimize the error (Fırat, 2002).

This process is called network training in the literature on ANN. With ANN, many different weight values are derived, and by using these weight values, output estimates are obtained with addition and activation functions. The process is completed at the point where the errors are minimum and the changes related to the errors are fixed by analyzing the variation of the errors calculated according to the observation values related to the output variable defined at the beginning with the predicted values obtained by ANN (Fırat, 2002).

It is stated in the literature that some normalization methods applied to ANN data will improve the performance and accuracy of the artificial neural network (Masters, 1993). Various normalization methods are used by researchers to solve the problems, and the Min-Max method is mostly preferred (Equation 1). With Min-Max normalization, the negative effects of extremely large and small data on the model are reduced by scaling the data between 0 - 1 (Öztemel, 2003).

$$x_{norm} = \frac{x_i - x_{min}}{x_{max} - x_{min}} \quad \text{(Equation 1)}$$

x_{norm}= Normalized data,

x_i= Input value

x_{min}= Smallest number in the input set

x_{max}= Largest number in the input set

RESULTS

Correlation analysis was conducted to determine the mutual relationships between variables. As a result of the correlation analysis, only a weak relationship was found between the bonitet index and elevation (R^2 = -0.45) and slope position (R^2 = 0.34).

Kurtosis-skewness values were determined in order to determine whether the data of the elevation and slope location and Bonitet Index in the modeling phase are normally distributed. When the skewness-kurtosis values are between -1.5 and 1.5, it is accepted to be a normal distribution (Tabachnick and Fidell, 2013). According to these results, it is seen that the data obtained show normal distribution (Table 1).

Table 1: Skewness and Kurtosis Values

Variables	**Skewness**	**Kurtosis**
Elevation	-1.31	1.49
Slope position	1.21	0.13

In order to determine the relationship between elevation and slope position and the variable Bonitet Index, first regression analysis was

performed. The R^2 value of the model created as a result of the analysis was determined as 0.21 (Table 2).

Table 2. Regression analysis results

Coefficients[a]

Model	Unstandardized Coefficients		Standardized Coefficients	t	Sig.
	B	Std. Error	Beta		
(Constant)	65,940	20,772		3,174	,002
Elevation	-,058	,020	-,387	-2,848	,006
Slope position	,079	,098	,109	,805	,424

a. Dependent Variable: Bonitet Index

After the estimated phase has switched to the regression model generated by ANN. In order to determine the relationship between the variables, Slope, Elevation, Slope position, General stoniness, Land form as output variables were selected as Bonitet index variable. 50 of the 70 cases presented of training data set, randomly selected as the test data set are 20 of them. Before starting the training of artificial neural networks, Min-Max normalization was applied to all data. Models were created according to the number of neurons with the training data, and the highest estimate was obtained from the model created with 5 inputs, 2 layers, 5 neurons.

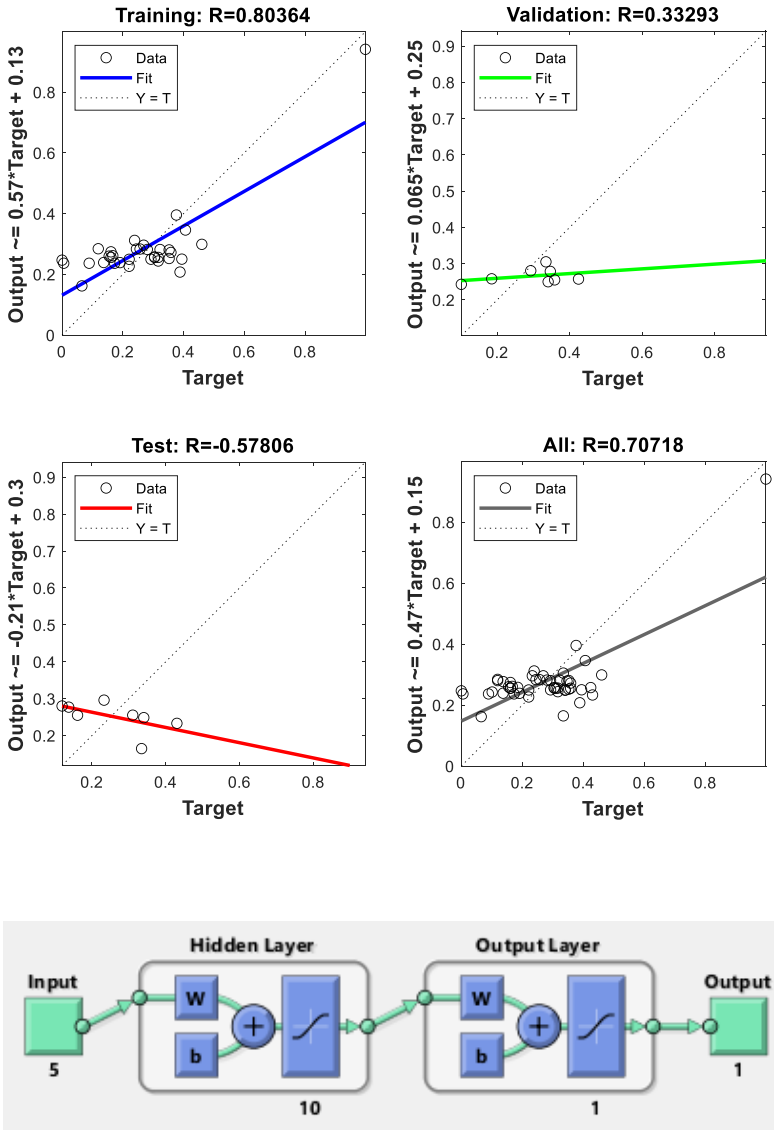

Figure 3. Training graphics of 2 layers 5 neurons

With the test data set, the site index of the model for the number of 2 layer 5 neurons was obtained. When the predictive power of the model was R^2 is 0.64 (Figure 4)

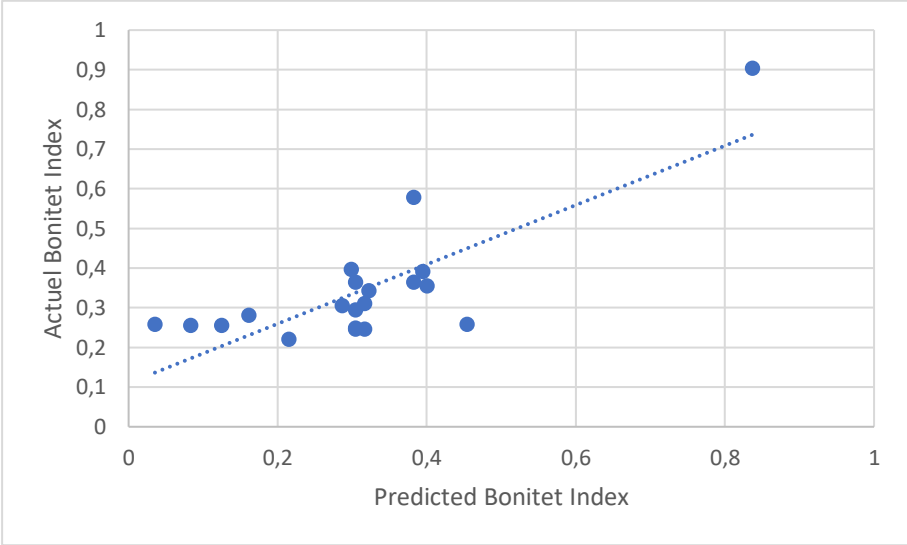

Figure 4. Relationship between actual and predicted bonitet index

When the estimation errors of the network are examined, the mean absolute error (MAD) value was determined as 0.44 for ANN model, the mean square error value (MSE) as 0.34, and the root mean square error (RMSE) as 0.58.The mean absolute percentage error (MAPE) was determined as 6.23 (Table 3).

Table 3: Statistics of model

MODEL	MAD	MSE	RMSE	MAPE	R^2
2K5N	0.44	0.34	0.58	6.23	0,64

CONCLUSION

In this study, it is aimed to determine some environmental factors affecting the productivity of the red pine stand spread in Dinar region and to investigate the mutual relations of these factors with productivity.

For this purpose, regression analysis and ANN technique, which is an application based on artificial intelligence, was used and the results obtained were compared. Correlation analysis was performed to determine the bilateral relationships between variables. As a result of the correlation analysis, when the bilateral relations of the variables with each other are examined, it was determined that the variables do not have high correlation with each other.

In order to determine the relationship between the independent variables and the dependent variable, the upper height variable, first regression analysis was performed. The R^2 value of the model created as a result of the analysis was determined as 0.21.

After the regression model was created, the estimation phase was started with ANN. In order to determine the relationship between the variables, Baku, Elevation, Slope location, General stoniness, Land form as output variables were selected as Bonitet Index variable.

A model consisting of 2 layers and 5 neurons was created with ANN. When looking at the predictive power of this model, R^2 value was

determined as 0.64.

In this study regarding the modeling of the productivity of Turkish red pine, the predictive power of the model obtained by the regression method (R^2 value 0.21) was found to be higher than the predictive power obtained by the ANN method (R^2 value 0.78).

SUGGESTIONS

Using the equation obtained as a result of the regression analysis while determining the potential afforestation areas of Turkish red pine, it should be aimed to obtain the highest profit by using the input variables here.

Bonitet Index = 65.9 - 0,058(elevation) + 0,079(slope position)

In this study the addition of unused site factors to the model can be obtained with high predictive power of re-testing.

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CHAPTER 6

NATURAL QUORUM QUENCHING MOLECULES

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INTRODUCTION

Since human beings were created, they have interacted with other living organisms as part of nature. Some microorganisms like bacteria, viruses and yeasts that use people as hosts to continue their lives cause many infections. Some of these infections can be treated easily, while others spread rapidly among humans, resulting in epidemics and pandemics (Habicht, Pate, Varotto, & Galassi, 2020). Diseases that spread within a particular region or population are defined as epidemics, while diseases that spread to a wider area or even around the world are called pandemics (Figure 1) (Kaur et al., 2020). Human society has been ravaged by pandemics, with lasting impacts on both the value of human life and the need for future stability. The COVID-19 pandemic, which affects the whole world today and has caused many people to lose their lives, has changed our entire lives and created a new world order. Although COVID-19 infection is a viral disease, it is seen that some pandemia seen throughout human history are caused by bacteria (Table 1) (Khan, Mehta, Arif, & Lakhani, 2020).

Figure 1. Difference Between Epidemic And Pandemic (Kaur et al., 2020)

Table 1. A Summary Of Notable Pandemics In The Human History (Khan et al., 2020)

Name	Infection causing agent and subtype	Dates	Possible region of origin	Reported deaths	Case fatality rate
Plague of Justinian	Yersinia Pestis	541-42	Unclear	25-100 million	Unknown
The Black Death	Yersinia Pestis	1347-50	Asia	75-200 million	Unknown
The First Cholera Pandemic	Vibrio cholerae	1817-24	India	1-2 million	Unknown
The Second Cholera Pandemic	Vibrio cholerae	1826-37	India	Unknown	Unknown
The Third Cholera Pandemic	Vibrio cholerae	1846-60	India	1-2 million	Unknown
Russian Flu or Asiatic Flu	Influenza A (Subtype unclear)	1889-90	Russia	1 million	0.1-0.28%
The Third Plague Pandemic	Yersinia Pestis	1894-1959	China	15 million	Unknown
Spanish Flu	Influenza A (subtype H1N1)	1918-20	Unclear	17-50 million	>2.5%
Asian Flu	Influenza A (subtype H2N2)	1957-58	China	1-4 million	<0.2%
HIV/AIDS Pandemic	HIV	1960-Present	Africa	32 million	100%
The Seventh Cholera Pandemic	Vibrio cholerae	1961-Present	Indonesia	155 thousand	Unknown
Hong-Kong Flu	Influenza A (H3N2)	1968-69	Hong-Kong/China	1-4 million	<0.2%

Antibiotics are the most important weapon used to fight bacterial infections. They affect the structures of bacteria such as cell membrane, cell wall, DNA, RNA, ribosome, proteins and show cytotoxic or cytostatic effects on bacteria. The era of antibiotics, which began with two names, Alexander Fleming and Paul Ehrlich, continued with the discovering of new antibiotics that affected many pathogenic bacteria from the 1950s to the 1970s (Mohr, 2016). In the period from the 1970s to the present day, there is a period of pause in the discovery of new antibiotics (Aminov, 2010; Zaman et al., 2017). In addition, as a result of the incorrect use of antibiotics today, a serious antibiotic resistance has occurred in bacteria (Lobanovska & Pilla, 2017). Thus, existing antibiotics are insufficient to treat bacterial infections, and scientists are striving to develop new approaches to treatment of infectious diseases. One of these approaches is to use the quorum quenching pathway that blocks the quorum sensing system, which is the communication network of bacteria (Grandclément, Tannières, Moréra, Dessaux, & Faure, 2015; Saeki, Kobayashi, & Nakazato, 2020; Whiteley, Diggle, & Greenberg, 2017). Information about natural quorum quenching molecules is given in this study.

1. QUORUM SENSING SYSTEM

A cell-cell communication mechanism used by microorganisms to control the behavior of microbial populations through the secretion of extracellular chemical signals is the Quorum Sensing (QS) system (Sharma, Singh, Sarmah, & Nandi, 2020). These signaling molecules called autoinducers, which are produced dependent on cell density, are

regulators for the expression of genes that play an important role in many metabolic pathways such as biofilm formation of cells, antibiotic resistance, sporulation, conjugal plasmid transfer, mobility, bacterial adhesion, bioluminescence, and virulence factor secretion (John & Ramesh, 2020). Receptor proteins can recognize these molecules after the signaling molecules produced by bacteria and secreted from the cell exceed a certain amount and pass the threshold value and, as a consequence, the expression of the target genes is regulated (Figure 2) (Zhang, Feng, Wang, Wang, & Zhang, 2019). There are four main signal molecule classes using for QS (Figure 3). The signal molecules used by gram negative bacteria are acyl homoserin lactones (AHLs), while gram positive bacteria use auto-inducing peptides (AIPs) (Sarkar & Das, 2019). Autoinducer-2 (AI-2) which is included in both gram negative and gram positive bacteria is responsible for communication. Pseudomonas quinolone signal (PQS) that was recently determined in *Pseudomonas aeruginosa* provides some pathogenic characteristics to this bacteria (Turan, Chormey, Büyükpınar, Engin, & Bakirdere, 2017).

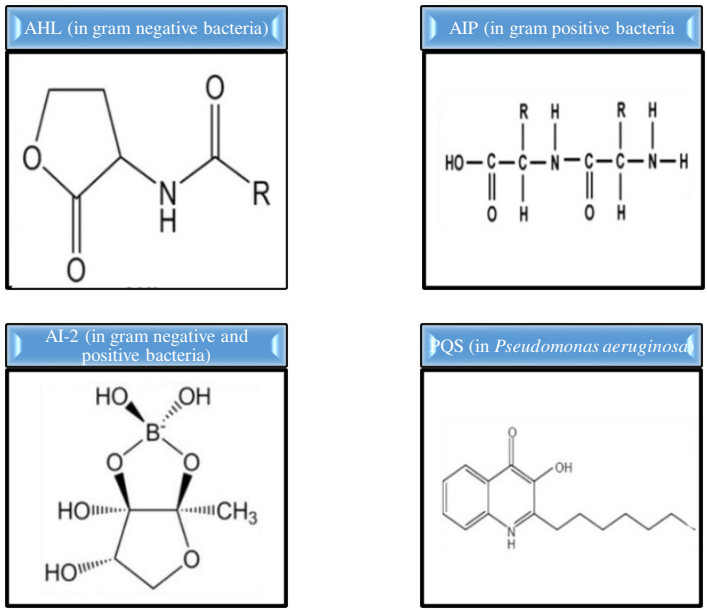

Figure 3. Main Classes of Signal Molecules Using for QS (Turan et al., 2017)

1.1. Quorum Sensing Mechanism in Gram Negative Bacteria

The main autoinducer molecule used by gram negative bacteria is acyl homoserin lactons (AHLs) with a well-preserved lactone ring bound to the acyl chain. Two components of this QS mechanism are LuxI protein (Autoinducer synthase) and LuxR protein (Autoinducer receptor). Autoinducer molecules (AIs) produced by autoinducer synthase are freely diffused out of the cell. When the density of AIs exceeds the threshold, the system is induced to produce more AIs. Produced AIs are linked to the receptor to form the AI-receptor complex. This complex binds to the promoter region to regulate QS gene regulation. If the amount of AIs increases a certain amount compared to the population density of bacteria, these molecules are taken into cells to regulate the expression of genes that provide the

properties of bacteria such as biofilm formation, virulence factor release, antibiotic production, and so on (Table4) (Asfour, 2018).

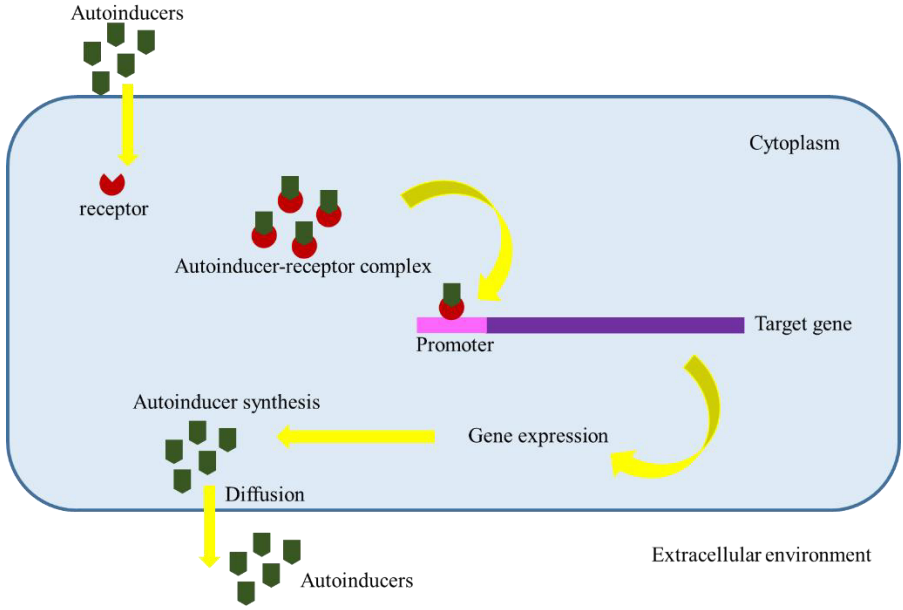

Figure 4. General Quorum Sensing Mechanism in Gram Negative Bacteria

1.2. Quorum Sensing Mechanism in Gram Positive Bacteria

The autoinducers used for QS in gram positive bacteria are autoinducing peptides (AIPs) created by processing from autoinducing peptide precursors. The modified AIPs are then transported out of the cell by ATP-binding cassette transporter complex. When the AIP concentration exceeds a certain amount, sensor kinase protein that extends out of the bacterial cell wall is activated. Then, this activated protein phosphorylate the response regulator protein. This regulator protein binds to the promoter region of the target gene and gene expression is activated (Figure 5) (Turan et al., 2017).

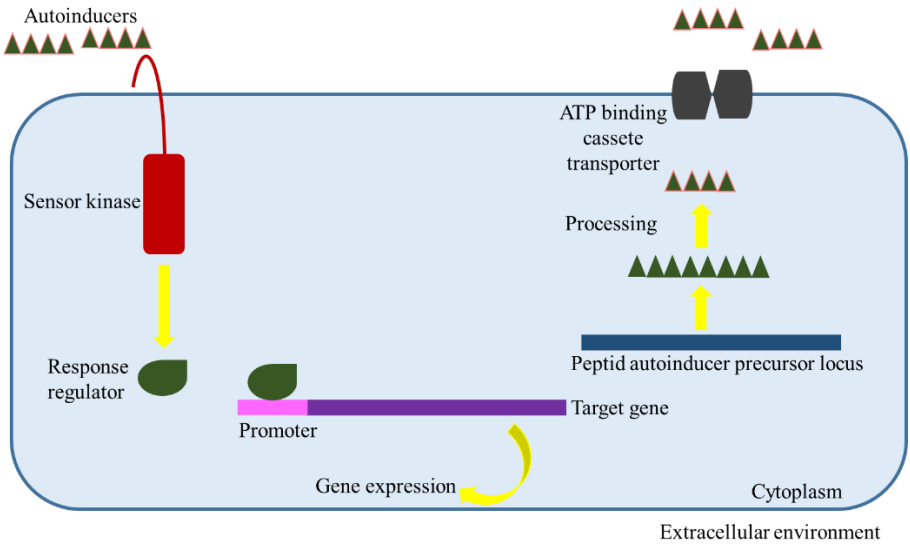

Figure 5. General Quorum Sensing Mechanism in Gram Positive Bacteria

2. QUORUM QUENCHING SYSTEM

The increase in bacteria with multiple antibiotic resistance in recent years has forced the development of new strategies for the treatment of infectious diseases. As a rational approach, targeting the quorum sensing system of bacteria in this respect has been used. The quorum quenching (QQ) mechanism interferes with the quorum sensing system of pathogenic bacteria, preventing the expression of genes associated with this system (Adak, Upadrasta, Kumar, Soni, & Banerjee, 2011; Stéphane Uroz, Dessaux, & Oger, 2009). Four main mechanisms of the QQ system are used to suppress the bacterial QS pathway (Figure 6). In the first mechanism, QS signal molecules are prevented from being formed while transporting these signals is inhibited on the second path. The degradation of the produced signal molecules is another process. In the last mechanism, it is aimed to

prevent signal molecules from binding to the receptor (Zhang et al., 2019).

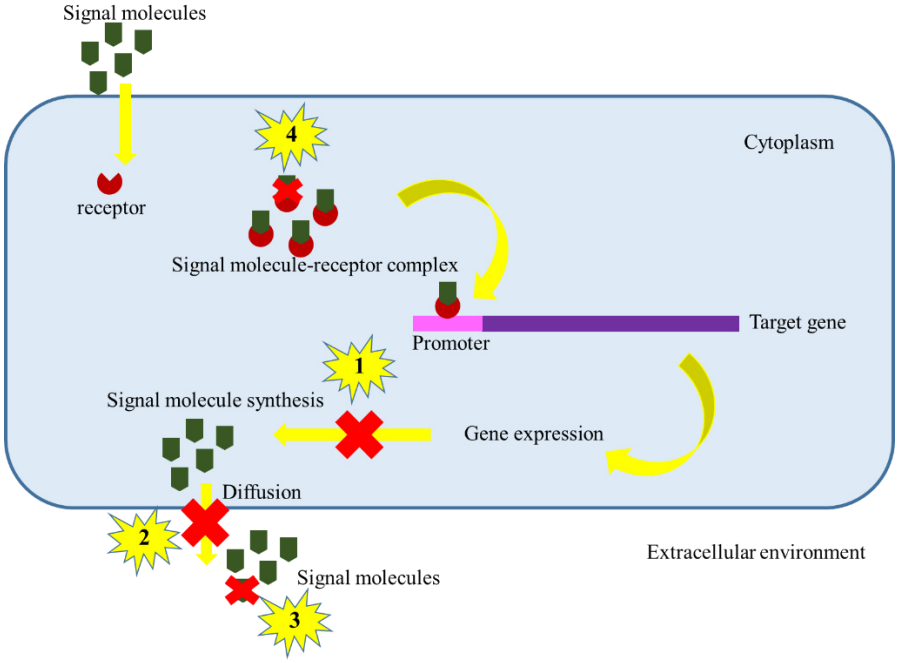

Figure 6. Main Quorum Quenching Pathways

Synthetic QQ molecules along with natural QQ molecules synthesized by various microorganisms, plants and animals are also being tested for QS inhibition in laboratories. it is possible to separate natural QQ molecules into two groups as small molecular QQ agents and macromolecular QQ agents according to molecular weight.

3. Classification of Natural QQ Molecules

3.1. Small Molecular QQ Agents

Different small molecules produced by plants, microorganisms and fungus able to inhibit the QS signals by using different mechanisms.

3.1.1. Plant Derivated QQ Molecules

Active components such as polyphenols, flavonoids, terpenoids or coumarins contained in many plants have a QQ effect by preventing the synthesis or receptor attachment of QS signal molecules (Asif, 2020; John & Ramesh, 2020). Some plants that synthesize QQ-characteristic molecules and the inhibitory molecules they produce are shown in table 2.

Table 2. Some Plant Derivated QQ Molecules (Zhang et al., 2019)

Plant	QQ Molecule
Andrographis paniculata (Burm. F) Nees	Andrographolide
Armoracia rusticana	Iberin
Allium sativum	Ajoene
Baccharis cassinaefolia	Benzopyran
Brassica oleracea	Erucin
Combretum albiflorum	Flavonoids
Centratherum punctatum	Sesquiterpene lactones
Curcuma longa	Curcumin
Grapefruit	Limonoids (obacunone)
Hamamelis virginiana	2,5-di-O-galloyl-D hamamelose
Houttuynia cordata	Houttuynin
Many plants	Cinnamaldehyde and its derivatives
Quercus	Tanic acid
Witch hazel	Witch hazel tannin

3.1.2. Marinal Organism Derivated QQ Molecules

Studies have shown that metabolites such as AHL analogues, phenethylamides, butenolides, produced by some marine bacteria have QQ activity (Borges & Simões, 2019). It is also known that marine algae synthesize QS inhibitor metabolites. Among these metabolites, the most studied molecules are furanones (Kalia, 2015).

3.1.3. Fungal Derivated QQ Molecules

Secondary metabolites such as patulin, penicillic acid, furanone synthesized by some fungus have been shown to inhibit the QS mechanism of some bacteria(Kalia, 2013; Turan & Engin, 2018).

3.2. Macromolecular QQ Agents

These group of QQ molecules include enzymes such as AHL lactonases, AHL acylases and AHL oxidoreductases which are effective on QS signaling molecules (Dong & Zhang, 2005). AHL lactonases cleave the lactone ring in the AHL molecule, causing the formation of acyl homoserin. AHL acylases break the amid bond in the AHL molecule, resulting in fatty acids and HSL (Figure 7) (Zhang et al., 2019). Oxydoreductases, on the other hand, modify the acyl chain by oxidation or reducing it instead of degradation the AHL molecule.

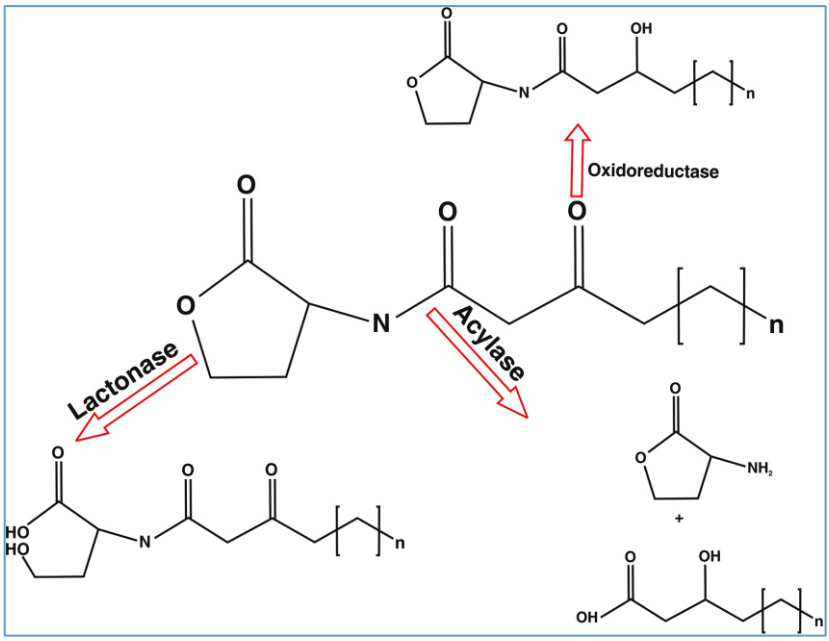

Figure 7. Structure of AHL Molecule and Enzymatic QQ Mechanisms (Gupta, Daroch, Harjai, & Chhibber, 2019)

3.2.1. AHL Lactonases

Lactonase enzymes produced by different bacterial species provide enzymatic degradation of AHL molecules (Dong, Wang, & Zhang, 2007). The homoserine lactone ring (HSL) ester bond is hydrolyzed by AHL lactonases to create the corresponding acyl-homoserines. Compared to the molecular structures of approximately 30 different AHL lactonases identified to date and these enzymes have been found to be in different prokaryotic enzyme families. First described AHL lactonase enzyme AiiA encoded by the aiiA gene was identified from a soil bacterial isolate *Bacillus* sp. 240B1 (Dong, Xu, Li, & Zhang, 2000). Metallo β-lactamases (Aiia, ahlD), paraoxanases,

phosphotriesterases (Ssopox, Sislac) and α/β hydrolase fold lactonases (AidH) are four protein families which AHL lactonases are involved in. AiiA is situated within the metallo-β-lactamase superfamily and hydrolyses the homoserine lactone ring of AHLs (Gupta et al., 2019). Some different types of AHL lactonases, the resources from which they are obtained and their protein families are given in Table 3.

Table 3. AHL Lactonases (Fetzner, 2015)

Enzyme	Source	Protein Family
AiiA	*Bacillus* spp. (*B. cereus* group)	Metallo-β-lactamase superfamily
AhlS	*Solibacillus silvestris* StLB046	
AhlD	*Arthrobacter* sp. IBN110	
AttM (AiiB)	*Agrobacterium tumefaciens* C58, M103	
QsdR1	*Rhizobium* sp. NGR234	
AhlK	*Klebsiella pneumoniae* KCTC2241	
AidC	*Chryseobacterium* sp. StRB126	
QlcA	Soil metagenome (acidobacterial origin)	
PPH, identical to Php of strain H37Rv	*Mycobacterium tuberculosis*	Phosphotriesterase like lactonase (PLL); amidohydrolase superfamily
MCP (MAP3668c)	*M. avium* ssp. *paratuberculosis* K-10	
QsdA (AhlA)	*Rhodococcus erythropolis* W2, SQ1, Mic1, MP50, CECT3008; *Rhodococcus* sp. BH4	
SsoPox	*Sulfolobus solfataricus* P2 (ATCC 35092)	
SisLac	*Sulfolobus islandicus* M.16.4	
PON1	Mammalian liver, serum	Paraoxonase (PON) family
PON2	All mammalian tissues	
PON3	Mammalian liver (and kidney), serum	
Bacterial PON	*Oceanicaulis alexandrii* HTCC2633	
AiiM	*Microbacterium testaceum* StLB037	α/β-Hydrolase superfamily
AidH	*Ochrobactrum* sp. T63	

3.2.2. AHL Acylases

Acylase enzyme is responsible for breaking the bond of acyl-amide, which provides the HSL used as a nitrogen source for bacterial growth and fatty acids. This enzyme was first described in Variovorax paradoxus (Turan & Engin, 2018). In addition, AhIM and AiiD enzymes in the AHL acylase family were found in *Streptomyces* and *Ralstonia* XJ12B respectively. These two enzymes were found to inhibit pyosiyanin production, elastase activity and swimming movement of *Pseudomonas aeruginosa* (Zhang et al., 2019). Studies show that in many microorganisms, the AHL acylase structure is similar and comes from the N-terminal nucleophile hydrolase family (Gupta et al., 2019). Some AHL acylases, the sources from which they are obtained and their protein families are given in Table 4.

3.2.3. AHL Oxidoreductases

Oxydoreductases, which were first shown its AHL reduction activity in the *Rhodococcus erythropolis* W2 strain, modify the AHL molecule by oxidation or reduction instead of breaking it down (S. Uroz, 2005). This modification of the AHL molecule distorts the specificity of the signal and as a result the expression of QS-related genes is blocked. Although there have been many studies on the chemical structure and mechanism of AHL lactonases and acylases, information on AHL oxydoreductases is very limited (Hong, Koh, Sam, Yin, & Chan, 2012; Tang & Zhang, 2014).

Table 4. AHL Acylases (Fetzner, 2015)

Enzyme	Source	Protein Family
not identified	*Variovorax paradoxus* VAI-C	not identified
AhlM	*Streptomyces* sp. M664	N-terminal nucleophile (Ntn) hydrolase family
AibP	*Brucella melitensis* 16M (ATCC 23456)	
AiiD	*Ralstonia* sp. XJ12B	
Aac	*Ralstonia solanacearum* GMI1000	
PvdQ (PA2385)	*P. aeruginosa* PAO1	
QuiP (PA1032)	*P. aeruginosa* PAO1	
HacB (PA0305)	*P. aeruginosa* PAO1	
HacB (Psyr 4858)	*P. syringae* B728a	
HacA (Psyr 1971)	*P. syringae* B728a	
Aac	*Shewanella* sp. MIB015	
AiiC	*Anabaena* sp. PCC7120	
AiiO	*Ochrobactrum* sp. A44	α/β-Hydrolase
QsdB	Soil metagenome	Amidase signature (AS) family

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CHAPTER 7

CATALYTIC HYDROTERMAL LIQUEFACTION METHODS OF PLANT BIOMASS

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INTRODUCTION

Biomass is a general name given to all organic substances of plant and animal origin. All substances that can be renewed in less than a century, including land and water-grown plants, animal residues, food industry and forest by-products, and urban waste are considered "biomass" (Saxena et al., 2009).

Biomass energy is the energy obtained from these organic substances. Biomass energy is formed by transforming biomass into useful energy forms by applying different processes. Transformation processes are particularly determined by the amount and type of biomass, the desired energy type, environmental standards, economic conditions and the characteristics of the applied processes. There are three basic processes to obtain energy from biomass: thermal, thermochemical and biochemical methods. Chemical methods are the fourth method to obtain energy from biomass (Gürsoy, 2017). Biomass can be converted into three main products: power/heat generation, fuel and useful chemical raw materials.

Although biomass resources are diverse, the most important ones used for energy purposes can be listed as follows:

- Wood,
- Herbal resources,
- Oilseed crops (sunflower seed, rapeseed, soybean, etc.),
- Carbohydrate crops (potato, wheat, corn, beet, etc.),
- Energy (C4) crops (eucalyptus, sweet millet, miscanthus, etc.),

- Aquatic crops (water hyacinth, algae, seaweed, some water grasses, etc.),
- Plant residues (branches, stalks, straw, roots, bark, etc.),
- Animal wastes,
- Urban and industrial waste.

Such biomass-based resources are passed through several transformation processes and used in heating as raw materials to prepare composite materials, in the production of biofuels such as ethanol, biodiesel, and in electricity generation. In addition to being renewable and sustainable, biomass energy is extremely important due to its easy availability, low storage and transportation costs, contribution to socio-economic development, and being defined as environmentally friendly (Özyurt, 2006).

Plant biomasses are hydrocarbon substances whose main components are C, H, O, and N, which are formed as a result of green plants transforming and storing solar energy into chemical energy through photosynthesis. In this academic study, some information will be provided and several statements will be made about the liquefaction (hydroxylation) methods of plant biomass.

Table 1. Plant Biomass and Types of Waste (Özyurt, 2006)

Industry	**Type**	**Example**
Forestry	Forest Forest By-Products	Trees (willow, poplar, eucalyptus) Wood waste, sawdust, etc.
Agriculture	Dry lignocellulosic plants Oily, sugary and starchy plants Agricultural waste	Oilseed plants (e.g. canola, sunflower, soy, etc.) Sugary plants (sugar beet, sweet sorghum etc.) Starchy plants (corn, wheat, potatoes etc.) Stalk, straw, pruning residues

The preparation of low-cost polyols from abundant and renewable plant biomass resources has long been an area of interest in producing industrial chemicals, especially polyurethane chemicals (Yao et al., 1996). Many scientific efforts to implement and extend the use of plant biomass are demonstrated in order to incorporate plant components such as starch, cellulose powders, lignocellulosic materials, lignin, etc. into polyurethane industrial chemicals (Hostettler, F., 1979; Hatakeyama et al., 1992).

Liquefaction of plant biomass to produce industrial chemicals is a method for using biomass resources. Research on this began with the liquefaction of wood pulp (Besteu et al., 1985; Maldas and Shiraishi,

1997; Vuori and Niemela, 1998; Yamada and Ono, 1999). However, the reaction conditions and its practical use were difficult and consumed a lot of energy. The researchers facilitated the reaction conditions and used the liquefied product to produce resin and foam (Alma et al., 2003; Lin et al., 1994).

The principles and methods of liquefaction of wood pulp have provided some ideas for using agricultural biomass wastes of similar composition with wood pulp (Wang and Chen, 2007). Some studies have been performed on the liquefaction of agricultural waste (Cinelli et al., 2013; Wang and Chen, 2007; Alma et al., 2003; Lin et al., 1994; Yao et al., 1993; Wang et al., 2013; Hakim et al., 2011).

Introducing biomass materials into industrial chemicals is accomplished by difficult steps that increase the biomass content, which affects the chain hardness, durability, chain length density, and thermal properties of industrial products (Ferrigno, 1967; Hsu and Glasser, 1979). It is known that cellulosic fibers contribute a set of benefits to the fiber structure such as high specific stiffness, durability, desired fiber aspect ratio, a flexibility that will not damage equipment during the process, low density, biodegradability, and low cost per unit (Shiraishi, 1992; Shiraishi et al., 1993).

Two different approaches are being tried to use biomass for the production of industrial materials (e.g polyurethane materials). The first method is a direct combination of biomass such as starch, lignocellulose, coffee grounds, etc. (Hostettler, 1979; Kohn and Rober, 1988; Yoshida et al., 1987). Hatakeyama et al., (2008) reported

that rigid polyurethane foams containing at least 50% biomass can be obtained by mixing biomass materials into petroleum-based polyols before foaming. (Hatakeyama et al., 2008). However, the majority of this biomass contributes to the structure of the foam as a solid filler rather than a reactive component, and as a result, there is a tendency for high-density fillers to emerge. The second method is to use the biomass via the hydroxylation reaction. Different types of biomass were studied, such as sorbitol (Hakim et al., 2011), sucrose (Pan et al., 2011), glucose (Cinelli et al., 2013), and starch (Barikani and Mohammadi, 2006). Polyols (raw material chemicals) having the desired level of hydroxyl and viscosity were obtained by this method.

Organic reagents (solvents) are used to liquefy biomass (Shiraishi, 1985). These organic reagents can be ethylene glycol, polyhydric alcohol, phenol, glycerol, as well as oils of natural soy and rape seeds. Organic reagents used in the liquefaction of biomass are advantageous due to their high liquefaction capacity and low cost (Yao et al., 1993; Kurimoto et al., 1992; Yao et al., 1996).

Most liquefaction reactions of biomass in the presence of organic solvents are carried out by conventional convective (convective conduction) and conductive (permeable conductor) heating sources such as water, oil, salt bath, fluid salt bath, and electric furnace. These methods are relatively slow and inefficient in transferring energy into the system since they are based on the thermal conduction of materials. Compared with the conventional heating method, the microwave heating method provides internal heating by applying

microwave energy to the reaction mixture consisting of catalyst, reactant, and solvent molecules through the in-core volumetric heating method (Zheng et al., 2011).

METHODS AND RESULTS

1. Liquefaction of Plant Biomass (Hydroxylation) in a Water Bath Method

The samples of various plant biomasses dried under the atmosphere or in the oven are placed in the reaction vessel after being milled in different sizes. The specified solvent and the appropriate amount of the selected catalyst are added thereto. The reaction mixture is pre-mixed. The hydroxylation reaction is carried out in the water bath at different temperatures and reaction times.

The hydroxylation process of plant biomass in the water bath takes place with a conventional convective (convective conduction) heating source. There are limitations since the hydroxylation reaction of plant biomasses in the water bath can be carried out at certain temperature ranges. Plant biomass degrades at higher temperatures due to their structural components ((Fidan, 2009; Wang and Chen,2007; Yao et al.; 1993).

(Reaction flask) (Water bath device)

Figure 1. Thermochemical Hydroxylation of Plant Biomass in The Water Bath.

2. Liquefaction of Plant Biomass (Hydroxylation) in Oil Bath Method

(Reaction flask) (Oil bath device)

Figure 2. Thermochemical Hydroxylation of Plant Biomass in The Oil Bath.

The samples of various plant biomasses dried under the atmosphere or in the oven are placed in the reaction vessel after being milled in different sizes. The specified solvent and the appropriate amount of the selected catalyst are added thereto. The reaction mixture is pre-

mixed. The hydroxylation reaction is carried out in the oil bath at different temperatures and reaction times.

The hydroxylation process of plant biomass in an oil bath is another example of the hydroxylation process performed with a conventional convective (convective conduction) heating source. The hydroxylation process of plant biomass in the oil bath has an advantage over the thermochemical dissolution method in which the water bath is used since it provides chemical dissolution in a higher and wider temperature range (Alma and Shiraishi, 1998; Yan et al., 2008).

3. Liquefaction of Plant Biomass (Hydroxylation) in Salt Bath and Fluid Salt Bath Method

(Reaction flask) (Salt) (Salt bath device)

Figure 3. Thermochemical Hydroxylation of Plant Biomass in Salt/Fluid Salt Bath.

The samples of various herbal biomasses dried under the atmosphere or in the oven are placed in the reaction vessel after being milled in different sizes. The specified solvent and the appropriate amount of the selected catalyst are added thereto. The reaction mixture is pre-mixed. The hydroxylation reaction is carried out in the salt bath/fluid

salt bath at different temperatures and reaction times. A salt bath assembly is created by using the mixture formed by dissolving various salts in solvents in the heating cabinet. Thermocouple consumption is common in salt bath use. Besides, the bath bed in which the salt solution was placed in the fluid salt bath was coated with various chemicals.

Although hydroxylation of plant biomass varieties in salt/fluid salt baths provides the opportunity to create reaction at very high temperatures, its cost is high due to the formation of corrosion in the assembly and the subsequent gas output (Pehlivan ve Taner, 2006).

4. Liquefaction of Plant Biomass (Hydroxylation) in Electric Furnace Method

(Reaction flask)

(Electric furnace)

Figure 4. Thermochemical Hydroxylation of Plant Biomass in an Electric Furnace.

It is the placement of hydroxylation reaction assembly in the electric furnace and the performance of the reaction at selected temperatures and times. The hydroxylation reaction is carried out with conductive (permeable conductive) heating sources. It is the execution of the reaction in the environment formed by the conversion of electrical

energy passing over the conductive wires inside the furnace to heat energy (induction transmission).

5. Liquefaction of Print Biomass (Hydroxylation) in Microwave Oven Method

It is another type of hydroxylation reaction performed with induction transmission sources. The microwave heating method provides internal heating by applying microwave energy to the reaction mixture consisting of catalyst, reactant, and solvent molecules through in-core volumetric heating (Zheng et al., 2011). It allows the hydroxylation reaction to take place at a lower temperature with less energy used (Gürsoy and Alma, 2017).

(Liquefaction reagents)

(Microwave oven)

Figure 5. Thermochemical Hydroxylation of Plant Biomass in a Microwave Oven.

With the microwave heating method, plant biomass hydroxylation reactions can be performed at the desired temperature, amount of electrical energy, reaction time, and mixing speed. In the microwave heating method, the pressure of the reaction flask where the reaction is carried out can be controlled by the condenser assembly connected to

the microwave oven equipment (Pan ve diğ., 2011; Cinelli ve ark., 2013).

6. Conductive Liquefaction of Plant Biomass (Bottom Transmission) Method

(Reaction flask)

(Conductive heater)

Figure 6. Thermochemical Hydroxylation of Plant Biomass with a Conductive Transmission Heater.

It is a method of liquefying plant biomass utilizing a conductive (bottom transmission) heater. The time to reach the temperature at which the hydroxylation reaction will take place is relatively longer (Wang ve diğ., 2008).

7. Liquefaction of Plant Biomass (Hydroxylation) in the Reactor Method

It is the hydroxylation method in which the hydrothermal liquefaction reactions of plant biomass are carried out in a high-pressure and temperature-resistant steel reactor. Atmosphere-/oven-dried samples of various plant biomass are placed in the reaction vessel after (or without) being milled in different sizes. The specified solvent and the

appropriate amount of the selected catalyst are added thereto. The reaction mixture is premixed and placed in the reservoir of the steel reactor. The temperature and pressure of the reactor are determined in accordance with the biomass hydroxylation.

(Reactor)

Figure 7. Thermochemical Hydroxylation of Plant Biomass in a Steel Reactor.

The liquefaction of plant biomass (hydroxylation) in the reactor method enables the hydroxylation of plant biomass both by milling and without milling (Durak, 2019, Elliot et al., 2015).

DISCUSSION

Biomass energy is a research area that has recently attracted attention with the search for alternative energy sources to fossil fuel reserves. Plant biomass resources constitute an important part of the total biomass. Plant biomass is composed of polymers made up of macromolecules containing C-C bonds. While the C-C bond forms the main skeleton of the polymers, there may be bonds consisting of C-O, C-H, C-N, C-S, or other elements. Cellulose is a high molecular weight non-polar long-chain polysaccharide with a high degree of

polymerization having a basic chemical unit of (C6H10O5)n, the solubility of which increases with temperature (Wang et al., 2017). Hemicellulose is a biopolymer containing pentose and hexoses in a branched, amorphous structure. Lignin is morphologically amorphous like hemicellulose but resembles cellulose with its low solubility characteristic (Gabir and Hameed, 2017). Starch is the polymer of both amylopectin and amylose glucose. It is a linear macromolecule and water-insoluble (Gürsoy and Alma, 2017). When the same liquefaction method is applied, plant biomass liquefies at different temperatures according to their structural properties, differences in their structural components, the type of solvent used, and the type of catalyst selected. In addition, they have different hydroxylation rates (Gürsoy 2018; Gürsoy 2018; Gürsoy 2018).

For the liquefaction reaction of plant biomass, the Plant Biomass Liquefaction in Water Bath (Hydroxylation) Method has limitations since it provides an environment until low temperature.

Although the Plant Biomass Liquefaction (Hydroxylation) in Oil Bath Method provides the required temperature environment, it has difficulties in its practical use and requires the milling of plant biomass.

The Plaint Biomass Liquefaction (Hydroxylation) in Salt Bath and Fluid Salt Bath Method is unnecessary because it can create a very high-temperature environment, although it provides the required temperature environment. It requires the milling of plant biomass. Different chemical salt types should be provided to be used in the salt

bath. Also, chemicals used in the fluid salt bath bed may corrode during the reaction and emit toxic gases.

Although the Plant Biomass Liquefaction (Hydroxylation) Method can be used in the Electric Furnace, it does not create a homogeneous heat dissipation environment during the hydroxylation reaction and does not provide regular mixing to the reaction mixture.

Plaint Biomass Liquefaction (Hydroxylation) in Microwave Oven Method is advantageous because it offers different temperatures, energy amounts, time, and mixing speed possibilities and it is easy to use in practice. On the other hand, it requires the milling of plant biomass and the use of water in the condenser (Gürsoy et al., 2014).

Although the Conductive (Bottom Transmission) Plant Biomass Liquefaction (Hydroxylation) Method allows the hydroxylation of plant biomass, the reaction time is longer, it does not provide homogeneous heat transmission, and it has difficulties in its use at rising temperatures. It requires the plant biomass to be milled but allows it to be mixed during the reaction.

Plant Biomass Liquefaction (Hydroxylation) in the Reactor Method allows hydroxylation of all types of biomass. The temperature and pressure of the reaction medium are at times high for plant biomass and creates more cost. However, it does not require the milling of plant biomass and the use of water in the condenser (Durak, 2019).

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CHAPTER 8

CHEMISTRY OF BODIPY DYES

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INTRODUCTION

The chemistry of organic dyes has attracted a great attention in recent years. The reason of this interest depends on the contemporary routes in organic chemistry, which lets to configurate the backbone of the existing chromophores or develop new molecular structures, with the requested replacement design, performing the concrete requisites of a desired application field (de Moliner, Kielland, Lavilla & Vendrell, 2017). Among these dyes borondipyrromethene (BODIPY), generally known as chromophores, are definitely in the forefront. According to the literature, bibliography of BODIPYs started with their discovery in the early 1900s by Treibs and Kruzer and they have been the focus of considerable research interest and rapidly growing (Treibs & Kreuzer, 1968). The main reason of this successive growing interest is depend on the photochemical, the chemical and the thermal stability of their boron-dipyrrin core, which procures powerful absorption and fluorescence spectral bands (Figure 1) (Bañuelos, 2016; Benstead, Mehl & Boyle 2011; Loudet & Burgess, 2007).

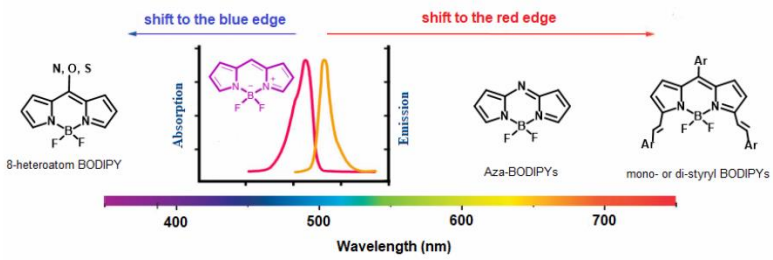


Figure 1. Molecular Structure Of BODIPY Core And The Basic Absorption And Emission Bands For Different Types Of BODIPYs.

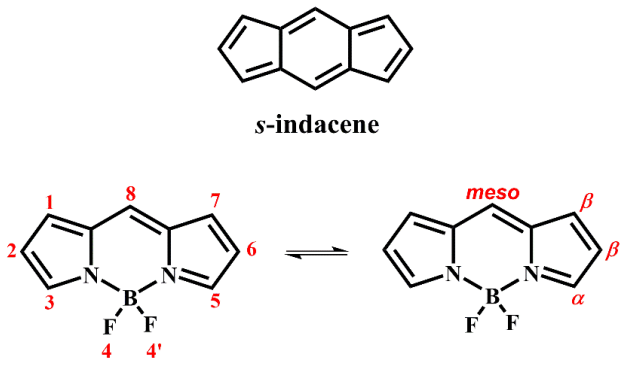
One of the important characteristics of BODIPYs is specified that they are strongly UV absorbing molecules and they emit radiation with high quantum yields (Loudet & Burgess, 2007). In addition, they have sharp fluorescence peaks. Other important property is that it is relatively insensitive to polarity and pH of their environment. This means that changing the solvent does not significantly affect the absorption and emission characteristics. In addition, they are stable to physiological conditions. New members of BODIPY family with shifted photophysical properties can be obtained by structural modifications. As a result of these modifications, they can be used in different application areas such as ion sensing, molecular logic gates, biomolecule labeling, drug delivery reagents, photosensitizers for solar cells and light harvesting systems, etc. (Loudet & Burgess, 2007). BODIPY creates absorb powerful visible region. Absorption profile of these dyes shows a strong and narrow absorption band indicating the S_0-S_1 (π-π^*) transition with a high energy's shoulder approximately 480 nm assigned to the 0-1 vibrational translation in the visible region. A much more impotent broadband approximately 350 nm shows the S_0-S_1 (π-π^*) transition (Meng, Velayudham, Smith, Luck & Liu, 2009). They have crucible molar absorption coefficients (40,000 to 80,000 M^{-1} cm^{-1}) and fluorescence quantum yields, sharp emission peaks and small Stokes' shift (~ 10 nm) (Descalzo, Xu, Shen & Rurack, 2008; Ulrich, Ziessel & Harriman, 2008). There is an equipollent narrow emission band of mirror image, which showed from the S_1 state over excitation to either the S_1 or S_2 states to the absorption spectra. A large number of BODIPYs generally exuded at wavelengths less than 600

nm, obtaining yellow color to green color emissions (500-590 nm) (Bonardi, Ulrich & Ziessel, 2008). Typical wavelengths for unsubstituted BODIPYs are nearly 500 nm for absorbing and around 510 nm for emitting. They indicate small Stokes' shifts and a mild change on the core after the vibrational relaxation and S_0-S_1 transitions (Benniston & Copley, 2008). Easy modifications can be applied to the BODIPY core for the requested performance at *α*-, *β*-, and *meso*-positions along with through substitution of the fluorine's (Figure 2). In addition to functional groups, at any situation of the BODIPY nucleus changes the photochemical configuration contingent upon the groups integrated (Bandichhor, Thivierge, Bhuvanesh & Burgess, 2006; Vos de Wael, Pardoen, van Koeveringe & Lugtenburg , 1977). Shifts from the red color to NIR are generally obtained in care of simple revision to the BODIPY core, expanding the π-delocalization's degree. Additionally, an electronic and steric interaction of the substituents affects the emissive behavior of BODIPY fluorophores. The shine and absorptive and emissive properties of BODIPYs are affected from rigidity of necklace of pendant components, much the same as their electron granting or withdrawing influencing on the conjugated core exceedinglly impresses (Hu *et al.*, 2009).

1. Structure of BODIPY Dyes:

BODIPY dyes are derived from the dipyrromethene ligand's complexation with an unsubstituted boron fragment, usually in the shape of BF_2, attained using boron trifluoride diethyl etherate ($BF_3.OEt_2$) (Loudet & Burgess, 2007; Ulrich *et al.*, 2008; Ziessel,

Ulrich & Harriman, 2007). The dipyrromethene ligand is comprised of the binding of α-position of two pyrroles by means of a methine bridge. This complexification generates a solid tricyclic system and avoids the dipyrromethene's geometric isomerization (cis/trans). Also permitting for the aggregation of π-electrons throughout the carbon-nitrogen backbone, which guides to extraordinarily high fluorescence quantum yields (Descalzo *et al.*, 2008). There have been several approaches accepted for the systematic called the BODIPY core. According to IUPAC system naming and numbering of BODIPY core is given in Figure 2 (Tram, Yan, Jenkins, Vassiliev & Bruce, 2009). In this system, carbons numbered as 3 and 5 are denoted as alpha (α), carbons numbered as 1, 2, 6 and 7 are denoted as beta (β) and carbon numbered as 8 is denoted as the *meso* (Descalzo *et al.*, 2008; Harreus, 2000).



4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (BODIPY)

Figure 2. Naming And Numbering Of BODIPY Based On *s*-indacene.

Considering the formal positive charge on one of the nitrogen atoms and the formal negative charge on the boron atom explains the bonding.

Single crystal BODIPY unit analysis indicates a three-ring fused framework showing strong π-electron delocalization across the nine carbons and two nitrogen collecting the two neighboring five-membered pyrrole rings and the central six-membered ring (Figure 3). B-N bonds breaks π-conjugation and shows that the BF_2 fragment plays hardly ever role in the systems expanded π-delocalization. The average bond length between N_1-C_4 indicates single bond form, whilst the N_1-C_3 indicates double bond form. Boron atom shows a formless tetrahedron BF2N2 conformation as noted by the F1-B1-F2 and N1-B1-N2 bond angles (Zheng, Xu & Prasad, 2008).

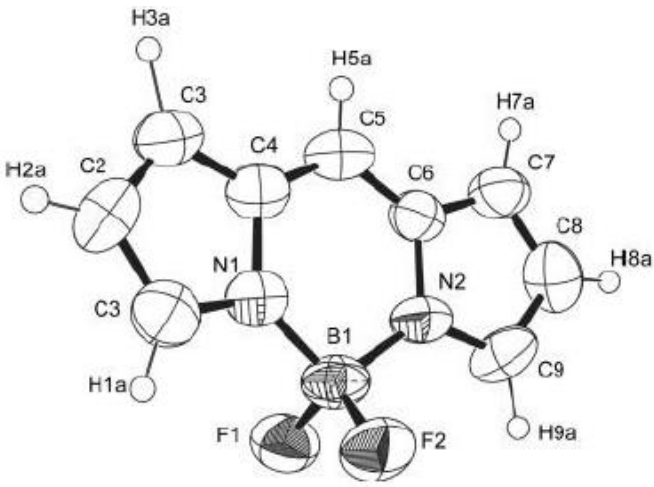
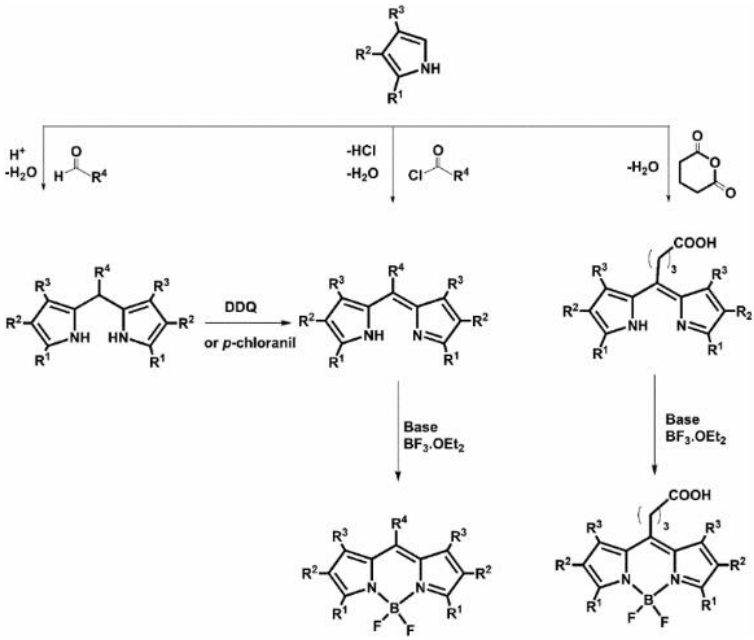


Figure 3. The Crystal Structure Of Unsubstituted BODIPY Elucidated By X-Ray Analysis (Tram *et al*., 2009).

2. Synthesis of BODIPY Dyes:

The first BODIPY was synthesized accidentally by the combination of acetic anhydride and 2,4-dimethylpyrrole towards $BF_3.OEt_2$ by Treibs - Kreuzer. Later, different methods derived for the synthesis of these fluorophores. A variety of dipyrromethene ligands are occurred by the pyrroles's interaction with excessively electrophilic carbonyl compounds. The complexation with BF_2 effects the BODIPY chromophore in aggrandizable outterns with using a secondary or tertiary amine as a base. There are different ways for synthesis of BODIPYs. A synthesis way was reported by Bruce *et al.* that the excessively reactive starting dipyrromethane was oxidized to the dipyrromethene (Tram *et al*., 2009). Oxidation by 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) was achieved at -78° under inert conditions tracked by in the relevant complexification with $BF_3.OEt_2$ to form the target BODIPY. Wild *et al.* reported the synthesis the target BODIPY with using a synthetic approach like assembling pyrrole and pyrrole-2-carbaldehyde towards an acid catalyst tracked by complexification of BF_2 towards a base (Schmitt, Hinkeldey, Wild & Jung, 2009). At the same time, Pena-Cabrera *et al.* reported a different way for synthesis the target BODIPY. They used the reaction between trimethylsilane and 8-thiomethyl BODIPY with the support of the catalytic amount of palladium in tetrahydrofuran and the stoichiometric amount of copper(I)thienyl-2-carboxylate mixed at 55°C around 45 minutes (Arroyo, Hu, , Merino, Tang & Pena-Cabrera, 2009).

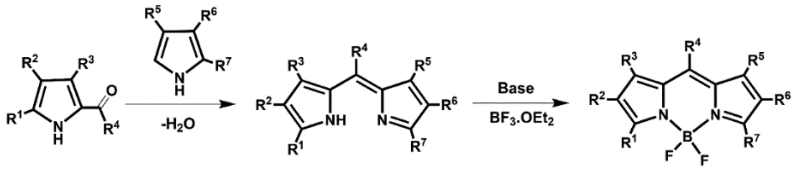
Two pyrroles's acid-catalyzed condensation with an anhydride, an acid chloride, or an aryl-aldehyde are used for synthesis of *meso*-substituted BODIPYs. Later, it should be oxidized to create the requested conjugated the dipyrrolic system. In order that avoiding the giving shape to the pyrrole chains longer than two unities, the precursor pyrrole have to get only one α-position suitable for the reactions (Goud, Tutar & Biellmann, 2006). Towards a tertiary amine reacting with $BF_2.OEt_2$, the dipyrromethene ligand is able to be complicated to shape BODIPY. *Meso*-substituted BODIPY indicates greater stability than its unsubstituted counterparts do (Wood & Thompson, 2007). The condensation of α-free pyrrole's two equivalence with an aryl aldehyde is the most common synthesis pathway of symmetrical BODIPYs. Before further complexation with BF_2 for transforming a dipyrromethane to dipyrromethene units, an oxidation step with using oxidizing agents such as DDQ or *p*-chloranil is required. A symmetrical structure including the aryl moiety at the *meso*-position is produced with using this method (Scheme 1). The condensation of two units of α-free pyrrole with an acid chloride is another widespread way for synthesizing symmetrical BODIPYs (Scheme 1). It's not necessary for an oxidation for dipyrromethene from dipyrromethane unlike the synthesis including aryl-aldehydes, frequently outcoming in major yields with less purification desired.



Scheme 1. General Procedure For The Synthesis Of Symmetrical BODIPY Via Condensation Of Two Pyrrole Units With An Activated Carbonyl.

Variations of the *meso*-position give compositionally incomparable compounds though the *meso*-group constantly has minor impact on the photophysics of BODIPY. Because of these variations, these dyes are able to be managed in many technics across many fields, from the medicinal chemistry to the materials chemistry (Loudet & Burgess, 2007). In addition, the acid anhydrides are able to be managed in symmetrical BODIPY synthesis for labeling biological molecules such as DNA, lipids and proteins (Li, Han, Nguyen, & Burgess, 2008; Li, Nguyen & Burgess, 2008).

When the dissimilar α-free pyrrole and the carbonyl-containing pyrrole to that of an acid chloride condensation gives an acid-catalyzed condensation, unsymmetrical BODIPYs produced (Scheme 2) (Sobenina *et al*., 2011). The desired BODIPY is afforded in superordinate outturns when the electron-rich pyrroles are managed by the condensation of 2-carbonyl-pyrrole with an α-free pyrrole under the catalytically acidic conditions. Pyrrole-2-carboxaldehyde's self-condensation is obligated outcoming in excessive outturns of an unwanted the symmetrical BODIPY when electron-deficient pyrroles are utilized. The resulting dipyrromethene is generally abstracted in its salt sharp that easily responds to $BF_3.OEt_2$ in the existence of the third-degree amine to afford the requested BODIPY. Formation of BODIPYs with this method including several functional fragments on the right and left hemispheres simplifying upward functionalization and bio conjugation.



Scheme 2. General Procedure For The Synthesis Of Unsymmetrical BODIPY From Carbonyl-Substituted Pyrrole.

3. Functionalization of BODIPYs for Desired Applications:

It is easy to synthesize and functionalize of electron-rich BODIPY compounds by virtue of their changeable reactivity compared to countless reaction types. BODIPY's absorption and fluorescence behaviors are excessively affected by the extent of the delocalization's

electron around the core and throughout conjugated substituents and because of this, their particular photophysical characteristics can be tuned. Owing to the chemical and photochemical features, these dyes has found wide applications in many research areas such as light harvesting molecules (Ziessel, Ulrich, Haefel & Harriman, 2013), solar cells (Ertan-Ela *et al*., 2008), photosensitizers for PDT (Mai *et al*., 2020), chemical sensors (Sun, Chen, Cheng & Marin, 2018), *etc*. (Figure 4). These applications depends on the functionalization of BODIPYs. The major BODIPY character is its chemical versatility, because its chromophores core is liable to a numerous of the reactions' chemical, which lets a detailed and generous substitution type (Figure 5). It is easy to functionalize the BODIPY core for obtain desired properties, and functionalization of BODIPY core from different positions give new compounds and open the doors for new application areas.

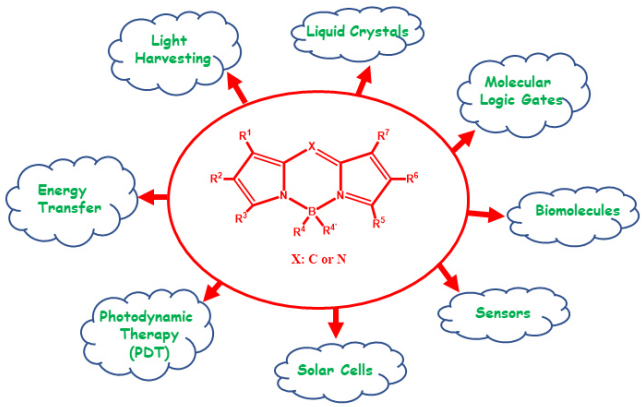


Figure 4. Most Common Application Areas For BODIPY Dyes.

Methyl's placed at the 1, 3, 5, and 7-positions are exceedingly nucleophilic owing to the electron density of the BODIPY core. Especially 3,5-methyls holding the most nucleophilic action that they are able to be easily deprotonated under temperate fundamental conditions. Generally, by the way of a Knoevenagel condensation, the deprotonated anionic methene is jointed to an electron-rich aromatic aldehyde to form the styryl group (Figure 5) (Dost, Atilgan & Akkaya, 2006; Galangau, Dumas-Verdes, Méallet-Renault & Clavier, 2010; Zhu *et al*., 2011). The functionalization from 3,5-positions denotes a bigger bathochromic shift (ca. 50 - 100 nm) than the functionalization from 2,6-positions. By the time all four methyls have turned into styryl groups it shows the greatest shift. The emission and absorption features of the BODIPY system most strongly affected by the expansion of the π-system throughout the 1, 3, 5, and 7-positions. However addition at 2,6-positions have an effect as well. In addition, chlorine or iodine which are good leaving groups occupy the 3,5-positions and allow electron-lacking BODIPYs to sustain nucleophilic the substitution reactions. A bathochromic shift in the absorption and emission spectra and fluorescence quenching in proportion to the parent dye is occurred because of halogen addition. This can be defined as heavy atom effect. By using of the palladium-mediated couplings for the aggregation of ethenyl, the ethynyl, and aryl-substituents for using in long wavelength BODIPY-basis fluorescent bio labels and bio sensing material is also consented by the existence of halogens at the 3,5-positions. 2- and 6-positions hold the least electrophilic and the least positive charge are by

electronic charting the study of the resonance structures of the BODIPY core. While leaving BF_2 chelate untouched, subsequent reaction takes place easily at this status. This modification's type is able to be managed as a pioneer for Pd-catalyzed coupling like Sonogashira (Goeb, & Ziessel, 2008), Suzuki (Zhai *et al*., 2012), Stille (Rohand, Qin, Boens & Dehaen, 2006) and Heck couplings (Chen, Mizumura, Shinokubo & Osuka, 2009).

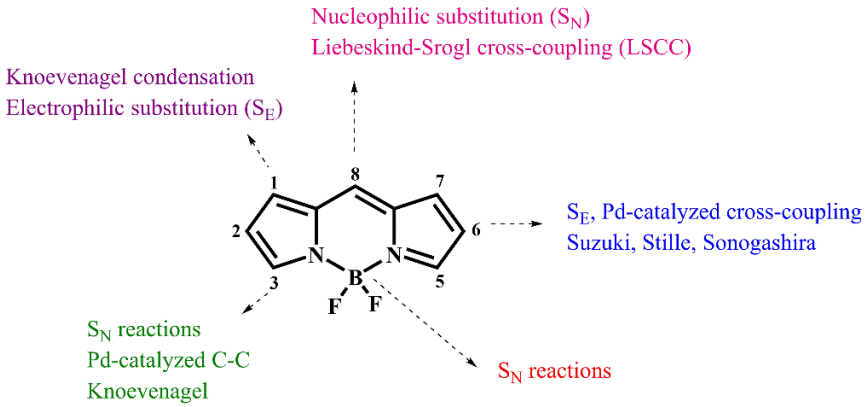


Figure 5. Main Organic Reactions Applied To BODIPY Core.

There are a few studies have reported for substitution the boron difluoride's fluorines chelate up to now. The photo's family stable, excessively luminescent redox-active *E*-BODIPYs, *O*-BODIPYs, and *C*-BODIPYs were obtained by fluorines' replacement by ethynyl, oxygen and carbon nucleophiles respectively (Loudet & Burgess, 2007). Stokes and Stability's shift of the fluorophore is enhanced by modification of the boron chelate. Generally, organometallic compounds such as an organolithium or Grignard complex is used for

substitution of fluorine. The large light-harvesting units' the grafting onto the core towards the boron chelate has been discovered to use as rapid energy transfer boxes via through-space energy transfer from the donor to the acceptor. Additions through the boron center do not affect the photophysical properties of the fluorophore like as the *meso*-substituent. These donor-acceptor systems have probable manages in molecular dyads, electroluminescent devices, energy transfer boxes, photovoltaic and supramolecular assemblies (Goze *et al.,* 2006; Goze, Ulrich & Ziessel, 2007; Harriman, Izzet & Ziessel, 2006).

A member of BODIPY family referred to as aza-BODIPYs are generated from changing of the *meso*-carbon with nitrogen (Figure 6). Like as BODIPYs, aza-BODIPYs have excessive molar extinction coefficients and temperate fluorescence quantum outturns (0.20 - 0.40). Aza-BODIPYs can be synthesized from cyanide Michael addition ammonia and products (Davies & Rogers, 1944; Knott, 1947; Rogers, 1943), and from nitroso bearing pyrroles via Michael addition products from formamide and chalcones. Aza-BODIPYs can be used as fluorescent labels (Palma *et al.,* 2009; Yoshii, Nagai & Chujo, 2010), near-IR emitting chemosensors, imaging probes (Li, Dolphin & Patrick, 2010; Loudet, Bandichhor, Wu & Burgess, 2008; Loudet *et al.,* 2008), and photosensitizers (Adarsh, Avirah & Ramaiah, 2010).

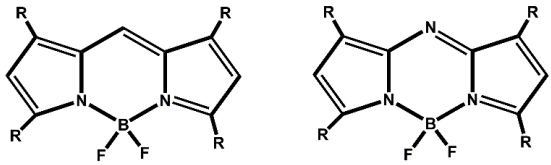


Figure 6. Structure Comparison Of BODIPY And aza-BODIPY Dyes.

According to the information given above, all of these different functionalization ways increase the versatility and possible uses of BODIPYs in different application areas immensely.

CONCLUSION

In this chapter, the fundamental structural properties, synthesis and functionalization methods and most common applications of BODIPY dyes introduced briefly. Because of their excellent structural and chemical properties these dyes are very popular especially in last decades. Up to now, various synthetic strategies was used to substitute the three available positions (alpha, beta and meso) of the BODIPY skeleton. These strategies showed that the optical, photophysical and photochemical properties of these dyes can be tuned for the desired applications by using different functional groups. Based on the recent studies on BODIPY dyes, it has seen that they have many application areas partically on biomolecules, sensors, light harvesting materials, and PDT, due to their outstanding photophysics. It appears that BODIPY dyes are still an opened door for better more efficient derivatives and will stay popular in the future.

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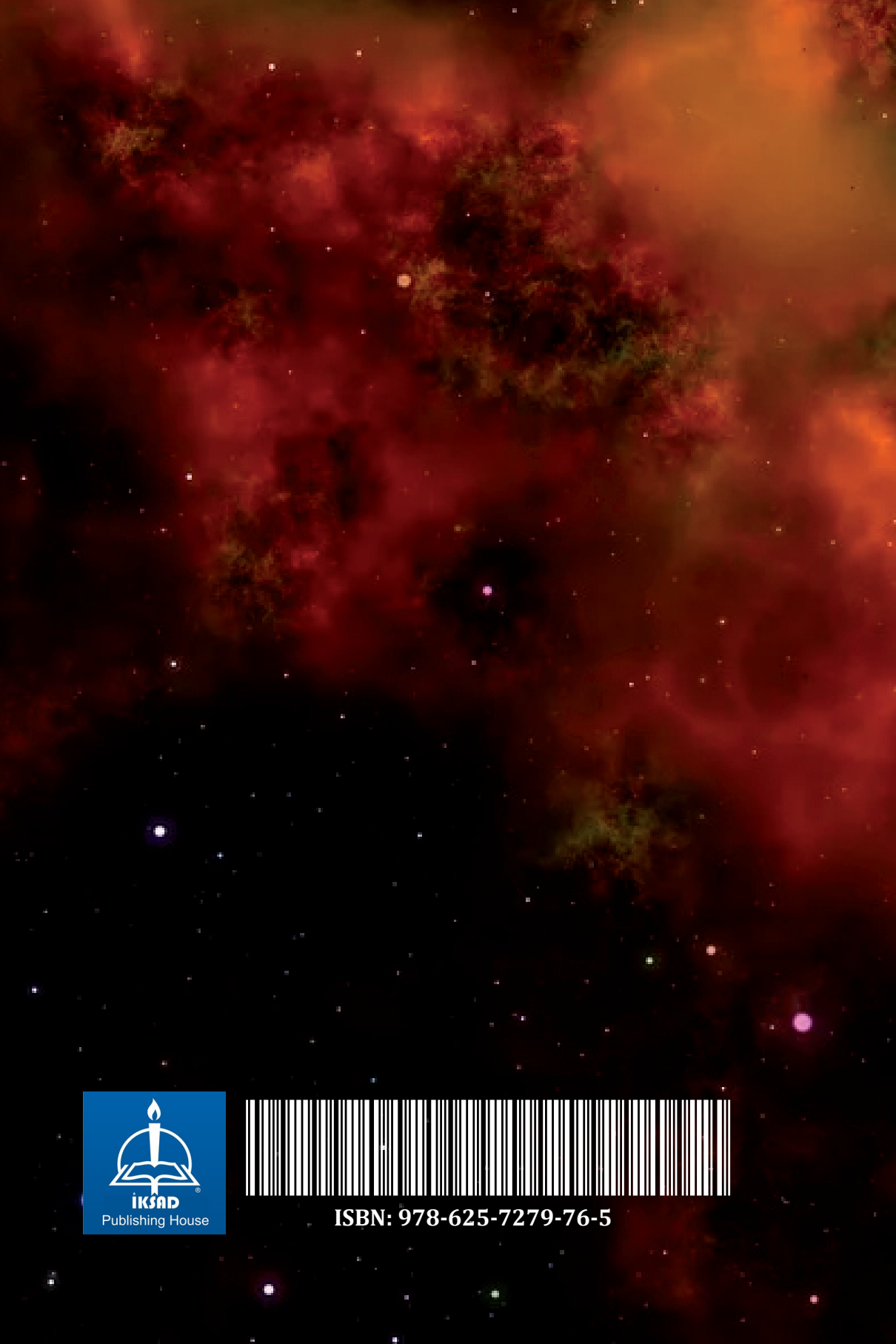

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