IN-SILICO INVESTIGATION OF TOXICITY AND FUNGAL LACCASE MEDIATED BIODEGRADATION OF BISPHENOL A AND PHTHALATE ESTERS

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ABSTRACT

Phthalates and bisphenol A, commonly used plastic additives are known to cause severe toxicity to the health and environment. They are one of the most prevalent chemicals in the environment often disposed from plastic and packaging industry. Bioremediation acts as an environment friendly approach, which holds immense potential in the degradation of these toxic chemicals. However, direct implementation of *in-vitro* bioremediation for the screening of potential micro-organisms is a time-consuming and resource extensive task. In-silico methodology provides a rapid, cost-effective approach for predicting the toxic hazards and potential microbes or enzyme for degradation. It accelerates the screening of enzyme against the pollutants and makes prediction based on binding affinity values. In this study, we have performed toxicity assessment of various PAEs and BPA using Toxtree tool and molecular docking was performed to analyse the degradation efficiency of laccase enzyme from *Thermothelomyces thermophilus* against the dimethyl phthalate (DMP), di-butyl phthalate (DBP), diethyl phthalate (DEP), benzyl butyl phthalate (BBP) and bisphenol A (BPA). Molecular docking analysis revealed high binding affinity of laccase towards BPA with binding score of 6.55 kcal/mol.

Keywords - Phthalates, bisphenol A, molecular docking, *in-silico* approach, biodegradation

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LIST OF SYMBOLS AND ABBREVIATIONS

DMP	Dimethyl phthalate
DBP	Dibutyl phthalate
DEP	Diethyl phthalate
BBP	Benzyl butyl phthalate
BPA	Bisphenol A
DEHP	Diethyl hexyl phthalate
PAEs	Phthalic acid esters
DIBP	Di-isobutyl phthalate
DPP	Dipentyl phthalate
MEP	Methyl ethyl phthalate
MiBP	Methyl isobutyl phthalate
PVC	Polyvinyl chloride
PET	Polyethylene terphthalate
PC	Polycarbonate
EDCs	Endocrine disrupting compounds
MnP	Manganese peroxidase
LiP	Lignin peroxidase
PDB	Protein data bank
QSAR	Quantitative structure activity relationship
RMSD	Root mean square deviation

CHAPTER 1

INTRODUCTION

Plastic pollution has emerged as a global environmental threat, imposing severe ecological and health hazards. The global plastic production has shown an exponential growth from 1.5 million metric tons in 1950 to 413.8 million metric tons in 2023. The degradation of plastic leads to the release of components such as microplastics and plastic additive [1]. With the global increase in the utility of plastic products, it has emerged as a persistent environmental pollutant [2]. Plastic additives are often utilized in production and processing of plastic materials to increase their flexibility and strength. Microplastic contamination in both terrestrial and aquatic habitat acts as carrier for plastic additives. Plastic additive are chemical compounds bound to the microplastics which gets released into the environment upon weathering and results in causing ecotoxicity. Various types of additives are utilized in plastic moulding process such as plasticizers, flame retardants, anti-oxidants, heat-stabilizers, foaming agents, colorants etc. Phthalate esters (PAE) and bisphenol A (BPA) are the most extensively used plastic additive utilized as plasticizers and stabilizers [3]. The migration of these additives from the environment to the food web is responsible for causing several health implications. Their accumulation in living organism causes endocrine disruption, neurological, carcinogenic and developmental toxicity [4]. Human body gets exposed to the microplastic mainly through oral intake and sometimes via inhalation and skin contact [5]. Due to the stable chemical structure and hydrophobic characteristics, PAEs and BPA cannot be degraded easily. Traditional approach towards remediation of plastics and associated additives are ineffective, since the physical and chemical methods are non-specific, and the process such as incineration leads to the formation of secondary toxic pollutants [6]. Lack of recycling infrastructure and mixed plastic waste are also one of the causes for ineffective management of plastic waste. Since, microbes possess intrinsic property of degrading various pollutants, this property is utilized in bioremediation process. Bioremediation offers the most sustainable and environment-friendly approach towards pollutant degradation. The process of biodegradation is defined as the microbes or enzymes assisted breakdown of polymeric materials into small oligomeric and monomeric forms using their metabolic mechanism. Fungal enzyme plays an important role in decreasing the toxicity level of plastic additive by transforming them into less toxic form [7]. Among various fungal enzymes, laccase has been widely studied for degradation of broad range of substrates including phenolic compounds [8].

Laccase, is a multicopper oxidase enzyme possessing oxidoreductase activity is capable of degrading various xenobiotic compounds [9]. It is secreted by various fungal species including *Trametes maxima, Melanocarpus albomyces, Aspergillus fumigatus* and *Trametes versicolor.* Laccase enzyme utilizes oxygen as electron acceptor to oxidise and degrade phenolic and non-phenolic contaminants.

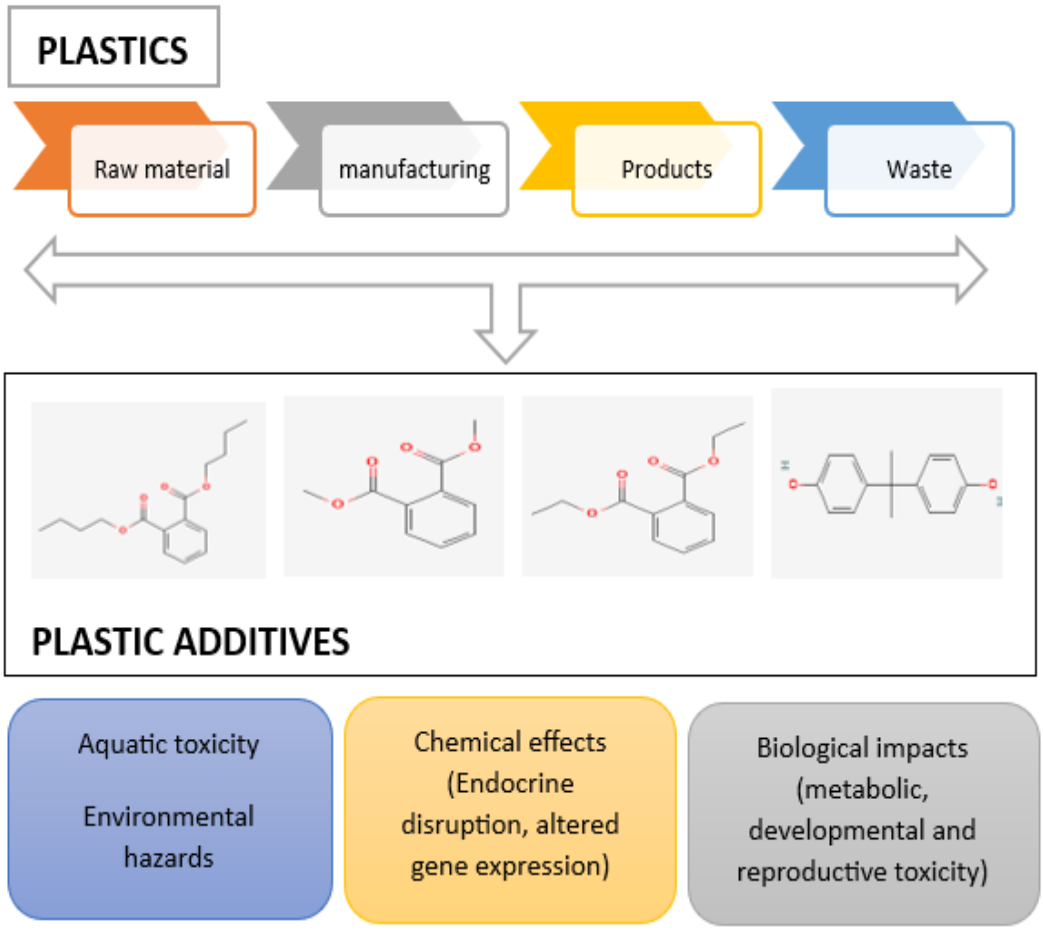

Figure 1- Sources and toxicological impacts of plastic additives

Despite efficient capability of degradation by microbial enzymes, bioremediation process has several disadvantages including incomplete degradation of pollutants, low efficiency of microbial strains and transformation of pollutants to more toxic intermediate [10]. Current bioremediation techniques do not provide complete solution towards mitigation of pollutant molecules. As we cannot assess the toxicity and

environmental consequences of intermediate metabolite and transformed compounds. Also, the experimental screening of microbes and microbial enzyme directly into the environment is a time and resource consuming method. In-silico framework provides an approach to analyse and predict the enzyme most capable of performing biodegradation. Various computational tools and software have been employed to understand the mechanism of biodegradation of plastic associated pollutants [10]. In-silico tools play a crucial role in determining the structure, function, evolutionary patterns and metabolic pathways of the microbial enzymes [11]. It helps to analyse the interaction between the various pollutants and microbial enzyme. Additionally, the toxicity prediction can also be performed using computational method [10]. Current studies on bioremediation suggests the implementation of molecular docking and molecular dynamics simulations provides insights into the binding efficiency of enzymes with various plastic additives [12]. Pathway prediction tools and algorithms are also used to predict accurate pathways for the degradation of various pollutants [12]. Therefore, computational techniques are known to provide foundational knowledge of enzyme-additive interaction at atomic level and aids the implementation of bioremediation strategy in-vitro. These techniques are utilized in screening and identification of microbial enzymes having potential to degrade the plastic additive pollutant. The persistence of plastic associated additives in the environment imposes significant health and environmental challenges. These pollutants raise concerns for both aquatic and terrestrial ecosystem. Conventional remediation strategies are not efficient enough to eradicate the toxicity and hazards caused by plastic additives. In-silico bioremediation approach serves as a sustainable, less-time consuming and eco-friendly approach. The aim of this study is to predict the toxicity and identify potential plastic additives that can be degraded using laccase enzyme from *Thermothelomyces thermophilus* using *in-silico* tools.

CHAPTER 2

LITERATURE REVIEW

2.1 Plastic additives

Plastic additive are chemical compounds bound to the microplastics which gets released into the environment upon weathering and results in causing ecotoxicity [13]. Since additives are not covalently linked to the parent plastic polymers, it can easily leach out of them. These chemical compounds are added to plastic in small amount to improve their processing and efficiency [14]. Although utilized in small amount they create a huge impact on quality of product and their utility. Recent studies signify approximately 10,000 chemical additives being used for various applications. The annual growth rate of plastic additive is expected to increase by 5.7% from 2021 to 2028. These chemicals are highly detected in water bodies and human food chain. are chemical compounds added to plastic in small amount to improve their processing and efficiency [14]. All of them possess unique characteristics that enhances the functional properties of plastics. The weak interaction between additive and the plastic polymer causes the leaching of additives, which further associates with toxic chemicals that persists in the environment as persistent environmental pollutants [15]. The toxicity associated with plastic additives gets transferred by their migration into the environment and food web [13]. The occurrence of plastic additives in the environment is caused by distinct sources including fresh water bodies, wastewater or sewage, marine habitats, and terrestrial environment. Among all, plasticizers and flame retardants accounts for the major part of additives [16]. The main purpose of adding plasticizers is to increase the plasticity and strength of polymeric plastic and reduce the production cost [16]. Phthalic acid esters (PAEs) are the most prominently used plasticizer in plastics especially PVC [17]. DEHP is the most commonly used plasticizers in PVC and for PET production DPP, DEHA, DEP, DIBP and DBP are preferred [14]. Di-ethyl hexyl phthalate and di-butyl phthalate are the most prevalently found phthalate esters in the river samples. Distinct plasticizers have been found in municipal waste water and sewage indicating them as a source of plastic additive [18]. Traces of phthalate-based plasticizers have been found in soil, dust and electronic waste recycling sites. Antioxidants are another type of plastic additives that helps in resisting the degradation of polymer when exposed to UV radiation. They are highly

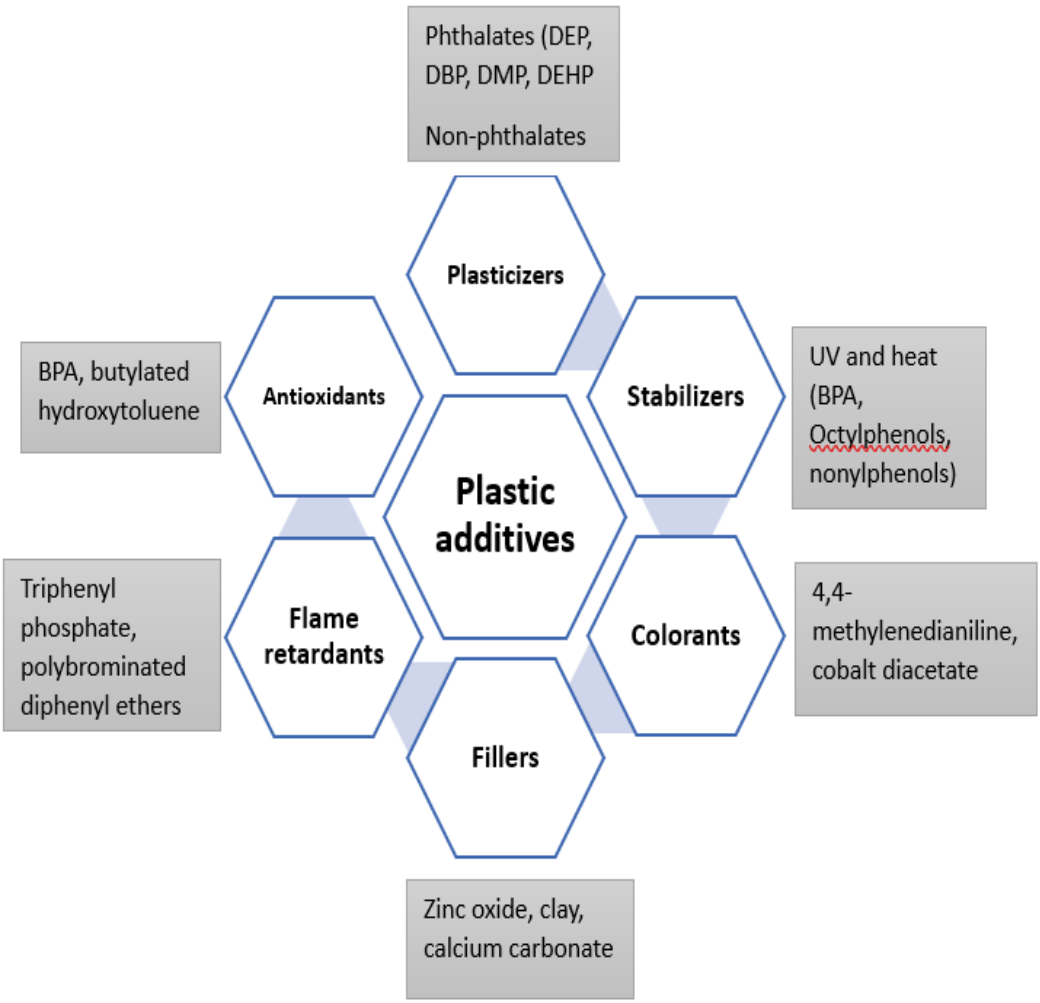

Figure 2-Types of plastic additives

utilized in food packaging products. Arylamines, phenolics and organophosphites are the some of the common antioxidants used. Flame retardants are responsible for restricting the combustibility of plastics [13]. They constitute 12-18% of plastic products. Various types of flame retardants used are halogenated flame retardants, phosphorus-based flame retardants, inorganic hydroxide containing flame retardants and melamine flame retardants. Stabilizers are the additives that restricts the breakdown of plastics. Two types of stabilizers are commonly used: UV and heat stabilizers [13]. Heat stabilizers prevent the thermal degradation of plastic polymer. Bisphenol A is a commonly used stabilizer and also utilized in monomeric form in polycarbonate plastics.

2.2 PAEs and BPA

Phthalic acid esters (PAE) and bisphenol A are present in significantly high concentration in the environment. Phthalates or phthalic acid esters (PAE), one of the most commonly found toxic chemicals in the environment is used in automotive products, pharmaceutical coating, cosmetics, toys, sprays, fragrance and insect repellents [18]. These industrial products are the major source of phthalate contamination in the environment which gets accumulated in air, soil, water and sediments. Phthalates being semi-volatile substance can easily migrate into air after leaching and cause its exposure to humans [18]. Apart from inhalation through air, humans get exposed to phthalates through food. Bisphenol A is used as both plasticizer as well as stabilizer. It is an organic chemical molecule utilized prominently in epoxy resins and polycarbonate plastic [19]. BPA containing plastics are extensively utilized in food packaging products such as disposable containers, water bottles and dairy product containing bottles and containers. Due to the contact between the food and packaging material, BPA sometimes releases into the food. This food acts as source for BPA exposure to human [19].

Table 1- Common additives and their uses

Additives	Uses	Used in polymers
Phthalates	Plasticizers	PVC, PS
Bisphenol A	Plasticizers and stabilizers	PVC, PC
Polycyclic aromatic hydrocarbons (PAHs)	Pesticides	All plastics
Dioxins	-	Various plastics
Polychlorinated biphenyls (PCBs)	Electronic equipment	All polymers
Nonylphenol	Surfactants	PVC

PAEs and BPA possess moderate to high environmental persistence, especially in anaerobic condition. Their degradation in the natural environment is a very slow process. Various parameters affect the degradation of these contaminants including the

degradative microbes, microbial activity, temperature, pH and photodegradation potential. However, some bacterial and fungal strains have been studied that degrade these contaminants to some extent, but the level of contamination in the environment is comparatively high. Moreover, the degradation intermediates formed during degradation can be more toxic and persistent.

2.3 TOXICOLOGICAL IMPACT OF PAEs and BPA

The ingestion of plastic causes both direct and indirect toxicity in the body by releasing toxic chemicals such as phthalic acid esters and bisphenol A. In-vitro studies have determined various toxicological effect of plastic additives. Some of the plastic additives are identified as endocrine disrupting chemicals (EDCs) and have been linked with disease such as diabetes, obesity, infertility, breast and prostate cancer.

Phthalic acid esters (PAE), have been noted to cause various health hazard including disruption of metabolic and immune response and skeletal development in aquatic organisms [20]. The exposure of PAE has also affected the human health from reproductive, cardiovascular, respiratory diseases to neurological disorder [21]. Multiple studies reflects that the exposure of PAE to human body causes reproductive disease by affecting the sperm count in males and precocious puberty and endometriosis in females. Precocious puberty is a condition in which early breast development and pubic hair occurs, and some association of PAE exposure in female has been linked to this condition. Some studies have reported increased concentration of phthalate esters such as MEP, MiBP, and MBP in affected individuals. Many phthalates even at a small concentration can induce critical side effects for example, PAE has been found to cause oxidative stress and further disturbs the hormonal balance of the body that can ultimately result in decrease in fertility. Hypertension, coronary heart disease and atherosclerosis are cardiovascular diseases caused due to PAE. Due to their persistence in the air, certain phthalate including DnBP, DEHP and MEHP are also responsible for causing respiratory diseases such as asthma, rhino-conjunctivitis and atopic dermatitis in children. Metabolic disorders such as diabetes, obesity has also been associated with phthalates exposure. PAEs including DBP, BBP, MBP and DEHP has been associated with attention deficit/hyperactivity disorder and other neurological disease.

Bisphenol A (BPA), in recent researches has been associated with large number of diseases including endocrine disruption, polycystic ovarian syndrome, obesity, cancer and diabetes mellitus [22]. Increased probability of neurological disorders was found

in animals due to ingestion of BPA. The exposure to minimal concentration of BPA can adversely affect the immune system, causing imbalance of microbiota [22].

Table 2-Toxic effects of BPA on human health

Type of toxicity	Impacts	Results	Reference
Male reproduction	Fertility	BPA showed negative correlation with testosterone	[23]
	Sperm quality	Decrease in sperm count and motility	[24]
Female reproduction	Hormone	Increase in Progesterone and luteinizing hormone (LH)	[25]
	Ovarian disease	Women with PCOS-high BPA concentration was found	[26]
Metabolic disorder	Type 2 diabetes	Fasting blood glucose ↑	[27]
	Cardiovascular disease	Increased risk of hypertension, systolic and diastolic blood pressure	[28]
	Obesity	Risk of abdominal obesity	[29]
	Insulin resistance	High insulin resistance	[30]
Immune function	Allergic disease	High risk of respiratory allergic diseases	[31]
	Thyroid autoimmunity	Autoimmune thyroid disease risk	[32]
Neurological disease	ADHD	↑ BPA exposure - ↑ ADHD	[33]
	Autism spectrum disorder	Positive corelation	[34]
Cancer	Breast cancer	Positive association with BPA	[35]
	Prostate cancer	Positive association with BPA	[36]

	Ovarian cancer	Positive association with BPA	[35]
Developmental disorder	Anogenital distance	Reduced anogenital distance in males	[37]
	Puberty	Delayed puberty in girls	[38]
Liver	NAFLD	Shows positive corelation with BPA	[39]
Kidney	Chronic kidney disease	Lower BPA level in CKD population	[40]

2.4 ENZYMATIC DEGRADATION OF PLASTIC ADDITIVES

Degradation of microplastics can be accomplished by both biotic and abiotic approach in the environment. However, biotic reactions that involves microbial degradation of contaminants is an environment friendly approach which makes the process of remediation less time consuming, efficient and cost-effective [41]. Microbial degradation of plastic additives is the ability of microbes or microbial enzymes to degrade or breakdown the polymeric plastic components. Microorganisms are considered as inherent decomposers of various natural polymers and also facilitate their bioconversion into value added products [42]. The degradability of plastic additives depends on various characteristics including crystallinity, molecular weight, kind of functional groups and also the additives used in plastic [43]. A large number of researches are being carried out to find novel microbes and their enzymes that facilitate the degradation of plastic [44]. The process of biodegradation involves 4 steps: biodeterioration, depolymerization, bio-assimilation and mineralization. Various bacteria and fungi have been studied for the degradation of plastic additives. These microbes are capable of producing natural surfactants and enzymes which helps them to utilize plastic additives as carbon source [45]. Biodegradation mechanism of plastic additives is carried out using 2 major types of enzymes: hydrolases and oxidoreductases [46]. The types of hydrolases used for microbial degradation include esterases, lipases, glycosidases and proteases. These enzymes undergo nucleophilic substitution reaction by adding water molecule to the substrate [47]. The major types of oxidoreductases used for biodegradation are oxidases, oxygenase, dehydrogenases and hemeperoxidases, which are involved in catalysing the oxidation-reduction reaction of the substrate [48].Extracellular enzyme initiates the biodegradation mechanism of microplastics through hydrolytic and oxidative process. The lateral chains of pollutants are cleaved using hydrolytic enzymes, whereas oxidative enzymes help in break-down of complex structure of plastic pollutants [49]. The complete biodegradation of pollutants is performed inside the cell using intracellular enzymes [45].

Phthalate esters and bisphenol A, the most commonly used plastic additives exist as persistent organic pollutant in the environment. Degradation of these organo-pollutants can be achieved by both aerobic and anaerobic microbes. Various bacterial species including *Arthrobacter* sp., *Acinetobacter* sp., *Pseudomonas* sp., *Bacillus* sp., *Rhodococcus* sp., *Sphingomonas* sp., have been studied for the degradation of phthalates. Also, Bisphenol A degrading microbes such as *Pandoraea* sp., *Serratia* sp., *Pseudomonas* sp., *Sphingomonas* sp., *Bacillus* sp., *Klebsiella* sp., have been identified in various habitats including soil, water bodies and waste water treatment plants [50].

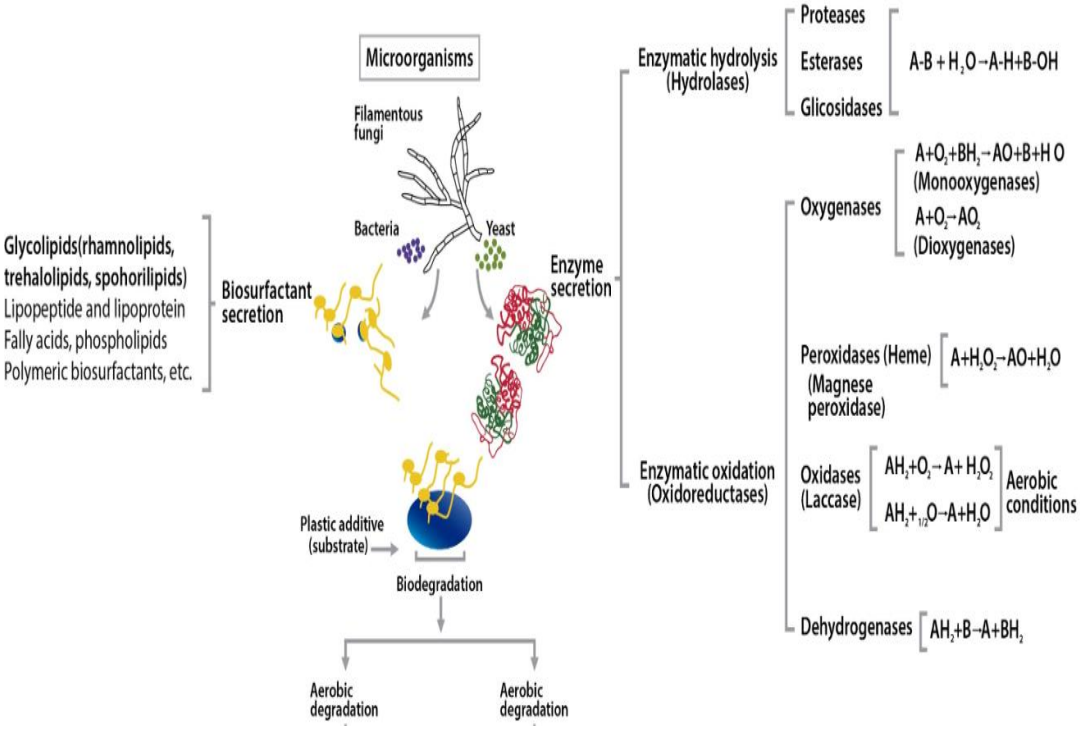

Figure 3- Classification of enzymes involved in degradation of Plastic additives (S'anchez et.al., 2021)

2.4.1 PAEs DEGRADATION BY FUNGI

The natural degradation of phthalate esters through abiotic factors such as UV light, hydrolysis, temperature is a very time-consuming process. It has been studied that the half-life of certain phthalates including DMP, BBP, and DEHP ranges between 0.3 to 2000 years. Since, degradation by micro-organisms holds immense potential, fungal species and enzymes plays a crucial role in the breakdown of hydrophobic contaminants such as phthalates. Fungal species are capable of secreting hydrolase

enzymes that helps in degrading the hydrophobic contaminants. Fungal species such as *Aspergillus niger*, *Trichoderma harzianum*, *Pleurotus ostreatus*, and *Saccharomyces cerevisiae* have been investigated for their bioremediation potential against phthalate esters.

Table 3- Recent studies on potential fungal strains for degradation of PAEs

PAEs	Fungal strains	Mechanism of action	Degradation rate (%)	Time	Reference
DEHP	*Aspergillus parasiticus*, *Fusarium subglutinans* and *Penicilliu m funiculosum*	Myco-adherence	70	14 days	[51]
DBP	*Fusarium culmorum*	Metabolism	99	9.5 days	[52]
DEHP	*Fusarium culmorum* and *T. harzianum*	Enzymatic	-	-	[53]
DEHP	*Fusarium culmorum*	Enzymatic	95	2.5 days	[54]
DBP	*Neurospora sitophyla*, *Trichoderma harzianum* and *Aspergillus niger*	Assimilation	-	-	[55]
DBP	*Fusarium culmorum*	Metabolism	99	9.5 days	[56]
DEHP	*Aspergillus niger, Penicillium sp., E. coli, Bacillus subtilis*	Metabolism	68.75	2.5 days	[57]
BBP and DiBP	*Trametes hirsuta*	Enzymatic	95	1 and 10 days	[58]
DMP	*Aspergillus versicolor*	Metabolism	100	2 days	[59]
DEP, DMP, BBP	*Pleurotus ostreatus*	-	100 for BBP	2 days	[60]
DBP	*Polyporus brumalis*	Adsorption	50	12	[61]
DPrP	*Fusarium oxysporum*	Enzymatic	70	2.5 hours	[62]

2.4.2 BISPHENOL A DEGRADATION BY FUNGI

Fungi are potential natural decomposers of various chemical compounds including BPA therefore, they are considered promising and eco-friendly biological tools for removal of BPA from the environment. Recent studies signify that filamentous and white rot fungi has played a crucial role in BPA bioremediation. They possess robust enzymatic mechanism [63]. The extracellular enzymatic system within the fungi comprises of distinct enzymes including laccase, manganese peroxidase (MnPs) and lignin peroxidases (LiPs) can degrade a broad range of phenolic and xenobiotic compounds like BPA [64]. The versatile substrate specificity of fungi makes them a potential tool in bioremediation research. These microbes are capable of surviving in harsh atmosphere with high temperature and even in nutrient deficient conditions. Examples of key enzyme involved in BPA degradation includes laccase, MnP and LiP [65]. Laccase is copper containing oxidase that catalyses the degradation through oxidation of phenolic and non-phenolic substrate.

Table 4- Fungal strains involved in BPA degradation

Fungal strains	**Enzymes**	**Efficiency (%)**	**Time**	**Condition**	**Reference**
Paraconiothyrium brasiliense	Laccase	100	10 days	30 °C, pH-7.5	[66]
M. roridum IM 6482	Laccase	100	72 hrs	-	[67]
Trametes hirsuta La-7	Laccase	100	5 days	-	[68]
Trametes polyzona WR710-1	Laccase	100	3 hrs	-	[69]
Aspergillus terreus (C10) And *A. flavus (G1)*	-	50,40	6 days	30 °C	[70]
Pleurotus ostreatus	Laccase	100	7 days	-	[71]
Myrothecium roridumIM 6482	Laccase	100	72 hrs	-	[72]

Chaetomium strumarium G5I, Thielavia arenaria CH9, Thielavia arenaria HJ22 and Thielavia arenaria SM1(III)	Laccase	100 (by *C. strumarium* G5I)	8 hrs	-	[73]
Coriolopsis gallica	Lacasse	100	3 hrs	Presence of HBT	[74]
Irpex lacteus	-	99.4	3 hrs	-	[75]
Pleurotus ostreatus	MnP	80	12 days	-	[76]

2.5 APPROACHES IN IN-SILICO BIOREMEDIATION

The advancement in computational biology and bioinformatics tool have equipped us with extensive understanding of molecular basis of interaction between plastic additives and microbial enzyme [77]. Therefore, In-silico approach is considered as an effective approach to validate the enzyme produced by micro-organism for biodegradation of polymer, and facilitates bioremediation research. This technique provides an extensive resource for prediction and analysis of cellular, molecular and genetic basis of biodegradation studies [78]. To implement the microbial degradation of pollutants in natural environment, the choice of microbial strain must be sustainable and efficient under various environmental conditions. Therefore, before executing the remediation in the *in-situ* and *ex-situ* environment, it is required to analyse the biodegradative strain or enzyme and various degradation pathways using *in-silico* approach. In-silico bioremediation involves multidisciplinary research involving genomics, proteomics, bioinformatics, mathematical model and molecular modelling. Various computational tools, databases and techniques are utilized for predicting biodegradation strategies of environmental pollutants. The 3-D structure of degradative enzyme plays an important role in understanding the mechanism of degradation which can be studied using distinct computational databases and tools such as Protein Data Bank (PDB), Molecular Modelling Database (MMDB), Class, Architecture Topology and Homology (CATH), PyMol, RasMol, Molecular Graphic Library (MGL) and Visual Molecular Dynamics (VMD). Toxicity prediction, molecular docking, molecular dynamic simulations and pathway prediction system are the most crucial techniques utilized in *in-silico* bioremediation

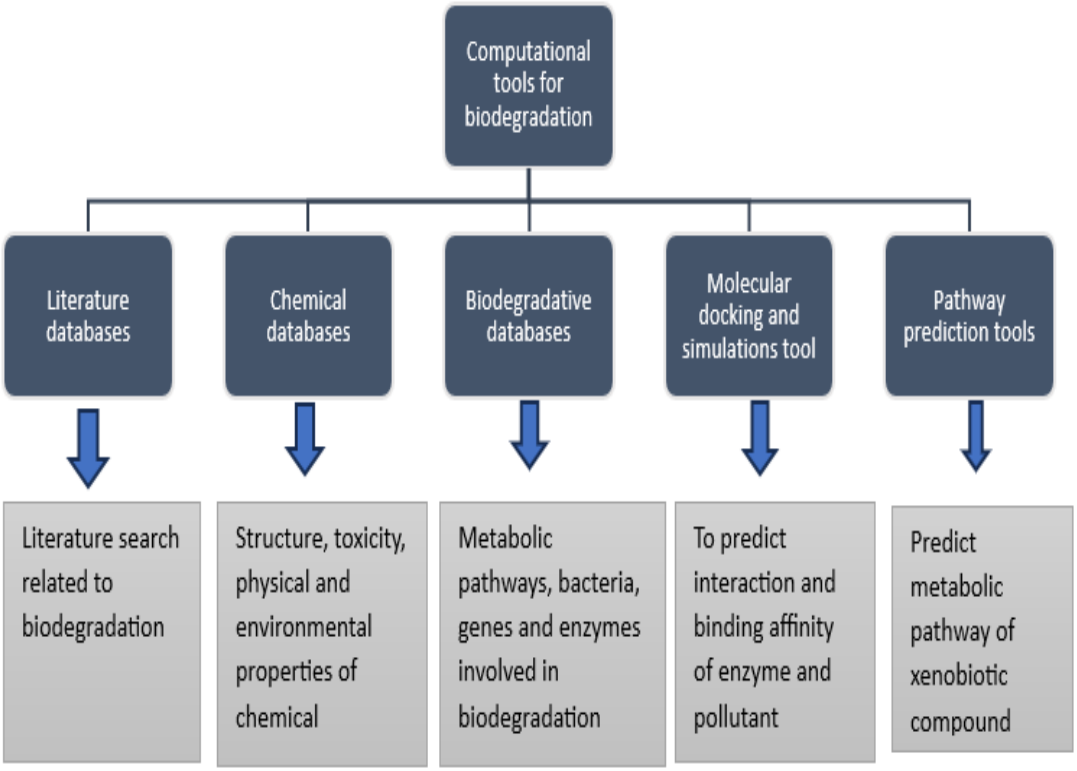

Figure 4- Computational tools and databases used to study biodegradation

Toxicity assessment, of chemicals in-vivo is costly and time-consuming method. In-silico tools serve as an efficient approach toward analysing and predicting the toxicity of various chemicals. These tools provide both qualitative and quantitative insights into toxicological data. Various computational approaches such as QSAR, Structural Alerts (SA) and rule-based method, Read Across, docking and Expert systems have been used for toxicity prediction each having certain applications and some limitations. Among other computational approach expert system consists of most convenient tools and software that utilizes various QSAR models for predicting the toxicity of distinct pollutants.

Molecular docking, a computational approach driven technique that determines the interaction between enzyme and substrate is employed for analysing microbial enzyme-pollutant interaction. The technique analyses various orientation of the substrate with enzyme and selects the one having most preferred conformation on the basis of scoring function. It also predicts the binding affinity and dissociation constant of enzyme with the substrate. In bioremediation process, the role of molecular docking

Table 5- List of computational databases and software used for biodegradation studies

TOOLS	DESCRIPTION	REFERENCES
NCLASS (The Nordic N class database on environmental hazard classification)	Information about chemicals that European commission has considered for environmental effects, classification and labelling.	[79]
OxDBase	Information about oxygenase derived from published literature and databases.	[79]
KEGG (Kyoto Encyclopedia of genes and genomes)	Description of genetic, metabolic, enzymatic and cellular progression	[80]
BioCyc	Has a collection of more than 2988 organism specific pathway/ genome database	
MetaCyc	Store information of more than 2097 experimentally designed metabolic pathways	[81]
Bionemo	Molecular understanding on the structure and function of biodegradation end product.	[82]
PathPred	Helps in predicting microbial degradation pathway for various pollutants	[83]
BNICE	Predicts new viable thermodynamic pathways based on the enzyme commission classification system's response laws	[84]
PMBD (Plastic microbial biodegradation database)	It focuses on microbial degradation of database.	[85]
EAWAG-BBD	It includes information of metabolic pathways, chemical reactions, bacteria, genes and enzymes involved in biodegradation.	[86]
ONDB (Organonitrogen database)	Provides information about chemical properties and biodegradation nature of organonitrogen compounds	[87]
ToxiPred	Used for prediction of aqueous toxicity of small chemical molecule	[88]

is to assess if the pollutant molecule binds within the active site of enzyme. To find the active site pocket of enzyme its 3-D structure is thoroughly studied. The 3-D structures of various enzymes can be retrieved using databases such as PDB and UniProt which consists of experimentally determined proteins. The unavailable 3D protein structure can be modelled using the available structural information. Further, the structure of ligand (pollutants) is retrieved using databases such as Pubchem, ChEMBL, ChemDB, ZINC and ChemSpider etc or the chemical structure can be drawn using different software.

Below is the diagrammatic representation of how *in-silico* bioremediation techniques can be utilized for toxic hazard prediction and for the investigation of potential enzyme for biodegradation. QSAR based toolset are often utilized for prediction of toxicological impact of pollutants or chemical at various biological endpoints. In-silico screening and validation of microbial enzyme is performed using techniques like molecular docking, molecular dynamics simulations and pathway prediction system.

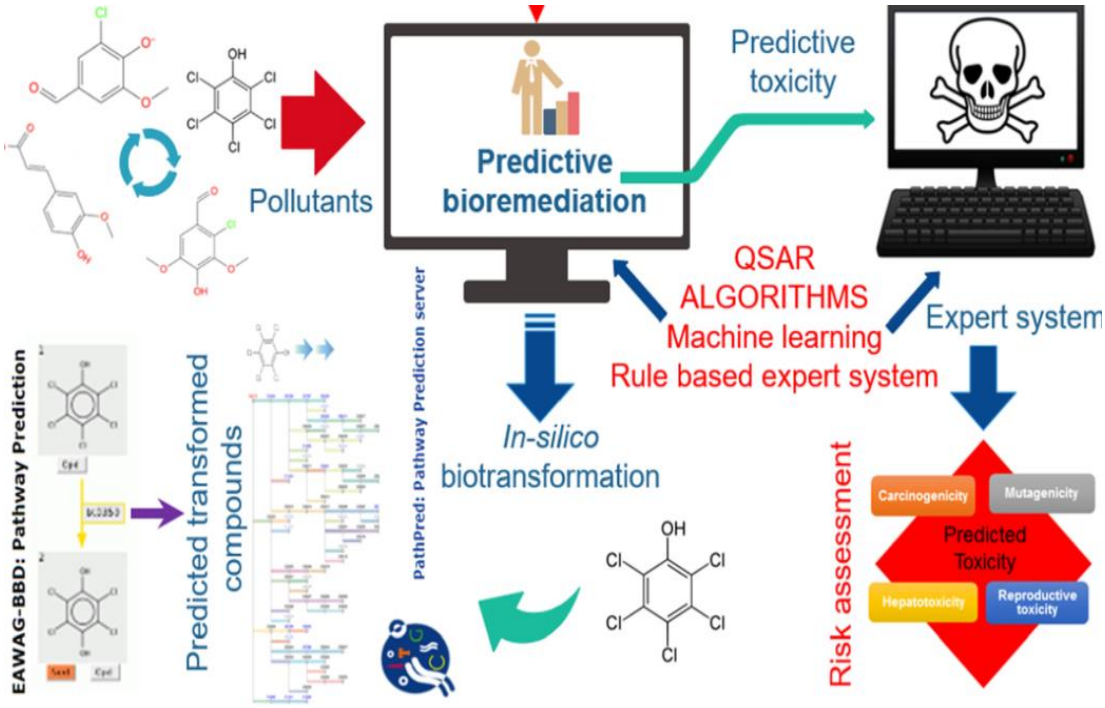

Figure 5- Diagrammatic illustration of in-silico techniques in bioremediation study (Singh et.al., 2021)

CHAPTER-3

METHODODLOGY

Plastic additives such as PAEs and BPA are highly toxic compounds found in the environment. They impose toxicity towards both health and ecosystem. In-silico toxicology is crucial component in predictive bioremediation studies that helps to investigate toxicological hazards at different end-points. Among various biocatalysts, laccase is a potential enzyme that has been studied to degrade a broad range of phenolic and non-phenolic structures. In this study computational approach was used to estimate the toxic hazard of PAEs and BPA and further the potential of laccase enzyme for the biodegradation of selected plastic additives was investigated.

3.1 Toxicity assessment using Toxtree

Toxtree is an open-source computational tool used to assess the potential risk and hazards of pollutants using decision tree approach. It classifies the compounds based on QSAR approach and implies Crammer classification to estimate the threshold of toxicology concern (TTC). There are three Crammer classes 1,2 and 3 assigned as least, intermediate and highly toxic. Other biological endpoints such as START biodegradability, carcinogenicity, mutagenicity, skin irritation, eye irritation, protein binding and DNA-binding alerts can also be explored using Toxtree. In this study, we have evaluated potential hazard of DMP, DBP, DEP, BBP and BPA on the basis of Crammer classification, START biodegradability, skin irritation and corrosion, protein binding alerts and DNA binding alerts. SMILES of these compounds were retrieved using PubChem database.

Table 6 -List of selected additives for the study and their SMILES retrieved from PubChem

Additives	SMILES
Dimethyl phthalate (DMP)	COC(=O)C1=CC=CC=C1C(=O)OC
Dibutyl phthalate (DBP)	CCCCOC(=O)C1=CC=CC=C1C(=O)OCCCC
Diethyl phthalate (DEP)	CCOC(=O)C1=CC=CC=C1C(=O)OCC
Benzyl butyl phthalate (BBP)	CCCCOC(=O)C1=CC=CC=C1C(=O)OCC2=CC=CC=C2
Bisphenol A (BPA)	CC(C)(C1=CC=C(C=C1)O)C2=CC=C(C=C2)O

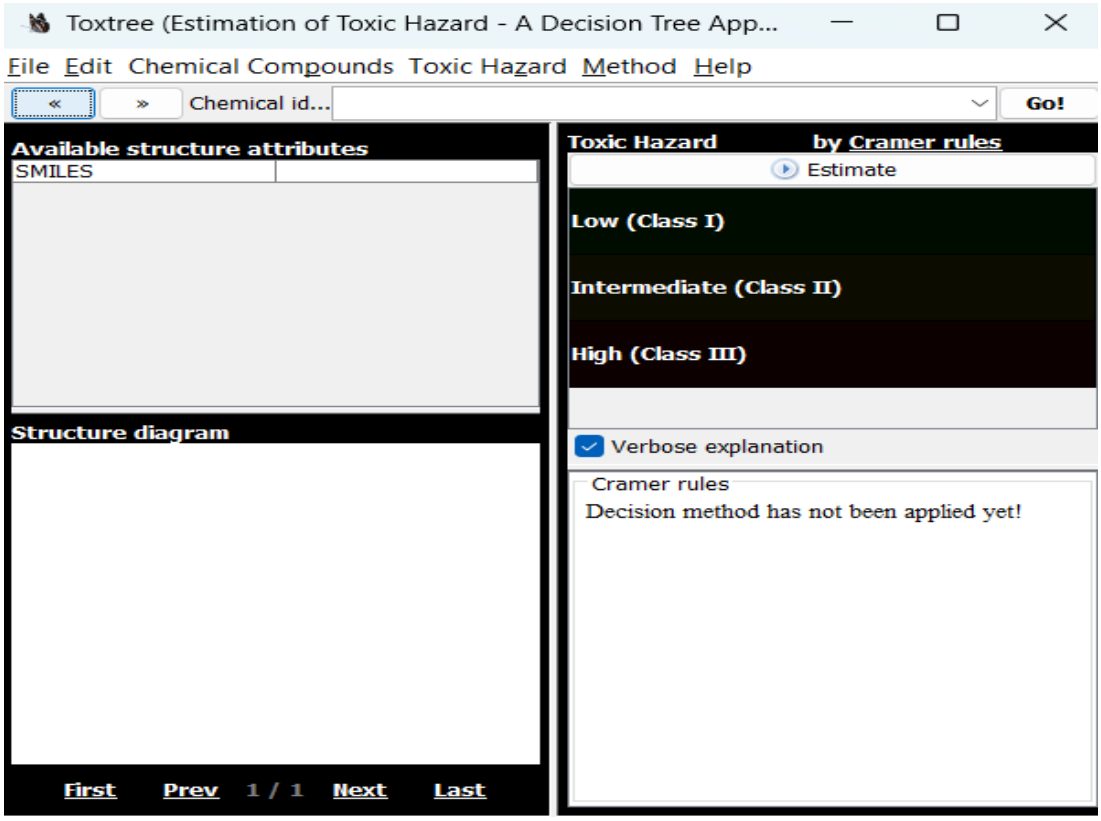

Figure 6- Search in Toxtree tool for estimation of toxic hazard

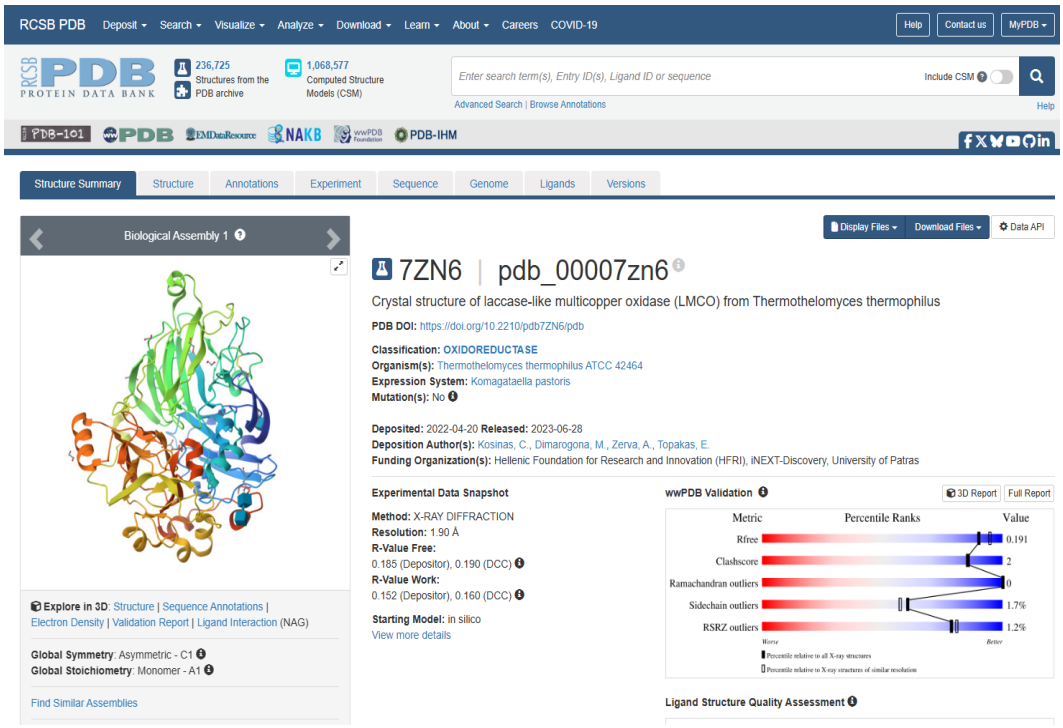

Figure 7- PDB search for laccase enzyme (PDB id - 7ZN6)

3.2 Enzyme retrieval and analysis

The crystal structure of enzyme- laccase like multicopper oxidase of *Thermothelomyces thermophilus* was retrieved from Protein Data Bank (PDB) for physiochemical analysis and molecular docking studies. The protein is annotated with PDB id- 7ZN6 and consists of two chains (A and B) and 650 amino acids. The physiochemical properties of laccase enzyme were determined using an in-silico tool Protparam which helps in analysing the chemical and biological parameters of the protein structure. Protparam tool uses UniProt ID to for the assessment of physiochemical parameters. Laccase enzyme used in this study has UniProt ID - G2QFD0. Physiochemical properties including molecular weight, atomic composition, amino acid composition, isoelectric point and extinction coefficient was determined.

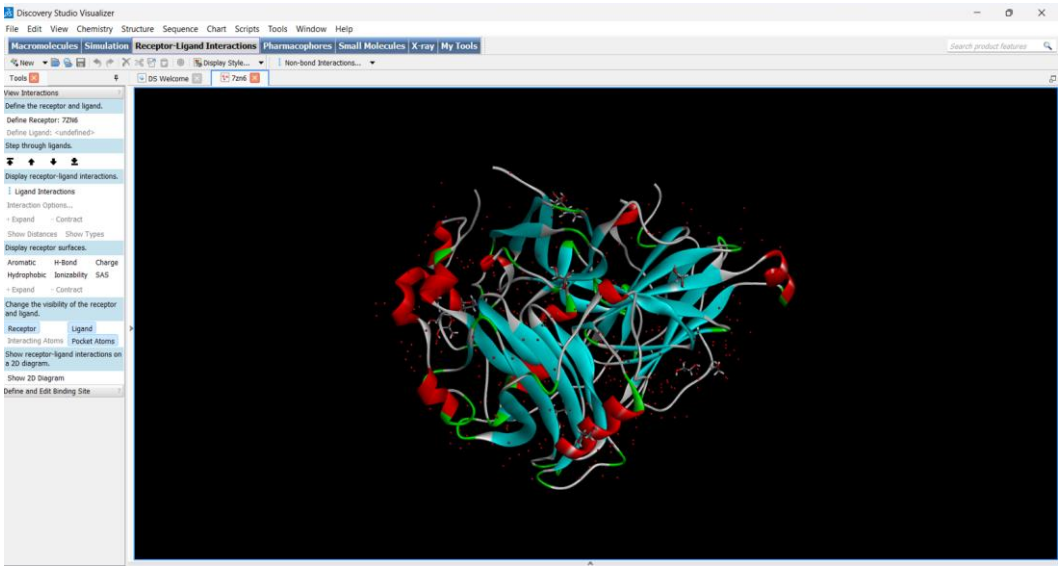

Figure 8- The PDB structure of laccase was visualized using Biovia Discovery Studio.

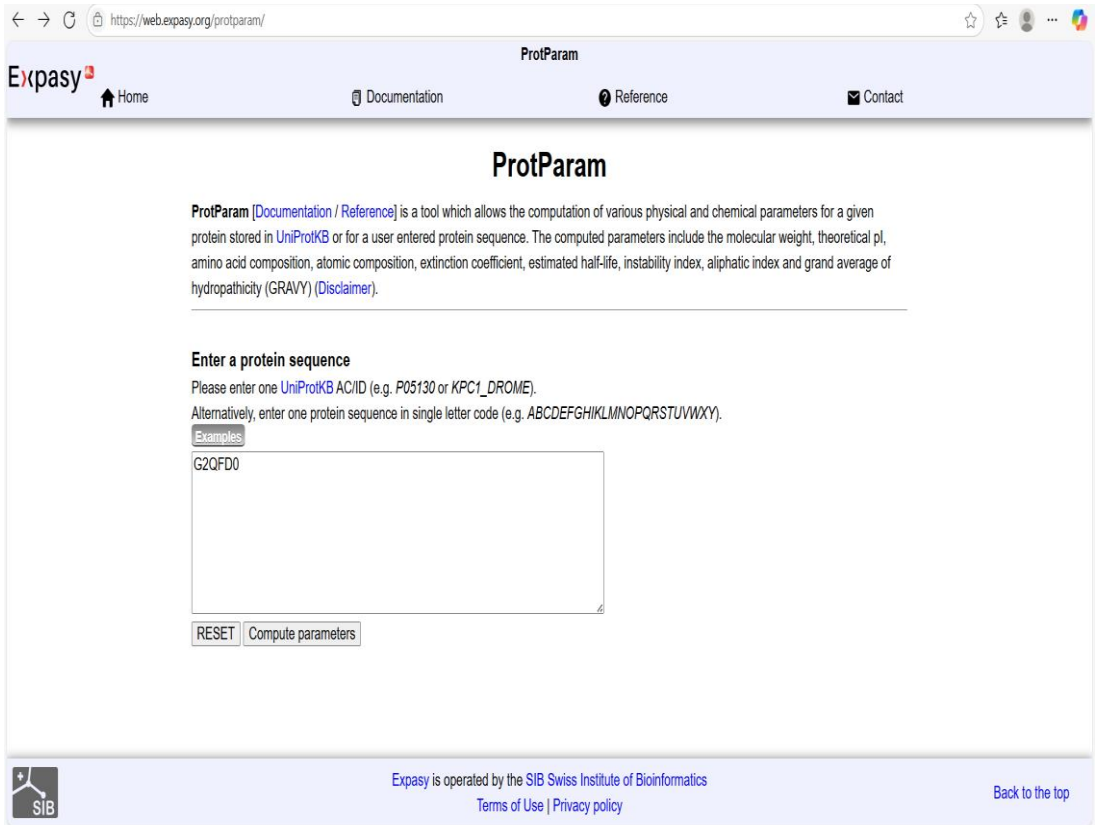

Figure 9- Protein search with UniProt ID in Expasy Protparam

3.3 Molecular docking

Molecular docking analysis requires 3-D structure of protein and ligand. They are retrieved from computational database such as UniProt, PDB and PubChem. Molecular docking was carried out using AutoDock and enzymatic interaction with target additives was visualized using Biovia Discovery studio. Among various classification of additives such as plasticizers, stabilizers, and flame retardants used in plastic processing, phthalic acid esters and bisphenol A were considered for this study. The 3-D structure of dimethyl phthalate, dibutyl phthalate diethyl phthalate, benzyl butyl phthalate and bisphenol A was retrieved from PubChem database. The structures were downloaded in structure data files (SDF) format having PubChem id 8554, 3026, 6781, 2347 and 6623 for dimethyl phthalate, dibutyl phthalate, diethyl phthalate, benzyl butyl phthalate and bisphenol A respectively. The chemical structure in SDF format was converted into PDBQT format using open babel GUI before docking. Laccase enzyme from *Thermothelomyces thermophilus* was used for docking against selected plastic additive molecules. To analyse the interaction between the plastic additives and laccase, molecular docking using AutoDockTools was performed. The enzymes were prepared by removing the interfering water molecules and heteroatoms further polar hydrogens and Kollman charge having value -44.748 was added to it. For the ligand preparation charges and polar hydrogens are added and number of torsions are selected.

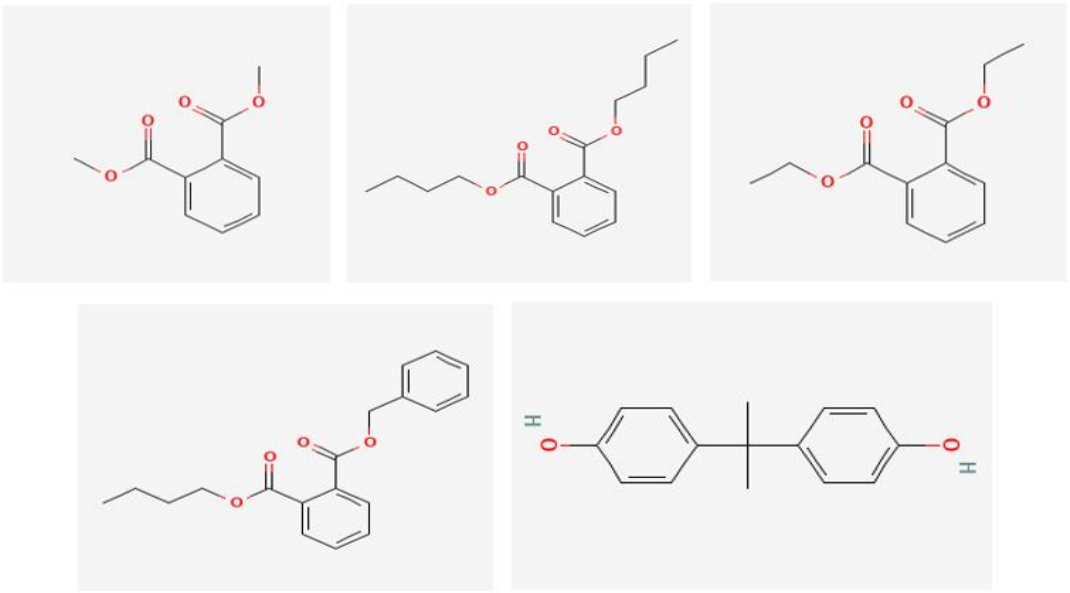

Figure 10- 2-D structures of 1. DMP, 2. DBP, 3. DEP, 4. BBP and 5. BPA

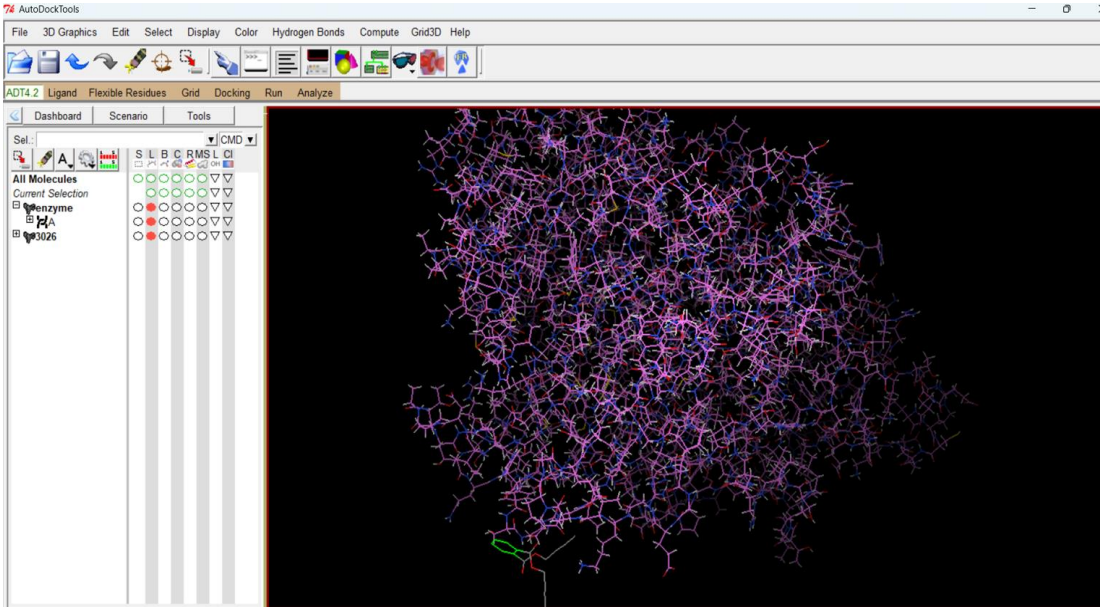

Figure 11- Protein and ligand preparation in AutoDock Tool

Blind docking of dimethyl phthalates, dibutyl phthalates, diethyl phthalate, benzyl butyl phthalate and bisphenol A against the three different enzymes was performed by setting docking parameter as:

Number of points in X-dimension -126

Number of points in Y-dimension – 120

Number of points in Z-dimension – 120

Spacing (angstrom) – 0.5

Centre Grid Box:

X center – 8.791,

Y center – 35.741,

and Z center - 0.324

The interactions having least binding affinity for all the three additives used in this study was analysed using BIOVIA Discovery studio. Both 2-D and 3-D interaction were visualized to determine the chemical bonds such as hydrogen bonds and hydrophobic interactions.

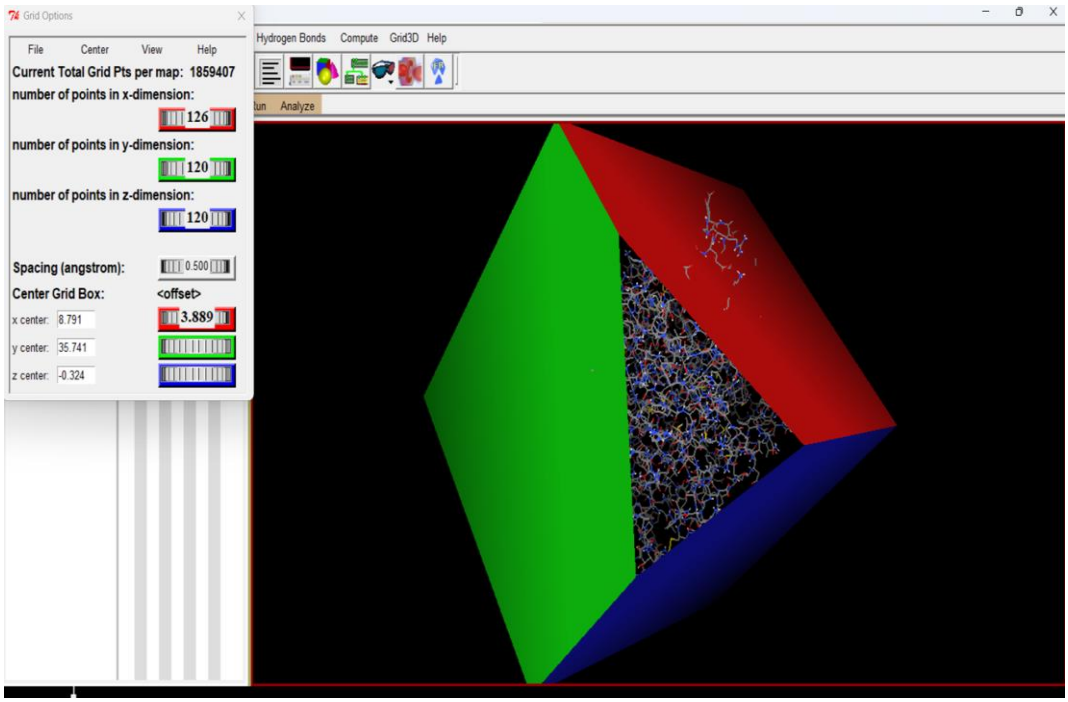

Figure 12- Setting grid box conformations for blind docking of ligand and receptor

CHAPTER-4

RESULTS

4.1 Toxtree assisted environmental hazard assessment

Toxtree version 3.1.0 was used to predict the toxic impact of phthalate esters (DMP, DBP, DEP, BBP) and bisphenol A. It supports the molecular structures of chemicals in the form of SMILES and also 2D structures can be drawn for the toxicity analysis. The crammer rule-based classification depicts Low (Class 1) for all the selected phthalate esters and High (Class 3) for Bisphenol A. Classification based on START biodegradability test suggests Class 1 (easily biodegradable chemical) for DBP, DMP, DEP and Class 2 (persistent chemical) for BBP and BPA. The toxtree analysis for Crammer class and START biodegradability is depicted in following figures.

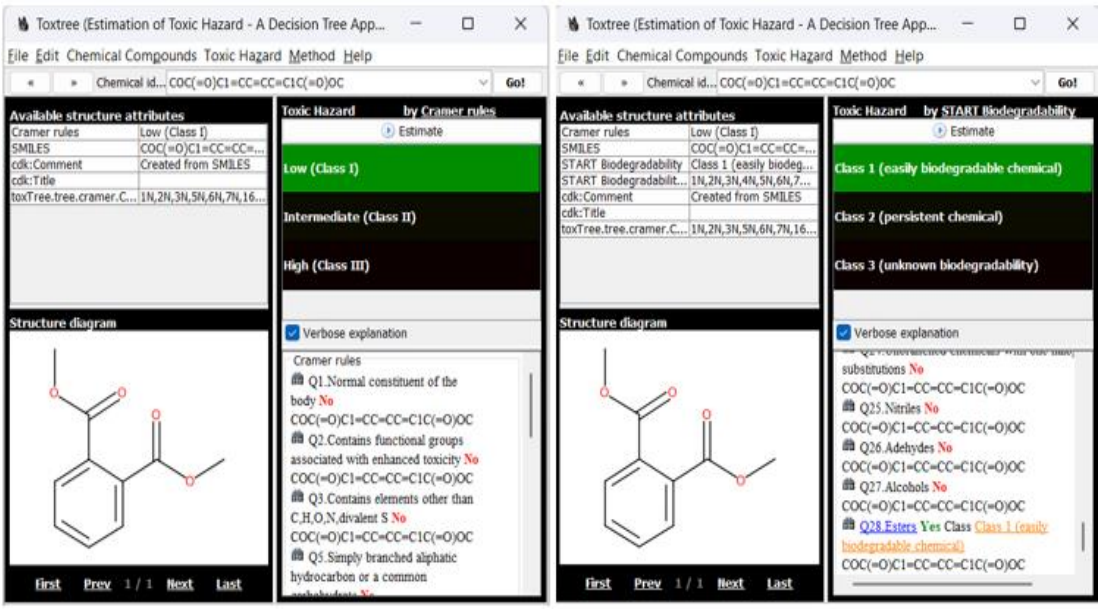

Figure 13-Crammer rules and START biodegradability prediction of DMP

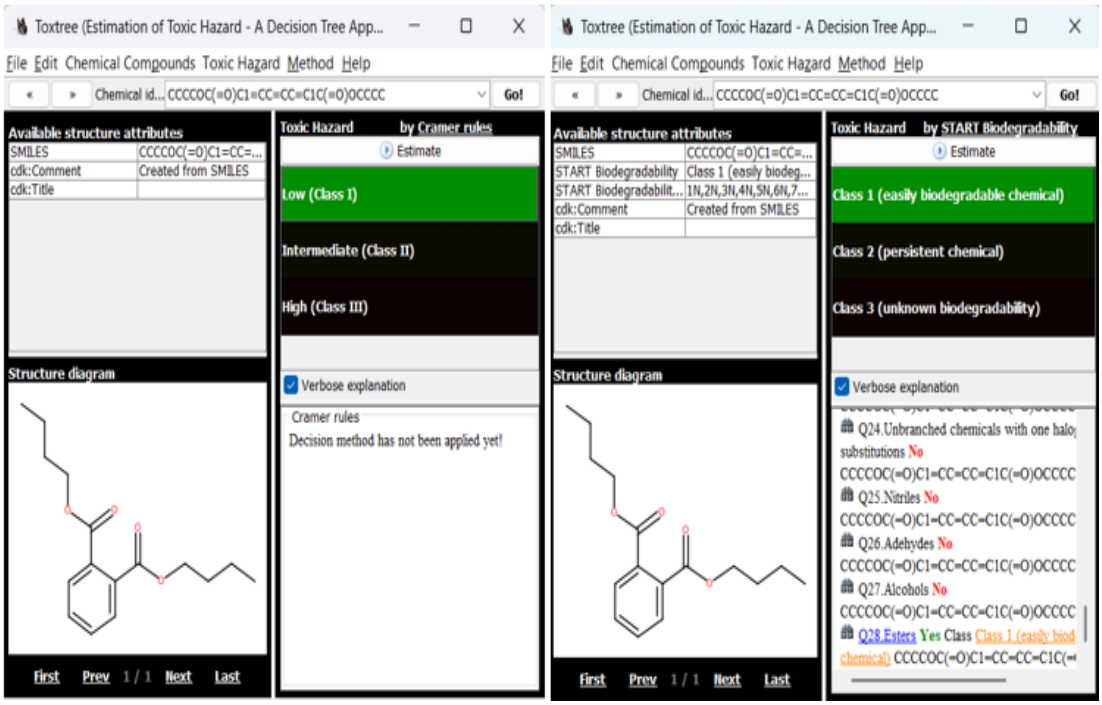

Figure 14-Crammer rules and START biodegradability prediction of DBP

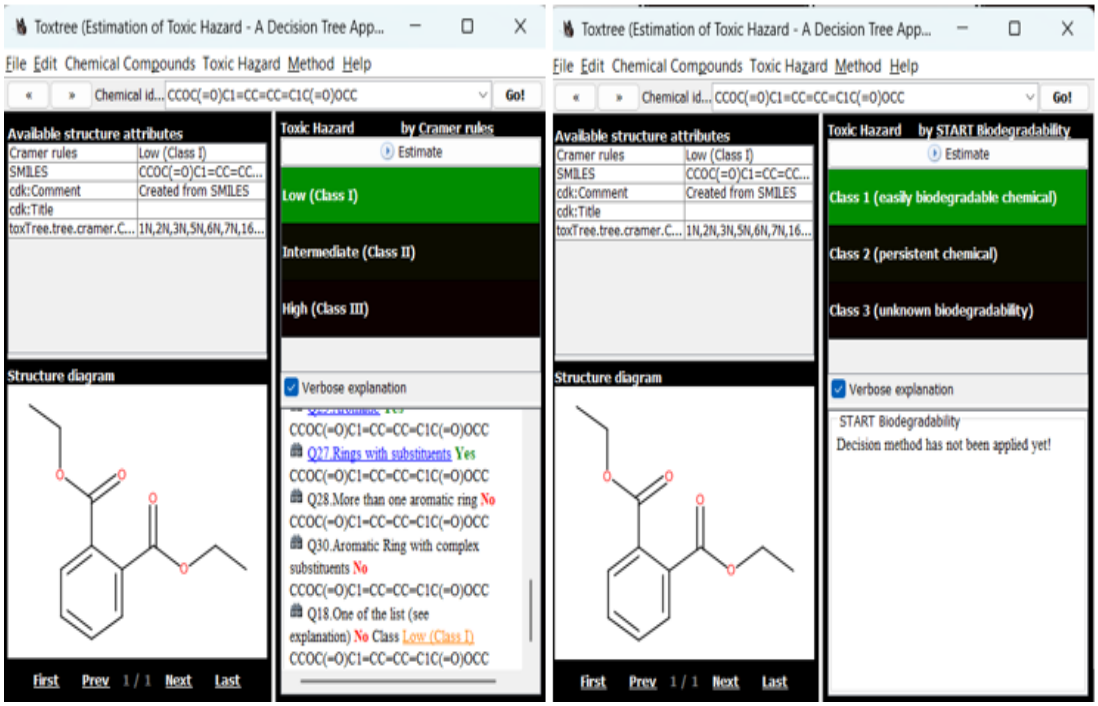

Figure 15- Crammer rules and START biodegradability prediction of DEP

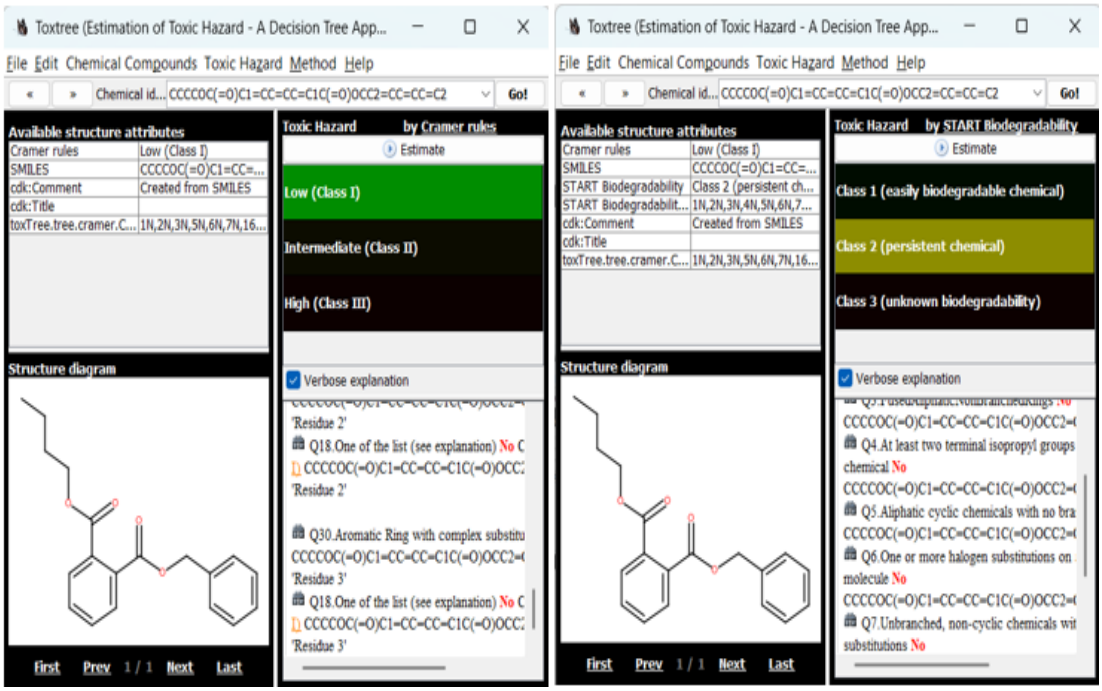

Figure 16-Crammer rules and START biodegradability prediction of BBP

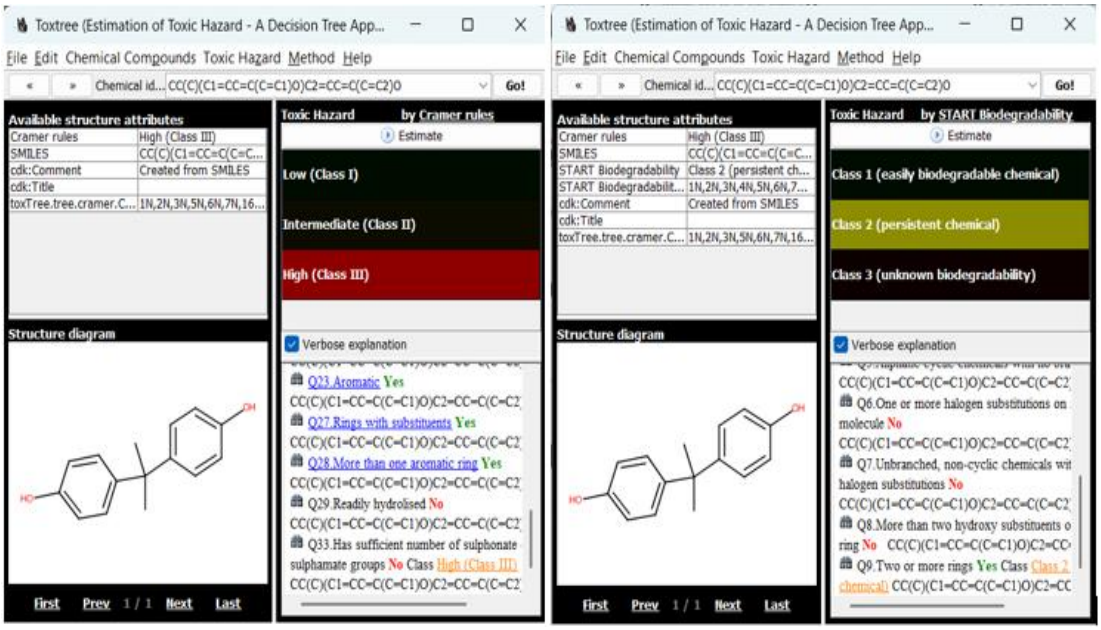

Figure 17-Crammer rules and START biodegradability prediction of BPA

Toxicity assessment on the basis of skin irritation/skin corrosion revealed DMP, DBP, DEP, BBP causes irritating to skin whereas BPA is irritating or corrosive to skin. Protein and DNA binding alerts signifies alert for Michael acceptor identified for BPA, DMP, DEP, DBP and for DNA binding alert on case of BBP alert for Michael acceptor

was identified and protein binding alert for SN2 and Michael acceptor both were identified.

Table 7-Toxic hazard estimation of various PAEs and BPA at different toxicological endpoints using Toxtree

Additives	Crammer rules	START biodegradation ability	Protein binding alert	DNA binding alert	Skin irritation and skin corrosion
DMP	Class 1 (Low)	Class 1 (easily biodegradable)	Alert for Michael acceptor	Alert for Michael acceptor	Irritating to skin
DBP	Class 1 (Low)	Class 1 (easily biodegradable)	Alert for Michael acceptor	Alert for Michael acceptor	Irritating to skin
DEP	Class 1 (Low)	Class 1 (easily biodegradable)	Alert for Michael acceptor	Alert for Michael acceptor	Irritating to skin
BBP	Class 1 (Low)	Class 2 (persistent)	Alert for Michael acceptor and SN2	Alert for Michael acceptor	Irritating to skin
BPA	Class 3 (High)	Class 2 (persistent)	Alert for Michael acceptor	Alert for Michael acceptor	Irritating and corrosive to skin

4.2 Analysis of physiochemical properties of Laccase

Protparam is an in-silico tool, which allows computation of physical and chemical properties of query protein. The query protein can be investigated in Protparam using its FASTA sequence or Uniprot id. Protparam calculates various parameters such as molecular weight, theoretical isoelectric point, amino acid composition, atomic composition, grand average of hydropathicity (GRAVY), estimated half-life, aliphatic index, instability index and extinction coefficient. The query protein in this study is Laccase like multicopper oxidase of *Thermothelomyces thermophilus* with UniProt id G2QFD0 having a total of 650 amino acids in its structure and leucine being the major constituent of 7.7% and alanine and threonine with 7.2% amino acid. The molecular weight of protein was found as 72049.69 Dalton and theoretical isoelectric point with 5.23. Total number of positively charged residues (Aspartate and glutamate) is 80 and

total number of negatively charged residues (Arginine and lysine) is 50. Total number of the atoms is 9946 with formula $C_{3214}H_{4863}N_{875}O_{970}S_{24}$. The instability index was computed as 37.70 signifying the protein to be stable. Aliphatic index and grand average of hydropathicity (GRAVY) was found as 73.82 and -0.368 respectively.

ProtParam - Results

LMCO1_THET4 (G2QFD0)

Description:

Laccase-like multicopper oxidase 1 precursor (EC 1.-.-.-) (LMCO)

Organism:

Thermothelomyces thermophilus (strain ATCC 42464 / BCRC 31852 / DSM 1799) (Sporotrichum thermophile)

The computation has been carried out on the complete sequence (650 amino acids).

Warning: All computation results shown below do **not** take into account any annotated post-translational modification.

[Documentation / Reference]

Number of amino acids: 650

Molecular weight: 72049.69
Theoretical pI: 5.23

Amino acid composition: CSV format

Ala (A)	47	7.2%
Arg (R)	32	4.9%
Asn (N)	24	3.7%
Asp (D)	44	6.8%
Cys (C)	8	1.2%
Gln (Q)	21	3.2%
Glu (E)	36	5.5%
Gly (G)	60	9.2%
His (H)	26	4.0%
Ile (I)	29	4.5%
Leu (L)	50	7.7%
Lys (K)	18	2.8%
Met (M)	16	2.5%
Phe (F)	24	3.7%
Pro (P)	44	6.8%
Ser (S)	39	6.0%
Thr (T)	47	7.2%
Trp (W)	14	2.2%
Tyr (Y)	28	4.3%
Val (V)	43	6.6%
Pyl (O)	0	0.0%
Sec (U)	0	0.0%
(B)	0	0.0%
(Z)	0	0.0%
(X)	0	0.0%

Total number of negatively charged residues (Asp + Glu): 80
Total number of positively charged residues (Arg + Lys): 50

Atomic composition:

Carbon	C	3214
Hydrogen	H	4863
Nitrogen	N	875
Oxygen	O	970
Sulfur	S	24

Formula: $C_{3214}H_{4863}N_{875}O_{970}S_{24}$
Total number of atoms: 9946

Extinction coefficients:
Extinction coefficients are in units of M^{-1} cm^{-1}, at 280 nm measured in water.
Ext. coefficient 119220
Abs 0.1% (=1 g/l) 1.655, assuming all pairs of Cys residues form cystines

Figure 18- Physiochemical parameter analysis of Laccase in Protparam

4.3 Molecular docking results

Molecular docking of enzymes against the pollutants is powerful technique which helps to predict their interaction. The analysis helps in validating the use of enzyme for bioremediation in the environment on the basis of their binding affinities. The molecular docking of dimethyl phthalate, dibutyl phthalate, diethyl phthalate, benzyl butyl phthalate and bisphenol A against the enzyme laccase was performed. The best binding interaction of all the docking performed was observed between laccase and bisphenol A showing binding affinity of -6.55 kcal/mol. The binding energy of laccase enzyme for different additives was found as DMP (-6.04), DEP (-5.19), DBP (-4.34), BBP (-5.54) and BPA (-6.55).

Rank	Sub-Rank	Run	Binding Energy	Cluster RMSD	Reference RMSD	Grep Pattern
1	1	8	-5.04	0.00	40.45	RANKING
2	1	5	-4.62	0.00	53.61	RANKING
2	2	9	-4.29	1.92	54.49	RANKING
2	3	3	-4.00	1.97	54.57	RANKING
3	1	6	-4.51	0.00	35.52	RANKING
4	1	2	-4.41	0.00	40.56	RANKING
5	1	1	-4.22	0.00	43.61	RANKING
6	1	10	-4.13	0.00	38.87	RANKING
7	1	7	-3.98	0.00	38.34	RANKING
8	1	4	-3.97	0.00	25.25	RANKING

Figure 19- Docking result of DMP with laccase

Best binding score for DMP was found in eight run as -5.04 kcal/mol with cluster RMSD value zero with partition function 10.07 and free energy as -1368.56 kcal/mol. The internal energy found was -4.32 kcal/mol and entropy of 4.58 kcal/mol/K.

Rank	Sub-Rank	Run	Binding Energy	Cluster RMSD	Reference RMSD	Grep Pattern
1	1	5	-5.19	0.00	40.79	RANKING
1	2	4	-5.07	0.82	40.97	RANKING
1	3	2	-5.05	0.76	40.73	RANKING
1	4	9	-4.96	1.04	40.53	RANKING
1	5	6	-4.80	0.96	41.18	RANKING
2	1	10	-4.51	0.00	40.37	RANKING
3	1	1	-4.10	0.00	20.67	RANKING
4	1	7	-3.98	0.00	24.77	RANKING
5	1	3	-3.94	0.00	21.94	RANKING
6	1	8	-3.55	0.00	36.90	RANKING

Figure 20- Docking result of DEP with laccase

Best binding score for DEP was found as -5.19kcal/mol in fifth run with partition function 10.08 and free energy -1358.75 kcal/mol. The internal energy was determined as -4.51 kcal/mol and entropy as 4.58 kcal/mol/K.

Rank	Sub-Rank	Run	Binding Energy	Cluster RMSD	Reference RMSD	Grep Pattern
1	1	6	-4.34	0.00	41.51	RANKING
2	1	2	-4.09	0.00	39.58	RANKING
2	2	8	-3.84	1.77	38.80	RANKING
2	3	3	-3.84	1.70	39.19	RANKING
3	1	4	-4.02	0.00	24.73	RANKING
4	1	5	-3.91	0.00	38.44	RANKING
5	1	9	-3.83	0.00	19.39	RANKING
6	1	7	-3.74	0.00	22.81	RANKING
7	1	10	-3.72	0.00	37.93	RANKING
8	1	1	-3.38	0.00	54.69	RANKING

Figure 21- Docking result of DBP with laccase

Binding score for DBP was found -4.34 kcal/mol in sixth run at cluster RMSD zero. The partition function predicted was found 10.07 and free energy as -1368.11 kcal/mol. The internal energy calculated is -3.78 kcal/mol and entropy as 4.58 kcal/mol/K.

Rank	Sub-Rank	Run	Binding Energy	Cluster RMSD	Reference RMSD	Grep Pattern
1	1	6	-5.64	0.00	36.35	RANKING
2	1	4	-5.40	0.00	39.74	RANKING
3	1	5	-5.15	0.00	54.56	RANKING
4	1	10	-4.86	0.00	37.91	RANKING
5	1	2	-4.62	0.00	36.43	RANKING
6	1	7	-4.23	0.00	37.54	RANKING
7	1	3	-3.89	0.00	43.69	RANKING
8	1	8	-3.84	0.00	48.10	RANKING
9	1	1	-3.78	0.00	39.29	RANKING
10	1	9	-3.47	0.00	53.55	RANKING

Figure 22- Docking result of BBP with laccase

Binding score for BBP was -5.64 kcal/mol in sixth run which is the least binding energy. The value of partition function found was 10.08 with free energy as -1368.73 kcal/mol. The internal energy found in -4.49 kcal/mol and entropy as 4.58 kcal/mol/K.

Rank	Sub-Rank	Run	Binding Energy	Cluster RMSD	Reference RMSD	Grep Pattern
1	1	10	-6.55	0.00	38.99	RANKING
1	2	2	-6.52	0.08	38.98	RANKING
1	3	6	-6.32	1.07	39.20	RANKING
2	1	8	-6.31	0.00	38.09	RANKING
3	1	9	-6.18	0.00	37.76	RANKING
3	2	5	-6.11	0.52	37.60	RANKING
4	1	3	-5.92	0.00	37.00	RANKING
4	2	4	-5.91	0.31	36.84	RANKING
5	1	7	-5.33	0.00	48.40	RANKING
6	1	1	-4.91	0.00	21.28	RANKING

Figure 23- Docking result of BPA with laccase

Binding score for BPA was -6.55 kcal/mol in tenth run of docking, showing the best binding affinity of enzyme laccase with BPA. The partition function found was 10.10 with free energy as -1370.25 kcal/mol. The internal energy and entropy was determined as -6.01 kcal/mol and 4.58 kcal/mol/K respectively.

4.4 Visualization of 2D and 3D interaction

Visualization tools help in analysing the interaction between biomolecules in various aspects, some of them includes determining H-bonds, hydrophobic interactions, alkyl bonds, charges and amino acids involved. BIOVIA Discovery studio is one such tool, utilized to interpret 2D and 3D interaction. The enzymes showing highest binding affinity for each plastic additive was analysed using BIOVIA Discovery studio. The 2D interaction gives clear insight into the chemical bonds and amino acid involved in the interaction. The 3D interaction additionally gives visual description of the binding region within the enzyme. Alanine, leucine, aspartate, asparagine and threonine were the amino acids present in enzyme laccase that showed interaction with DMP as shown in Fig 24. H-bonds and pi alkyl bonds were prominently involved in binding of laccase and DMP. Alanine, tyrosine and valine were the amino acid that interacted with enzyme laccase showing binding towards DBP with conventional H-bonds, alkyl and pi-alkyl interactions as shown in Fig 25. In the binding of DEP with enzyme laccase, the amino acids involved were alanine, valine, glycine, leucine and tyrosine. Alkyl, pi-sigma and conventional H-bonds were typically involved in this interaction as given in Fig 26.

BBP interacts to laccase enzyme by amino acids including proline, arginine, threonine, leucine, alanine, asparagine. Alkyl bonds and H-bond interactions are mainly depicted in the interaction. For the BPA interaction with laccase enzyme amino acids involved are arginine, tyrosine, alanine and valine through conventional H-bonds, Pi-sigma and alkyl bonds.

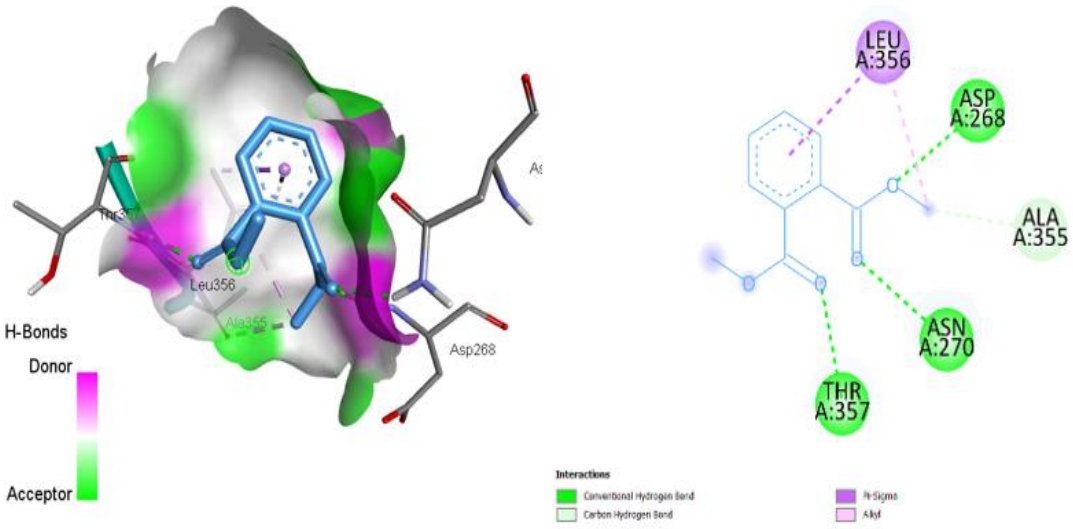

Figure 24- Visualization of 2D and 3D interaction of DMP with laccase enzyme

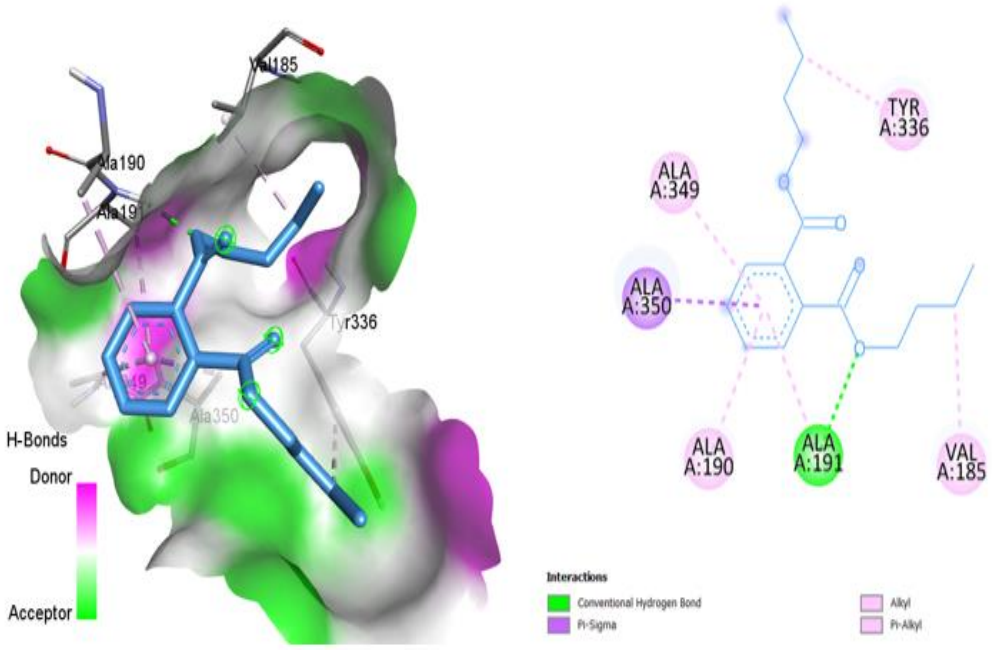

Figure 25- Visualization of 2D and 3D interaction of DBP with laccase enzyme

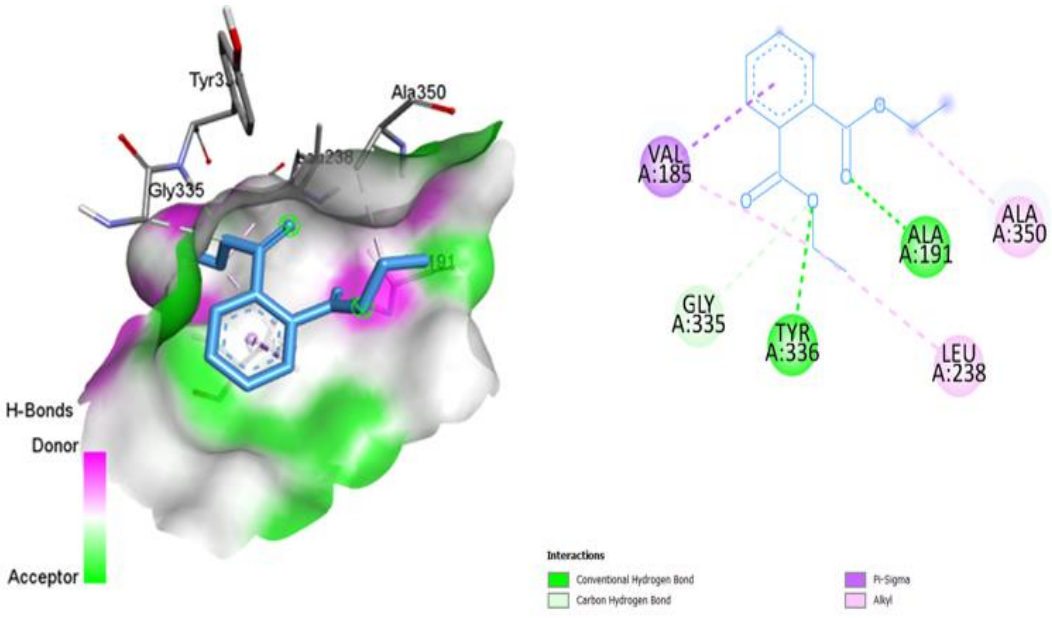

Figure 26-Visualization of 2D and 3D interaction of DEP with laccase enzyme

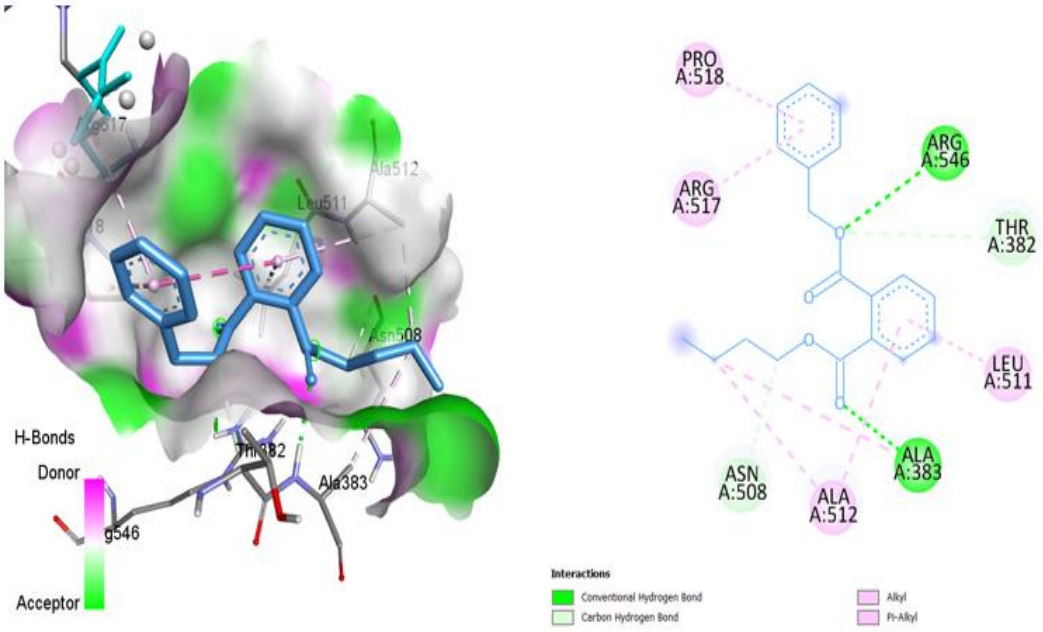

Figure 27- Visualization of 2D and 3D interaction of BBP with laccase enzyme

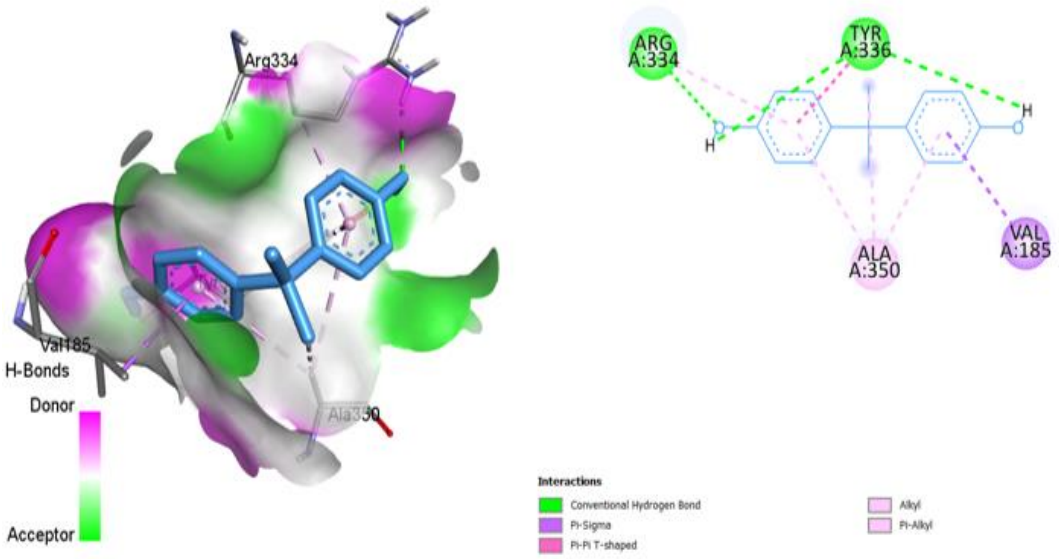

Figure 28- Visualization of 2D and 3D interaction of BPA with laccase enzyme

CHAPTER-5

FUTURE PROSPECTS

The current study highlights the use of computational approach in prediction of toxicity impacts and bioremediation techniques for degradation of toxic plastic additives including PAEs and BPA. Since, the result of molecular docking signifies strong interaction between laccase from *Thermothelomyces thermophilus* and BPA, it provides various future prospects for future research.

The study can be explored to identify different fungal and bacterial strains or enzymes that holds potential for degradation of these recalcitrant pollutants. Various enzymes such as cutinase, esterase, mono-oxygenase, di-oxygenase and peroxidase can be investigated to find the most efficient degradative enzyme for bioremediation. For further validation, molecular dynamics simulation can be implemented to evaluate the stability and conformation of enzyme and ligand over time.

To further expand the scope of research other toxic additive molecules can be investigated that are persistant and hazardous to the environment. Apart from this, metagenomic data can be expored to identify novel strains of microbes from various environment such as soil, wastewater, sludge and marine ecosystem. Genomic sequencing and analysis of such microbes can lead to the identification of novel biocatalyst for bioremediation.

Additionally, Artificial intelligence and machine learning can be employed to predict efficient degradative enzyme based on sequence and pathway analysis. AI/ML based model can also be utilized to predict biodegradation pathway and toxicity prediaction based on QSAR analysis. These models are accurately trained by large datasets of ligand enzyme interaction.

Synthetic biology approaches also plays an important role in bioremediation strategy by utilizing genetically modified strains for degradation.

CHAPTER-6

CONCLUSION

The persistence of plastic additives in the environment imposes significant health and environment hazards. Being highly resistant to degradation through environmental factors, their migration causes toxicity to both marine and terrestrial ecosystem. Human body often gets exposed to these additives through contaminated food or inhalation due to their presence in air. The remediation of these toxic pollutant in the environment is a primary requirement. Utilizing computational techniques can significantly enhance our understanding of the biodegradation mechanism since, specific bioremediation databases and tools have been designed. In this study in-silico investigation of biodegradation potential of laccase enzymes was performed against 5 different plastic additives. Due to the lack of prior knowledge of the active site, blind docking was performed. Binding affinities oxidoreductase enzyme (laccase) with plastic additives including dimethyl phthalate (DMP), dibutyl phthalate (DBP), diethyl phthalate (DEP), benzyl phthalate (BBP) and bisphenol A (BPA) was determined. It was found that laccase showed overall highest binding affinity, i.e., -6.55 towards bisphenol A. For other additives, the binding affinity of laccase was -5.04, -4.34, -5.19, -5.64 for DMP, DBP, DEP, and BBP respectively. Further, the configuration with best binding affinity was visualized and analysed in Biovia Dis covery studio software. The amino acids interacting with the target additives can be visualized in 2-D interaction analysis. Chemical interaction such as hydrogen bonds, alkyl bonds and pi-alkyl interactions were found prominently. However, the binding affinity of all the enzymes against the targets were significantly good, therefore all these enzymes can be used for further investigation of their specificity towards distinct pollutant molecule and also to design novel bioremediation strategies. Since, the binding affinity of laccase was highest for both bisphenol A, therefore it can be concluded that laccase holds immense potential in transforming a distinguished toxic compound. The enzymes used in this study can be utilized for experimental validation in future for the bioremediation of plastic additives.

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