ANNEXURE-I

ADVANCES IN MICROBIAL BIOREMEDIATION OF PETROLEUM HYDROCARBONS AND BLOCKER SEARCH TO OPTIMIZE CYTOCHROME P450 IN CONTAMINATION CLEANUP

Thesis submitted
in partial Fulfilment of the requirements for the
Degree of
MASTER OF TECHNOLOGY
in
BIOINFORMATICS
by
Pratik Kakde
(2K22/BIO/03)
Under the Supervision of
Prof. JAI GOPAL SHARMA

Department of Biotechnology
DELHI TECHNOLOGICAL UNIVERSITY
(Formerly Delhi College of Engineering)
Shahbad Daulatpur, Main Bawana Road, Delhi, 110042, India
May, 2024

ACKNOWLEDGEMENT

I would like to express my heartfelt gratitude to Professor Jai Gopal Sharma for his invaluable guidance, support, and mentorship throughout the journey of this thesis. His expertise, encouragement, and insightful feedback have been instrumental in shaping my research and academic growth. I am truly grateful for his unwavering dedication, patience, and belief in my abilities.

I would also like to extend my deepest appreciation to my parents for their endless love, encouragement, and unwavering support throughout my academic pursuits. Their belief in me and their sacrifices have been a constant source of inspiration. I am grateful for their unwavering faith in my abilities and for always being there to provide guidance and encouragement.

Furthermore, I would like to express my gratitude to the faculty members of the department for their knowledge sharing and for providing a conducive learning environment. Their passion for teaching and dedication to fostering academic excellence have had a profound impact on my education.

This thesis would not have been possible without the support and contributions of these individuals, and I am deeply grateful for their involvement in my academic journey.

ANNEXURE-II

DELHI TECHNOLOGICAL UNNIVERSITY

(Formerly Delhi college of engineering)
Shahabad Daulatpur, Main Bawana road, Delhi, India

CANDIDATE’S DECLARATION

I Pratik Kakde hereby certify that the work which is being presented in the thesis entitled “ADVANCES IN MICROBIAL BIOREMEDIATION OF PETROLEUM HYDROCARBONS AND BLOCKER SEARCH TO OPTIMIZE CYTOCHROME P450 IN CONTAMINATION CLEANUP” in partial fulfillment of the requirements for the award of the Master’s degree in Bioinformatics, submitted in the Department of Biotechnology, Delhi Technological University is an authentic record of my own work carried out during the period of January to Junne under the supervision of Prof. Jai Gopal Sharma.
The matter presented in the thesis has not been submitted by me for the award of any other degree of this or any other institute.

Pratik Kakde

This is to certify that the student has incorporated all the corrections suggested by the examiners in the thesis and the statement made by the candidate is correct to the best of our knowledge.

Prof. Jai Gopal Sharma **Signature of External Examiner**

ANNEXURE-III

DELHI TECHNOLOGICAL UNNIVERSITY

(Formerly Delhi college of engineering)
Shahabad Daulatpur, Main Bawana road, Delhi, India

CERTIFICATE

Certified that **Pratik Kakde (2k22/BIO/03)** has carried out their search work presented in this thesis entitled "**Advances In Microbial Bioremediation of Petroleum Hydrocarbons and Blocker search to Optimize Cytochrome P450 in Contamination Cleanup**" for the award of Master of Technology from department of Biotechnology, Delhi Technological University, Delhi, under my supervision. The thesis embodies result of original work, and studies are carried out by the student himself and the contents of the thesis do not form the basis for the award of any other degree to the candidate or to anybody else from this or any other University/Institution.

Prof. Yasha Hasija	**Prof. Jai Gopal Sharma**
Head of Department	**Supervisor**
Department of Biotechnology	**Department of Biotechnology**
Delhi Technological University	**Delhi Technological University**

ANNEXURE-IV

DELHI TECHNOLOGICAL UNIVERSITY

(Formerly Delhi college of engineering)
Shahabad Daulatpur, Main Bawana road, Delhi, India

PLAGIARISM VERIFICATION

Title of the Thesis ADVANCES IN MICROBIAL BIOREMEDIATION OF PETROLEUM HYDROCARBONS AND BLOCKER SEARCH TO OPTIMIZE CYTOCHROME P450 IN CONTAMINATION CLEANUP

Total Pages ____38____ Name of the Scholar__________Pratik Ramchandra Kakde

Supervisor (s)

(1) Prof. Jai Gopal Sharma
(2) ____
(3)____

Department___________BIOTECHNOLOGY, DELHI TECHNOLOGICAL UNIVERSITY

This is to report that the above thesis was scanned for similarity detection. Process and outcome is given below:

Software used:______Turnitin________ Similarity Index:____3%__ Total Word Count: 14621

Date:

Pratik Kakde **Prof. Jai Gopal Sharma**

ABSTRACT

One major issue with petroleum hydrocarbons is contamination of soil. We need practical solutions to address this significant environmental challenge and lower ecological dangers. This thesis delves deeply into the state of our knowledge on petroleum hydrocarbon contamination of soil. We are examining various sources and extent of pollution. To truly understand mechanism of contamination we are investigating a wide range of hydrocarbon molecules This includes aliphatic and aromatic components. This study primarily focuses on microbial bioremediation methods. When it comes to cleaning up soil that has been contaminated with petroleum hydrocarbons. These methods have demonstrated great potential. and sustainability Three microbial processes—biodegradation, bioaugmentation and biostimulation—are covered in detail in the thesis. We dissect each technique's intricate mechanics. To provide you with a thorough knowledge. We also highlight recent advances in molecular biology and genetics That shed light on metabolic processes and microbial interactions involved in hydrocarbon breakdown [42]. Still that's not all. We also investigate efficacy of phytoremediation—a type of plant-assisted bioremediation. This technique improves removal of petroleum hydrocarbons from contaminated soils. It combines strength of specific plant species with bioaugmentation and stimulation. We examine ways in which rhizosphere interactions between microorganisms and plants can have significant impact. Therefore, this thesis provides all the information you need. It helps understand more about how to deal with petroleum hydrocarbon-related soil pollution. It's brimming with insightful analysis. Research can assist us in resolving these urgent environmental problems.

Furthermore, paper evaluates influence of environmental variables. These include soil composition, temperature and moisture content. All affect effectiveness of bioremediation techniques. It offers valuable perspectives on enhancing remediation efficiency through optimal conditions. The possibility for enhancing conventional bioremediation techniques through the incorporation of cutting-edge technology like nano-remediation is also explored. After in depth understanding of microbial bioremediation the paper tries to provide a lead compound for blocking of Cytochrome P450CAM enzyme produced in an elevated amount by the bacterium *Pseudomonas putida* in the presence of petroleum hydrocarbons in the surrounding. While the enzyme has been proven to deal with the hydrocarbon contamination, its regulation in alter fractions of process is preferred because, the oxidation of hydrocarbons caused by the enzyme leads to the formation of various byproducts whose accumulation may cause depletion in oxygen, increase in Ph and ultimately cell toxicity. The lead compound presented in this paper with CHEMBL ID of CHEMBL330775 showed the most inhibition potential towards the enzyme CYT P450CAM. The use of this blocking agent in the later stages of bioremediation process would cause reduction in the pace of the oxidation of hydrocarbons and provide the bacterial consortium time to degrade the byproducts before accumulation, thus reducing the subsequent toxicity and avoiding cell death. Molecular docking a showed the result of 4.3 which can be considered as a decent docking score which confirms that the molecule has certain level of affinity towards the enzyme Cytochrome P450.

Keywords: *Bioremediation, microbial degradation, total petroleum hydrocarbons, phytoremediation, soil contamination, oil spills, Cytochrome P450, Pseudomonas putida.*

TABLE OF CONTENT

Sr. no.	Title	Page no.
I	**Certificate**	
II	**Abstract**	
III	**Acknowledgement**	
IV	**List of Tables**	
V	**List of Figures**	
VI	**List of Symbols and Abbreviations**	
1	**Chapter 1- Introduction**	1
	1.1 Effects of PHCs on various aspects of environment and human health	
	1.2 Types of Petroleum Hydrocarbon contamination	
2	**Chapter 2- Literature Review**	8
	2.1 Bioaugmentation	
	2.2 Biostimulation	
	2.3 Cytochrome P450 enzyme in bioremediation	
	2.4 Coupling of Phytoremediation with bioaugmentation	
3	**Chapter 3- Methodology**	21
	3.1 Search of Lead molecule	
	3.2 Docking of Cyclohexylamine to Cytochrome P450 CAM	
	3.3 Alternate lead molecule analysis and comparison with cyclohexylamine to study its in-silico effects	
4	**Chapter 4- Result**	31
	4.1 Comparison of Studied Bacteria	
	4.2 Factors influencing Bioremediation efficiency	
	4.3 Blocker Molecule for Cytochrome P450 CAM	
	4.4 Various proteins produced by microbes which have been proven effective in controlling petroleum contamination	
	4.5 Molecule docking between cyclohexylamine and cytochrome P450 CAM	
	4.6 Docking results of other searched molecules and their comparison with the molecule under study	
5	**Chapter 5- Future Prospect**	40
6	**Chapter 6- Conclusion**	41
7	**References**	43

List of Tables

Table title	**Page no.**
Table 1- Characteristics of most common hydrocarbon pollutants and its effects on human health	9
Table 2- Degradation of different components of petroleum hydrocarbon with various microbial preparations and comparison of their effects.	14
Table 3- Structures of the major enzymes involved in enhancing bioremediation of hydrocarbons and the bacterial species they are associated with.	18
Table 4- Table depicts ChEMBL IDs, molecular structures, and IC50 statuses (active or intermediate) of lead compounds, highlighting their potential as candidates for Cytochrome P450.	25

List Of Figures

Figure Title	Page no.
Figure 1- Most Observed failures that turns out to be major contributors to land oil spills.	2
Figure 2- Effects of PH contamination on environment as well as human health adapted from (Teklit et al. 2022)	5
Figure 3- Physical factors and bioremedial choices that affects the biodegradation of petroleum hydrocarbons adapted from (Teklit et al.)	19
Figure 4- List of all the Molecules whose effects have been studied on Pseudomonas putida and are available on PubChem obtained using Jupyter Notebook.	21
Figure 5- Specific molecules that show affinity mainly towards Cytochrome P450 CAM were separated using Google Colab.	22
Figure 6- The molecules were further arranged on the basis of their IC_50 value, which was labeled as Standard_Type.	23
Figure 7- The structure of Cytochrome P450 was obtained from PDB database with the id of 6WPL.	26
Figure 8- The structure of the protein visualized with the help of PyMol and cleaned of any additional molecules like water.	26
Figure 9- OpenBabel helps you convert and process chemical data formats easily when performing any pharmaceutical study. Here it was used to convert the smiles structure of the ligand to a 3D structure	28
Figure 10- The prepared ligand or the molecule of interest (CHEMBL330775) in a 3D structure observed using PyMol.	28
Figure 11- Visualized structures of the obtained molecules with CHEMBL id as CHEMBL120057, CHEMBL 116766 and CHEMBL120774.	29
. Figure 12- Most potential molecules depicted as their ChEMBL id. The IC50 value is a measure of the inhibitory potency of a compound, representing the concentration required to inhibit 50% of the enzymatic activity or biological response under study, thus indicating the compound's effectiveness as an inhibitor.	33
Figure 13- Visualization of completely cleaned and transformed protein and ligand in Autodock	35
Figure 14- The grid box dimensions were observed as this are necessary for the molecular docking programme.	35
Figure 15- The result of Molecular Docking performed with the help of Autodock Vina to determine the binding affinity of Cyclohexylamine with respect to Cytochrome P450.	36
Figure 16- Docking result of molecule named N-methyl-2-pyridylamine and was obtained from CHEMBL.	37
Figure 17- Molecular docking result of 1-(Cyclohexylmethyl)-1H- imidazole (CHEMBL120057)	38
Figure 18- The molecule with Id as CHEMBL120774 was found to be named 2-(4-ethylpiperazin-1-yl)pyrimidine and the docking results seems to determine its affinity to be moderate with the protein	39

List of Symbols and abbreviations

1. PAH: Polycyclic aromatic hydrocarbons
2. PH: Petroleum Hydrocarbons
3. EDB: Exogenous degrading bacteria
4. VOC: Volatile organic compounds
5. Cyt P450: Cytochrome P450
6. TPH: Total Petroleum Hydrocarbons
7. DDT: Dichloro diphenyl trichloroethane
8. IC: Inhibitory Concentration
9. PDB: Protein Data Bank
10. RMSD: Root Mean Square deviation

CHAPTER I
INTRODUCTION

In today’s time it’s not hidden from anyone that Environmental pollution is a big concern as a health hazard for the human civilization. The standard of living for people has increased during the last few centuries as a result of the expansion of human understanding. Environmental pollution is caused by factors such as population increase, industrialization, urbanization, deforestation, and the advancement of modern technology. The release of the toxic substances like nuclear waste, greenhouse gases, heavy metals and hydrocarbons released by the above factors are of great concern [15]. These factors all have a large negative impact on the natural processes taking place in the environment.[6] Exposure of the human or any other species to this polluted water sources, air and soil has led to the death of 8.4 million people worldwide in the year 2012. Other leading causes of death such as HIV/AIDS are responsible for about 6.9 million less deaths per year worldwide when compared to environmental pollution. The mass majority of this affected population are from Low- and Middle-income countries (LMICs) This happens because of the lack of dealing methodology against the problem. Poor countries also suffer the pollution caused by the immigrant industries. The industries empty disproportionate amount of various toxins in the atmosphere causing the local population to suffer.[1] Some major contributor to air pollution is seen to be the particulate matter from the power plants and the emission caused by the motor vehicles. Children's underdeveloped lungs induced with indoor and outdoor air pollution, possess an increased risk of respiratory infections. Asthma and allergic rhinitis have become more common in children and young adults. Ranging from minor inconvenience to death causing diseases air pollution can be responsible for them all. Looking into the WHO reports, we can say that around 2.4 million people succumb to the consequences of air pollution all around the world [2,4].

A land oil spill refers to the unintended release of petroleum hydrocarbons onto terrestrial environments, often resulting from incidents such as pipeline ruptures, storage tank leaks, or transportation accidents as shown in *Figure 1*. When oil is spilled on land, it can infiltrate soil and may percolate into groundwater, posing environmental hazards. The spilled oil undergoes physical and chemical transformations, with its components interacting with soil particles, affecting soil structure and permeability. These hydrocarbons can persist in the environment, leading to soil contamination and potential adverse effects on vegetation, microbial communities, and groundwater quality. Mitigating the impact of land oil spills involves prompt containment, cleanup efforts, and remediation strategies to minimize soil and groundwater contamination, as well as the associated with ecological and human health risk. The Exxon Valdez oil spill on March 24, 1989, was one of the significant incidents that raised public awareness of petroleum contamination. An estimated 11 million gallons of crude oil leaked into the pristine waters of Prince William Sound, Alaska, after the oil tanker Exxon Valdez grounded on Bligh Reef. The local ecology was severely damaged by the spill, with significant harm done to coastal habitats, marine life, and the livelihoods of communities who depended on fishing and tourism. In addition, the incident caused major changes in exploitation usage and transportation policies of the petroleum hydrocarbons

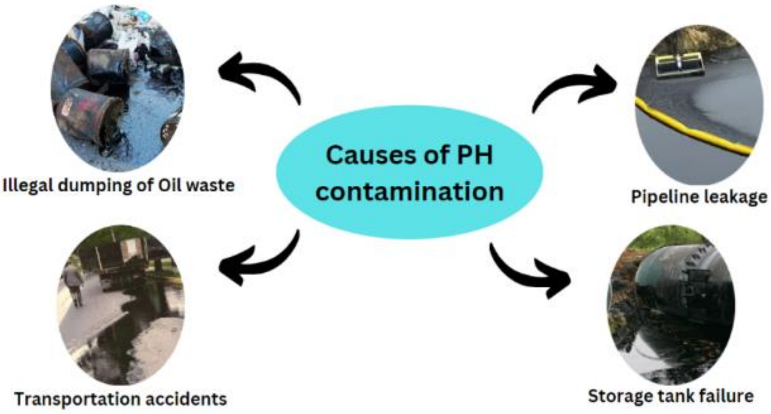


Figure 1- Most Observed failures that turns out to be major contributors to land oil spills.

Nigerian soil, groundwater, vegetation, and streams have long been contaminated by land oil spills, with spill extension being the main cause of many difficulties. Because of these, soil restoration is absolutely necessary in the host communities around oil fields and pipelines. As a result, this research offers information about the state of land oil spills today and the efficacy of on-site remediation strategies in various localities. The Shell Petroleum Development Company registered 44 land oil spill occurrences, totaling ≥ 500 bbl between 2011 and 2019, or 53,631 bbl. These accidents were mostly ascribed to 83% of the overall sabotage. The 53,631 bbl of spills were not fully cleaned up from the spill sites, negatively affecting farms, fishponds, rivers, and residential areas. Land oil spills and leakage incidents as bad they are, come in recognition because of the major health effects it can put on the population [7]. On the other hand, sea water oil spills are not taken care of as promptly as the land spills.

As the population increases, we are in constant need of fuels like petroleum. This has led us to over exploitation of the available resources mainly ocean oil fields. The systems of extraction are quite efficient but the leakage of the oil and dumping of the burnt petroleum waste into the ocean is inevitable taking into consideration the land left with us. The human dependence on petroleum and its byproducts has played a significant role in the occurrence of oil spill pollution. The extraction, transportation, and use of petroleum come with inherent risks that can lead to accidental releases and spills leading to heavy oil deposition on the sea surfaces. The deposition has been increased to such high numbers that the government is obligated to look into the methods to reduce the deposition. One of the methods that were earlier used seemed to be optimal at first but it possessed its own risks. Some of these techniques involved Mechanical containment and recovery technique [3].

By the research and data collection, it has been proven that the contamination due to the accumulation of petroleum hydrocarbons (PHCs) is the leading cause of soil contamination. This issue is so widespread because of the increased demand in the petroleum production, its storage and its utilization all over the globe. The PHCs are organic compounds and are heterogeneous in nature with carbon and hydrogen arranged in a specific pattern while it also consists of some non-hydrocarbon elements like nitrogen, sulphur, and halogens. PHC- based Pollutants with various physio-chemical properties exert a risk on human health and pose a serious environmental threat [16].

Global environmental pollution is a result of growing industrialization, mining, oil and gas extraction, and chemical extraction, all of which continuously release significant volumes of contaminants into the environment. Heavy metals and petroleum hydrocarbons (PHCs) are two of the main pollutants that are considered to be a substantial environmental danger. Of these, pollutants based on polycyclic aromatic hydrocarbons (PHCs) are causing significant harm to ecosystems, flora, and fauna, as well as unfavourable economic outcomes. These PHC-based pollutants are also extremely stable, heterogeneous organic molecules with a spectrum of physico-chemical characteristics made up of different carbon and hydrogen atoms organised structurally. Furthermore, some hydrocarbons have a few non-hydrocarbon components connected to them, such as sulphur, oxygen, and nitrogen. The two primary classifications into which it can be further divided are aromatic and aliphatic hydrocarbons [31]. Total petroleum hydrocarbons (TPH) is a term used in analytical methods that designates the quantifiable amount of petroleum hydrocarbons in environmental matrices. It also serves as a regulatory benchmark. Rapid, accurate, on-site, and reasonably priced methodologies are frequently needed for a typical assessment of TPH contamination in soil and sediment in order to support risk assessment procedures and help with site management and remediation. Many approaches of quantifying TPH and its constituent parts have been investigated. Total petroleum hydrocarbon as petrol (TPHg) and total petroleum hydrocarbon as diesel (TPHd), based on the carbon range, are the two forms of TPH that are now analysed in soil and sediment. PHCs are generally composed of around 57% aliphatic hydrocarbons, approximately 29% aromatic hydrocarbons, and approximately 14% asphaltenes with various polar components. Furthermore, aliphatic hydrocarbons exist in both saturated and unsaturated forms as linear or branched-chain hydrocarbons. the increased demand for petroleum oil has resulted in hydrocarbon contamination in soil, particularly agricultural lands, and many other ecosystems worldwide. An example of this is the persistent polycyclic ring seen in aromatic hydrocarbons, such as benzene. Although there are issues with cost-effectiveness, physical and chemical treatments are useful methods for removing large amounts of contamination from small regions [18].

Petroleum hydrocarbons encompass a vast array of compounds, each with its unique structure. The most primary structures that forms up the PHCs are alkanes (Paraffins), alkynes (Acetylenes) and alkenes (Olefins). Alkanes have a linear or branched structure, consisting of single carbon-carbon bonds and saturated with hydrogen atoms. An example is methane (CH4), where a single carbon atom is bonded to four hydrogen atoms. Alkenes contain at least one carbon-carbon double bond, leading to a degree of unsaturation in the moleculeethene (C2H4) serves as a basic example. Since it has at least one carbon-carbon double bond, it is an alkene. It is hence quite unsaturated. Ethyne is an additional illustration of an alkene (C2H2). These hydrocarbons are classified as aliphatic. Contrarily, a class of organic substances known as cycloaliphatic hydrocarbons possesses closed-ring structures together with aliphatic characteristics. Put otherwise, the arrangement of the carbon atoms is cyclical. Cycloaliphatic hydrocarbons are saturated and only contain single bonds, in contrast to aromatic hydrocarbons. There are several different types of cycloaliphatic hydrocarbons, including cycloalkanes, cycloalkenes, and cycloalkynes. Without any double or triple bonds, cyclic hydrocarbons are known as cycloalkanes. Whereas cycloalkynes feature triple bonds between carbon atoms, cycloalkenes have one or more double bonds in the ring. Aromatic chemicals have fascinating electrical characteristics that differ significantly from aliphatic chemicals in both stability and reactivity in the last, particularly. However, they are critical to synthetic organic chemistry and serve as primary feed-stocks for many industrial chemicals, drugs, and polymer syntheses. The investigation of the aromatic system is therefore important in leading our attempt to comprehend reactivity in relationships to molecular structure and attributes.

1.1 Effects of PHCs on various aspects of environment and human health:

The contamination of the soil by petroleum hydrocarbon has varied but related effects on the environment which exert damage to the ecosystem and the services they provide. The introduction of hydrocarbons alters soil physicochemical properties, leading to reduced soil porosity, water retention, and nutrient availability, thus compromising the overall soil quality. The persistence of certain hydrocarbons can impede microbial activity essential for nutrient cycling, disrupting ecological processes. Additionally, the toxicological effects on soil biota, including bacteria, fungi, and higher organisms, may result in reduced biodiversity and ecological imbalances. The mobility of hydrocarbons in the soil matrix facilitates their leaching into groundwater, potentially contaminating drinking water resources and aquatic ecosystems. Furthermore, the phytotoxicity of hydrocarbons may inhibit plant germination and growth, affecting primary productivity and disrupting trophic interactions. Persistent hydrocarbon contamination in soil contributes to long-term ecological stress, challenging the resilience and sustainability of affected ecosystems. These conditions being deteriorating for all the flora and fauna doesn’t exempt from damaging the human health in a direct or indirect way.

Petroleum hydrocarbon contamination of soil poses significant risks to human health through various exposure pathways. As depicted in *Figure 2*, Inhalation of volatile organic compounds (VOCs) released from contaminated soil can lead to respiratory irritation, exacerbate pre-existing respiratory conditions, and potentially cause systemic health effects. Direct dermal contact with contaminated soil may result in the absorption of hydrocarbons through the skin, causing skin irritation and, in some cases, dermatological disorders. Moreover, the potential migration of hydrocarbons into groundwater can contaminate drinking water sources, exposing humans to toxic compounds through ingestion. Moreover, certain hydrocarbons, such as benzene, toluene, ethylbenzene, and xylene, are carcinogenic and long-term exposure to them can lead to the development of cancer. Finally, because hydrocarbons in soil are gradually accumulated in the food chain, some crops grown in such land will contain a higher concentration of them, which will further increase the likelihood of human exposure. Thus, effective sources of risk analysis and damage prevention are essential to reduce the hazards of in using of soil contaminated with petroleum hydrocarbons for human health and protect the people who have been in contact with the substances.

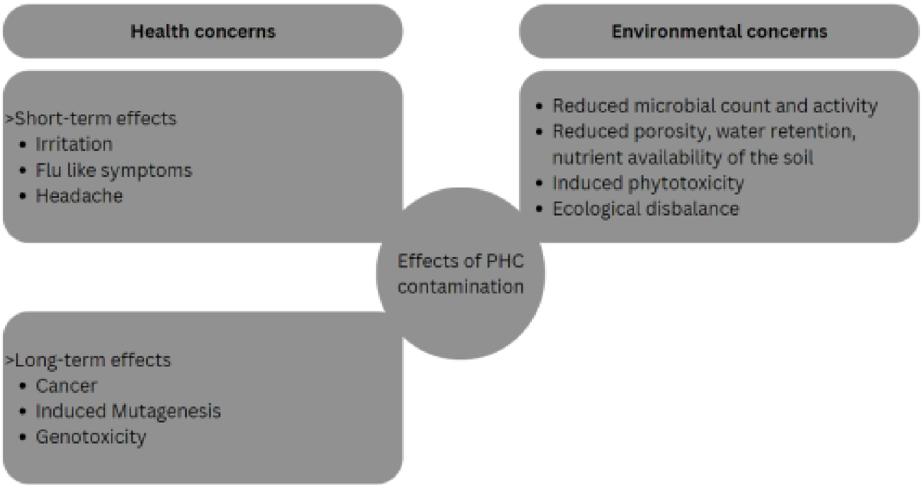


Figure 2- Effects of PH contamination on environment as well as human health adapted from (Teklit et al. 2022)

1.2 Types of Petroleum hydrocarbon contamination:

Petroleum hydrocarbons are composed of a variety of substances such as crude oil, refined products, polycyclic aromatic hydrocarbons (PAHs), and chlorinated hydrocarbons which all possess different characteristics and environmental implications. We will discuss four major types of petroleum hydrocarbon contamination including crude oil, refined petroleum product contaminants, PAH contaminants and chlorinated hydrocarbon contaminants from the perspective of their specific attributes and impacts. The Table 1 below is represented by these main harmful pollutants in major petroleum categories that have affected various aspects of biodiversity. Soil gets contaminated with crude oil when unrefined petroleum is released into the environment often through oil spillage during extraction, transportation or storage. Crude oil is made up of aliphatic and aromatic hydrocarbons that occurs as a complex mixture containing straight chain alkanes, alkenes, PAHs among others. Spilled oil can modify both the structure and composition of soil resulting in reduced fertility and altered microbial activity. Persistence of some components like PAHs poses long term ecological risks to humans [27].

Contamination of refined petroleum products includes the discharge of processed petrol and its derivatives, like benzene, gasoline or diesel fuel. Such chemicals have a spectrum of hydrocarbons including paraffins, aromatics as well as additives. Refined petroleum products can enter soil rapidly, altering its physical and chemical characteristics. Vocalization of certain components such as BTEX (benzene, toluene, ethylbenzene and xylene) compounds mainly causes air pollution while any other remnants in the soil may cause persistent pollution even through groundwater. PAH contamination is associated with polycyclic aromatic hydrocarbons which are made up of several fused aromatic rings. PAHs are often generated by incomplete combustion processes for organic materials e.g fossil fuels and wood. Their presence in soils permanently and bio-magnification pose risks towards terrestrial ecosystems as well as human health [29]. Carcinogenic properties are known for PAHs that can result to long term health effects when an individual is exposed through contaminated soils. Chlorinated hydrocarbon contamination involves compounds where chlorine atoms replace hydrogen in hydrocarbons. Common chlorinated hydrocarbons include trichloroethylene (TCE) and perchloroethylene (PCE), often used in industrial processes. These compounds can persist in soil and groundwater, posing significant risks to human health. TCE, for instance, is associated with adverse effects on the nervous system, liver, and kidneys, emphasizing the importance of addressing chlorinated hydrocarbon contamination through rigorous remediation strategies.

In the current scenario, the most frequently used remediation strategies for petroleum contamination include physical and chemical remediation, where physical remediation focuses on stabilization/solidification and pyrolysis and chemical remediation makes use of oxidation and surfactant leaching, the field of bioremediation is also being utilized. These techniques include processes like microbial remediation and phytoremediation etc. Even while PHs can be swiftly removed from soil through physical and chemical remediation, it still needs a large amount of labour and supplies [22]. Following cleanup, land productivity and soil organic matter will decline, which will have a negative impact on soil ecology. It is commonly and simply assumed that antagonistic relationships with native bacteria and soil heterogeneity are what produced the long-term and inconsistent results that the exogenous degrading bacteria (EDB) utilized for microbial remediation showed [21]. The process relies on metabolic activities of certain bacteria capable of utilizing hydrocarbons as carbon and energy source. Bacteria possess enzymes such as hydroxylases and oxygenases. These initiate degradation of hydrocarbons by introducing oxygen into molecular structure. To aid in degrading microbial bioremediation concentrates on cleaving carbon-hydrogen (C-H) bonds in petroleum hydrocarbons. Enzymes are used in first step, activating these C-H bonds making them more pliable. This helps transformation. The two main categories of enzymes essential for cleaving these bonds are oxygenases and hydroxylases. In particular, these enzymatic proteins play crucial role in breakdown of petroleum hydrocarbons. Specifically during microbial bioremediation. Because they catalyse hydrolytic reactions including bond-breaking like amide and ester in hydrocarbon molecules. Hydrolases play crucial role in this. The hydroxyl groups introduced by this enzyme activity increase hydrocarbons' reactivity. This prepares them for more oxidation.

On the other hand, oxygenases are instrumental in directly incorporating molecular oxygen into the hydrocarbon structure. Mono-oxygenases and dioxygenases, two major classes of oxygenases, catalyse the introduction of one or two oxygen atoms, respectively, into the hydrocarbon framework. The hydrocarbons' structural classification is summarised in the *Table 1* along with their effects on the environment. Although they are generally less hazardous, aliphatic hydrocarbons—which have open-chain structures—can aid in the natural attenuation of pollution. With one or more aromatic rings, aromatic hydrocarbons are more enduring and may be harmful to aquatic life. Chlorinated hydrocarbons are extremely stable compounds that can cause cancer, reproductive problems, liver and kidney damage, and substitution of chlorine atoms for hydrogen atoms. Low boiling point chemicals known as volatile organic compounds (VOCs) can irritate the respiratory tract, give rise to headaches, and have long-term negative health effects. They are also a major source of air pollution. Multiple fused aromatic rings make up polycyclic aromatic hydrocarbons (PAHs), which can cause cancer as well as problems with development and reproduction.

These enzymatic transformations enhance the hydrophilicity of the hydrocarbons, facilitating their degradation by microbial communities. Microorganisms can start the process of breaking down hydrocarbons' resistant C-H bonds by working along with oxygenases and hydrolases. This will eventually lead to the mineralization of hydrocarbons into less harmful products for the environment, like water and carbon dioxide. It is essential to comprehend the complexities of these enzymatic activities in order to optimise microbial bioremediation techniques for sites contaminated with hydrocarbons.[22].

CHAPTER II
LITERATURE REVIEW

As our civilization continues to strive for growing industrialization and growth, toxins are inadvertently being released into the air, water, and soil, negatively impacting the environment. A few antiquated techniques are still in use to clean up these problems. We offer pump and treat, chemical treatment, physical treatment, and excavation services. The primary goal of excavation is to physically remove contaminated material or soil from a location. In essence, we excavate it and transport it to a specialised location for disposal or treatment. When the contamination is localised or near the surface, this technique is quite effective at eliminating heavily contaminated soil or silt. As we look into the chemistry involved, Petroleum hydrocarbons in soil, water, or sediment are broken down or neutralised using chemical treatments, that is to say, chemicals. Oxidation is a common chemical treatment technique that converts hydrocarbons into less hazardous molecules using substances like potassium permanganate or hydrogen peroxide. Hydrocarbons can be broken down by chemical treatments, although they can be costly and lead to the creation of new contaminants. Furthermore, the type and quantity of pollutants present determine how well a hydrocarbon may be treated with chemicals; not all hydrocarbons respond well to this approach. Other useful approach would be Pump and Treat method. The main focus of this one is clearing contaminated groundwater. The dirty groundwater is pumped out first, treated to remove pollutants, and then either reinjected into the ground or returned to the environment. Activated carbon adsorption, air stripping, and biological treatment are some of the techniques used in pump-and-treat systems. Hydrocarbons can be removed from groundwater using pump-and-treat method. This can be costly and take some time to get the desired effects. It also has limitations. The capacity of treatment systems and challenges with sites that have complex hydro-geology or high groundwater flow rates make it difficult.

Innovative and sustainable ways to pollution control are necessary. Traditional methods frequently prove inadequate and ineffective in tackling these concerns. Microbial bioremediation is one method that has attracted a lot of interest. This innovative field uses microbes to lessen and even reverse effects of pollution on environment. Microbial bioremediation offers a promising way to protect ecological and human health by utilising natural capacities of bacteria fungus and other microorganisms to degrade or change contaminants. This thesis delves into various approaches and uses of microbial bioremediation. Examining how these tiny heroes are transforming way we fight pollution and clear path for a more hygienic and sustainable Earth. Microbial remediation is practical and environmentally safe way to clean up areas contaminated by petroleum hydrocarbons (PHCs). Biological removal is currently most widely used method for removing hazardous chemicals from land. In recent years we have made significant progress. Our understanding of how microorganisms break down hydrocarbons has advanced. Efforts to increase the effectiveness of bioremediation have shown promise. We now understand primary microorganisms involved in breakdown of petroleum hydrocarbons are bacteria, fungus and archaea. The most researched and effective of these three species at decomposing hydrocarbons is bacteria. Due to their diverse metabolic capacities, they can obtain carbon and energy from a variety of hydrocarbon molecules. Some of the key bacterial groups known for their ability to degrade hydrocarbons include *Pseudomonas, Acinetobacter*, and *Rhodococcus*.

Table 1- Characteristics of most common hydrocarbon pollutants and its effects on human health

Type	Structure	Impact	Reference
Aliphatic Hydrocarbons	Open chain Contains C-C, C-H bonds Made of alkanes, alkenes and alkynes	Relatively less toxic but can cause natural attenuation of contamination.	Joao Jesus et al.
Aromatic hydrocarbons	One or more aromatic rings (Typically benzene) Alternating double and single bonds	More persistent. BTEX is toxic to aquatic life. Carcinogenic to human and can also cause hematological and immune system disorders.	Hashem et al.
Chlorinated hydrocarbons	Hydrogen atom substituted with chlorine Aliphatic (Chloroalkanes) and aromatic (Chlorobenzene) Structure	Persistence and accumulation. Highly stable. Liver and kidney damage, reproductive disorders and carcinogenicity.	Xing et al.
Volatile organic compounds (VOCs)	Diverse group of organic components Low boiling point, High pressure Aliphatic or aromatic HCs coupled with nitrogen, oxygen, sulphur functional group	Contributes to air pollution. Respiratory irritation, Headache. Chronic health effects, including liver and kidney damage.	Yoshikawa et al.
Polycyclic aromatic hydrocarbons (PAHs)	Multiple fused aromatic ring Primary functional group being other aromatic ring	Carcinogenic. Causes developmental and reproductive issues.	Ali et al.

As the knowledge in the field of bioremediation increased, there were several methods deployed to manage the effects of this pollutants. Several studies focus on bioremediation using microbes, one such study includes *Aeribacillus pallidus*. Because *Aeribacillus pallidus* is a thermophilic bacterium that prefers high temperatures, it is especially well suited for bioremediation in hot climes or high-temperature situations like oil reservoirs or thermal vents. The capacity of this bacteria to break down different types of hydrocarbons, such as the aliphatic and aromatic hydrocarbons present in crude oil and petroleum products, has been investigated. As part of its hydrocarbon degradation process. A. pallidus produces enzymes including dioxygenases and alkane hydroxylases. These start breakdown of hydrocarbon molecules into smaller easier-to-manage chemicals. Because of how well these enzymes break down complex hydrocarbons. A. pallidus is good option for bioremediation applications.

2.1 BIOAUGMENTATION

Bioaugmentation is remediation technique that involves introducing specific beneficial microorganisms (such as bacteria fungi or algae) into contaminated environment to enhance degradation of pollutants. It uses tailored approach for degradation of target pollutant. This pollutant is dominant in the region. These microorganisms have ability to break down or metabolize pollutants into less harmful substances. Bioaugmentation is commonly used in situations where indigenous microorganisms are insufficient or ineffective at degrading the contaminants. For example, introducing oil-degrading bacteria into an oil spill site to accelerate the breakdown of hydrocarbons. One of the most interesting bioaugmentation techniques is the immobilization of the added augmented microbial strains on a biocarrier, which shows potential future applications for the improved and speedy biological treatment of diverse pollutants. There is a wide range of microorganisms that are able to degrade a variety of pollutants but some pollutants are just resistant to the biodegradation, this resistance may be caused due to the low bioavailability, high stability, high toxicity or low biodegradability of the pollutants [44].

Although bioaugmentation has been shown to be effective in cleaning up locations that have been contaminated by aromatic chemicals, there are still numerous environmental issues to be solved. The persistence of strains added to soil is one of the most challenging problems. The number of external microbes has been seen to decline quickly after soil inoculation. Numerous studies have demonstrated that biotic and abiotic elements interact to determine how effective bioaugmentation is. For example, *Burkholderia sp.* FDS-1 was used to study how temperature and pH affected the degradation of the nitrophenolic pesticide fenitrothion. It was discovered that the best conditions for bacterial activity were 30°C and a slightly alkaline pH, whereas 10°C and 50°C and highly acidic conditions were inappropriate for pesticide detoxification [45]. To avoid such misconduct bioaugmentation is generally uses genetically engineered or modified bacteria. The process has been tested in labs several times and on various microbial strains.

Bioaugmentation, as a concept within environmental biotechnology, is currently in the phase of evolving from theoretical applications to practical implementation in real-life scenarios. While the widespread deployment of bioaugmentation techniques may not have reached full fruition, there is considerable potential for its utilization in various fields. Laboratory tests have demonstrated successful outcomes with various strains of microorganisms, showcasing the feasibility of the approach in controlled settings. Various studies have been done which deals with degradation of petroleum hydrocarbons by addition of required microbial concussions to enhance the degradation. Bioaugmentation procedures are mostly coupled with biostimulation processes to get better outcomes like in the study aimed to assess the efficacy of permanganate pretreatment in tandem with subsequent bioaugmentation for the degradation of petroleum hydrocarbons in unsaturated soil, marking a significant departure from conventional approaches. Diesel-contaminated unsaturated soil underwent pretreatment with 0.5-pore-volume (5%) potassium permanganate using both solution pouring and foam spraying techniques, the latter incorporating a surfactant. Total petroleum hydrocarbon (TPH) removal efficiencies were measured at

37% and 72.1% for solution pouring and foam spraying, respectively. The subsequent application of permanganate-bioaugmentation foam resulted in a final TPH concentration of 438 mg/kg, indicating an impressive 92.1% total reduction. Notably, the experiment avoided soil mixing or disturbance. This led to the findings like the PP foam, in conjunction with bioaugmentation foam, showcased a remarkable TPH removal efficiency, attributing its success to increased soil pH and effective remediation agent infiltration facilitated by foaming. The application of PP foam elevated the soil pH in acidic conditions, fostering enhanced microbial activity and contributing to a first-order biodegradation rate of 0.068 d-1 post-PP oxidation. Chromatogram peak analysis verified the removal of 94% of relatively persistent hydrocarbons (C18-C22) through PP-bioaugmentation. Physicochemical parameter evaluations demonstrated that PP, when combined with bioaugmentation foam, significantly improved TPH degradation and biodegradation rates in unsaturated diesel-contaminated soil.

However, the translation of these successes to large-scale, real-life projects remains a challenge that demands further attention. The prospect of enhancing specific biological processes by introducing carefully selected microorganisms into targeted environments, such as wastewater treatment plants, holds promise. Although challenges and research gaps exist, the successful completion of laboratory tests with various strains provides a foundation for optimism. Bioaugmentation is still in its early stages of development, and more research is needed to address scaling up issues and optimize methods for real-world use. It seems possible to include bioaugmentation into large-scale projects with sustained effort, which could lead to a paradigm change in biological and environmental engineering. The benefits of bioaugmentation in addressing environmental issues and increasing process efficiency are becoming more and more possible as scientists and engineers tackle current obstacles. The reviewed research has shown that TPH pollution (up to 380,000 mg kg-1) in clay, silt, and sandy soils can be effectively treated with composting technique. With a maximum of 99%, the majority of these investigations recorded removal efficiencies of greater than 70%. TPHs are used as carbon and energy sources by the microorganisms throughout the composting process [26].

Table 2 includes a list of microbes and microbial consortium, which work together to give varied efficiency in degradation of the hydrocarbon pollutants processes like biostimulation when applied in conjunction to phytoremediation have also been shown to have positive effects in degradation of the contaminant.

2.2 BIOSTIMULATION

The approaches most commonly employed for in situ bioremediation of unintentional spills and persistently contaminated sites worldwide involve the addition of pre-grown microbial cultures in order to speed the breakdown of undesirable compounds (bioaugmentation) and/or the introduction of nutrients and other additional materials to the native microbial population to encourage faster propagation (biostimulation). In bioremediation, biostimulation is a technique used to support the natural processes in contaminated environments—like soil or groundwater—that aid in the disintegration or transformation of pollutants. Unlike bioaugmentation, which involves the introduction of specific microorganisms, biostimulation focuses on optimizing the conditions for the native microorganisms already residing in the contaminated site. The primary objective of biostimulation is to stimulate the growth and metabolic activity of these indigenous microorganisms, enabling them to more effectively break down or convert contaminants into less harmful substances [21]. In accordance with several reports, specific types of microorganisms have the metabolic pathways needed to break down contaminated substances. Some of the most investigated microbial species for their capability for bioremediation include *Pseudomonas* species, *Mycobacterium* species, *Haemophilus* species, *Rhodococcus* species, *Paenibacillus* species, and *Ralstonia* species [33,34]. These strains can break down petroleum hydrocarbons, aromatic hydrocarbons like benzene, toluene, ethylbenzene, and xylene, as well as polyaromatic hydrocarbons like naphthalene, phenanthrene, anthracene, pyrene, and the extremely carcinogenic benzo[alpha] pyrene (Pseudomonas, Haemophilus, and Mycobacterium). Biostimulation mostly depends on supplying the optimal conditions to this native bacterium for faster and better decontamination of the polluted sites. This factor can be the presence of water, ph of the soil, utilizable nitrogen, phosphorus source etc. The rate of decontamination is accelerated corresponding to biostimulation as the system's ability to degrade contaminants is improved by the addition of one or more rate-limiting nutrients [21].

Various techniques have been employed to make the environment optimal for the microbial species that would be responsible for the biodegradation of the contaminants. nutrients such as carbon, phosphorus, nitrogen which can stimulate the microbial growth. Co-substrate addition is another such technique. For some microbes, certain pollutants may act as co-substrates. These co-substrates can promote the development of microorganisms that can biodegrade the intended contaminants. It should be kept in mind that the added contaminant is not harmful and can be controlled when the work is done.

Biostimulation is employed across a diverse range of environmental scenarios where the objective is to heighten the natural processes carried out by microorganisms for the degradation or transformation of contaminants in polluted environments. The effect of biostimulation was effectively studied by Omar Mata et al. The study showed that the contamination of soil by residual automotive oil is a pressing global environmental issue, and biostimulation stands out as a highly suitable technology for effectively addressing this problem. The biostimulation study made the use of enzymes, surfactants, and vermicompost. The study employed a factorial design with various factors, including time, pH, temperature, and biostimulation strategies involving enzymes and additives. Specifically, enzymes derived from *Ricinus communis L.* seeds, along with commercial vermicompost and Triton X-100 surfactant, were utilized. The results revealed that the most remarkable removal efficiency, reaching an impressive 99.9%, was achieved after a 49-day period, under conditions of pH 4.5, a temperature of 37°C, and biostimulation involving enzymes and vermicompost (3% w/v–5% w/w). Surprisingly, the addition of the surfactant did not significantly contribute to the enhancement of removal efficiency. Particularly useful bioremediation method in this study was proven to be vermicompost. There have been studies that make both use of biostimulation and bioaugmentation to control the contamination in a particular zone. A study performed by *Yang Bai et al.* Showed that the simultaneous introduction of a TCC-degrading *Ochrobactrum* sp. TCC-

2 and electrode into the TCC and PAHs co-contaminated sediments resulted in the proposal of a novel electro-biostimulation and bioaugmentation combination remediation system. According to the results, the combined system's PAHs and TCC degradation efficiencies were 2.9–3.0 and 4.6 times, respectively, greater than those of the control group (which received neither electro-biostimulation nor bioaugmentation treatments). The enhanced electroactive bacteria and PAHs degraders (such as *Desulfobulbus*, *Clostridium*, and *Paenarthrobacter*) and the introduced strain TCC-2 worked together to accelerate the breakdown of TCC and PAHs. By raising the functional gene abundances associated with different metabolic activities, the preferential elimination of the TCC inhibitory impact with bioaugmentation treatment could restore microbial functioning [24]. Coupling various bioremediation process have been proven of value as shown in *Table 2.*

2.3 CYT P450 ENZYME IN BIOREMEDIATION

Pseudomonas putida is another bacterium that has been extensively researched in terms of bioremediation. Because Pseudomonas putida has a wide range of enzymes and metabolic pathways, it can efficiently break down the aromatic and aliphatic hydrocarbons found in petroleum products. Its great degree of plasticity allows it to flourish in a range of environments with varying temperatures, pH levels, and oxygen concentrations [6]. Its adaptability allows it to live in a range of hydrocarbon-contaminated conditions. Pseudomonas putida has the ability to produce biofilms, which are collections of bacteria stuck to surfaces. Biofilms can speed up the breakdown of hydrocarbons by providing a safe environment for bacterial growth and encouraging genetic material transfer between cells.

Table 2- Degradation of different components of petroleum hydrocarbon with various microbial preparations and comparison of their effects.

	Type of HC contamination	Method of Bioremediation	Efficiency	Reference
Rhodococcus sps.	TPH	Bioaugmentation	Effective in wide range of temperature, salinity, ph, and other variables.	Xin chen et al.
Bacillus methylotrophicus	TPH	Biostimulation	80.24% aged TPH removed.	Lunge rong et al.
Indigenous consortium+ Sophorolipids	TPH	Biostimlation	100% degradation is achievable in 6 days	Farzaneh Fanaei et al.
Athrobacter globiformis+ **rhamnolipids+ mixed surfactants**	PAH+ DDT	Biostimulation	60.7% PAH and 29.3% DDT degradation higher than control	Xiaoxu wang et al.
Pseudomonas brassicacearum MPDS	Napthlene+ PAH	Bioaugmentation	31.1% napthlene removed and inceased PAH degradation.	Zhenshi Chen et al.

Seretia marcescenes+ Bacillus megaterium+ Aeromonas hydrophilia	PAH (acenephthalene, Fluorine)	Bioaugmentation	98.92% fluorine degradation and 98.16% acenephthalene degradation was obtained.	Alegbeleye et al.
Panicum virgatum+ **Bacterial strains**	TPH	Phytoremediation	Increased degradation of TPH. Most efficient in saturated anaerobic environment.	Patrick Macintosh et al.
Acetinomycetales+ *Melilotus officinalis*	TPH+PAH	Phyto+ microbial bioremediation	Increased TPH degradation by 32% and the degradation of other HCs were found.	Teresa Steliga et al.
Rhizosphere	TPH+ Aliphatic HCs	Bioaugmentation	Improved TPH degradation by 32% and the degradation of other HCs were found.	Paikhomba Singha et al.
Rhodococcus sp. Sejongia sps.	PAH	Bioaugmentation+ Biostimulation	Increased PAH degradation	Holeyar et al.

CYT P450 CAM are Haem monooxygenases which are well-known for their capacity to reduce one atom of molecular oxygen to water while introducing another atom into a remarkably wide spectrum of substrates [1]. P450s, or cytochrome P450 enzymes, are widely distributed in living things and are essential for the biosynthesis of natural products, the breakdown of xenobiotics, the creation of steroids, and the metabolism of drugs. Due to their ability to catalyse a wide range of reactions and substrate architectures, P450s are regarded as the most versatile biocatalysts found in nature. Particularly, P450s are effective biocatalysts in the synthesis of fine or bulk chemicals, flavours, fragrances, bioremediation agents, and commodity pharmaceuticals because they can catalyse the regio- and stereoselective oxidations of nonactivated C-H bonds in complex organic molecules under mild conditions [8]. Significant work has gone into creating better P450 systems that get beyond the inherent drawbacks of the natural enzymes. A variety of families of ring hydroxylating dioxygenases and alkane monooxygenases are primarily found in strains of Firmicutes, Proteobacteria, Actinobacteria, and Fungi [2]. In hydrocarbon-degrading bacteria like *Pseudomonas putida*, the cytochrome P450 enzymes can recognize and bind to hydrocarbons using hydrophobic interactions. This recognition can also be based on active site complementarity. After binding enzyme reacts with molecular oxygen (O2) to form hydroperoxo intermediate or peroxo. These are examples of reactive oxygen species. Subsequently, an oxygen atom is inserted into hydrocarbon molecule. This occurs when this activated oxygen species is introduced into hydrocarbon substrate.

The formation of an alcohol or epoxide intermediate which is dependent on cytochrome P450 isoform. Employed and the hydrocarbon substrate's structure, is a characteristic of oxidation processes. These intermediates are far more polar. Reactive chemically than initial hydrocarbon substrate. Since these intermediaries are typically more dangerous. Less quickly metabolised than parent hydrocarbons. They may stop further breakdown processes. Moreover several enzymes and metabolic pathways within microbial cell can metabolise this alcohol or epoxide intermediate [7] The next step in this process entails oxidation and reduction reactions. Finally cause hydrocarbon backbone to split. Producing smaller, more readily metabolised compounds like organic acids and aldehydes. The viability of the bioremediating cultures may be limited by these substances, which in high concentrations may be toxic to microbial cells and may interfere with cellular functions. The degradation of hydrocarbons consumes oxygen, which can lead to oxygen depletion in the environment. This can limit the activity of aerobic microorganisms involved in bioremediation. By controlling the metabolism activity of the cytochrome p450 cam we can achieve enhanced biodegradation of petroleum hydrocarbons. This can be done by manipulating the external factors like Ph, temperature of the surrounding as well as by nutrient manipulation. Or it can be obtained through more tedious procedure like genetic manipulations like gene under-expression, but this method takes more precision and modifying microbes specifically for the later stages is not always possible. The method that seems to be most suitable is to find a ligand or a molecule that binds to the overproduced Cytochrome P450 and disables it for the time being to provide the microbial consortium more time to degrade the metabolites produced by the degradation of hydrocarbons by cytochrome P450. The paper tries to do just that. It is an attempt in finding a plausible lead molecule for binding with the enzyme and provide more time for the degradation of the metabolites.

2.4 COUPLING OF PHYTOREMEDIATION WITH BIOAUGMENTATION

Phytoremediation is a sustainable and eco-friendly approach used to clean up environmental pollutants from soil, water, or air by harnessing the natural abilities of certain plant species. Phytoremediation started to become a research area in the early 1980s by emerging studies on metal uptake by hyper-accumulating plants and the reports of toxicity caused by the pesticides in crops came out. The exposure to heavy metals may occur through polluted soil, phytotoxicity-induced reduction in the quality of food crops, decreased land production due to agricultural practices leading to food insecurity, and a variety of other land issues [26]. Given that plants cover the majority of the planet's surface, it makes sense that they have a significant impact on the fate and movement of chemicals and xenobiotic substances. Numerous neutral hydrophobic compounds in soil underwent phytoremediation, primarily by bacteria in the rhizosphere rather than within the plant. The root zone provided a favourable environment for microbes, who seemed to benefit from exudates, dissolved oxygen, and secondary metabolites released by plants.19–21Exudates functioned as supplementary substrates for the catabolism of aromatic compounds, such as long-chain alkanes in petroleum hydrocarbons, polynuclear aromatic hydrocarbons (PAHs), and benzene/ toluene/ ethylbenzene/ xylenes (BTEX).

Given that exudates were a highly accessible and degradable carbon source, they occasionally inhibited metabolic degraders, causing diauxic or catabolic suppression in the breakdown of target molecules. Rhizosphere bacteria have a major role in TPH phytoremediation, however plant uptake and BTEX chemical transformation may also play a role. Among the most prevalent families are Actinobacteria, Proteobacteria, and Bacteriodetes. It has been effectively proved that petroleum hydrocarbons can be permanently removed from soil at former tank farm sites by phytoremediation [28]. While plant absorption and BTEX chemical transformation may contribute to TPH phytoremediation, rhizosphere bacteria are primarily responsible for the process. Actinobacteria, Proteobacteria, and Bacteriodetes are among the dominant families. It has been effectively proved that petroleum hydrocarbons can be permanently removed from soil at former tank farm sites by phytoremediation.

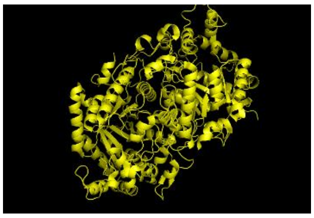
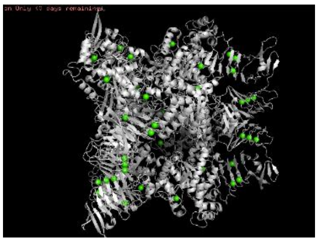
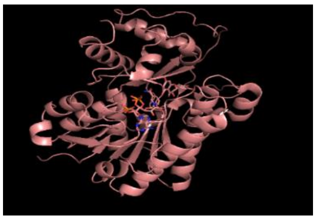
The simultaneous combination of phytoremediation and bioaugmentation in environmental remediation results in a synergistic fusion, which utilizes the innate abilities of plants and microbial communities to achieve a more effective purification of polluted ecosystems. Phytoremediation, based on the Phyto-extraction potential of plants, can bind metals and certain organic poisons. This botanical skill is improved when it is integrated with biological augmentation, a biotechnological process where some microbes are introduced knowingly into an environment so that they can metabolize stubborn pollutants. The deliberate interaction between plant roots and introduced microorganisms in the rhizosphere (i.e., the area around roots) raises contaminant translocation rates as well as microbial activities [30]. This merger increases the range of rehabilitation since phytoremediation focuses on metal absorption while bioaugmentation targets organic pollutants; thus, multiple types of contaminants can be addressed. Furthermore, nutrient availability is enhanced through such introduced microbial communities leading to increased plant healthiness and strength. This combined approach's flexibility is demonstrated by its ability to customize the composition of the microbial community to site-specific soil conditions, ensuring a customized and effective remediation plan. The combination of these techniques results in faster breakdown of contaminants since bacteria and plants have a strong symbiotic relationship that works to speed up the cleanup process.

Moreover, the long-term effectiveness of this combination is demonstrated by the creation of a long-lasting, sustainable solution, whereby bioaugmentation maintains microbial activity in the rhizosphere and phytoremediation offers persistent plant-driven contaminant uptake. Together, both phytoremediation and bioaugmentation may offer an adaptable and sustainable method to mitigate environmental pollutants across different ecological matrices; showcasing a scientifically intricate process. Further research on the combination of phytoremediation and bioaugmentation is expected to focus on improving the symbiotic interactions between microbial communities and plants. An increased understanding is needed of the mechanisms that precisely control the ability of plants to tolerate this additional plant microbe partner in the rhizosphere [36] which, more specifically, will allow increases in pollutant breakdown efficiency. Moreover, a few of the sustainability aims demand-controlled plant-microbe interactions that are not yet feasible to research such as construction of innovative artificial plant-microbe partnerships created for the purpose of targeting an additional journey or soil habitat requirementsModern molecular and genetic techniques can be used to develop plants and microbes with enhanced cleanup capabilities [40]. Additionally, research may examine the sustainability and long-term ecological effects of integrated techniques, taking into account elements like the possibility of unforeseen repercussions and the development of resilient ecosystems following cleanup. The scientific foundations and real-world applications of integrated phytoremediation and bioaugmentation techniques for efficient and sustainable environmental cleanup are expected to be furthered by future research in this area. The combined effort of nanotechnology and microbial degradation has also enabled us to make significant progress in contaminant bioremediation. Nanotechnology has revolutionised subject of bioremediation by providing new methods for enhancing efficiency of microbial degradation processes. Nanomaterials such as nanoparticles (NPs) and nanotubes enhance activity. They more effectively transfer hydrocarbon-degrading microorganisms to contaminated areas when paired with bioaugmentation. One of the primary advantages of nanotechnology is its ability. It increases surface area available for microbial adhesion. Activity in bioremediation [4]. Moreover nanoparticles have ability to transport nutrients. And other agents that promote growth. Enabling a sustained release of these materials that increase microbial activity.

This strategy can help overcome nutritional constraints and promote growth of bacteria that degrade hydrocarbons in contaminated environments. In a study by Zhang et al. (2016) soil contaminated with diesel fuel was cleaned up using hydrocarbon-degrading bacteria. Nano zero-valent iron (nZVI) particles. The bacterial group was mixed with nZVI particles. These acted as bacteria's transporters and were added to lab-scale bioreactors holding the soil contaminated by diesel. When compared to merely employing bacteria, this approach proved far more effective. Breaking down the diesel fuel In order to spread bacteria throughout the soil. The nZVI particles were essential in providing more channels for the breakdown of hydrocarbons. This study highlights how nanoparticles have potential to greatly improve efficiency of bioremediation processes for soil polluted with petroleum hydrocarbons. The proteins produced by some major bacteria for bioremediation of petroleum hydrocarbon is given in table 3.

Table 3- Structures of the major enzymes involved in enhancing bioremediation of hydrocarbons and the bacterial species they are associated with.

Bacterial Species	Enzyme Type	Enzyme	Reference
Rhodococcus Sps.	Monooxygenase	Cytochrome P450 	Xin Chen et al.

Bacillus Methylotrophicus	Rhamnolipids		Thaís Strieder Machado at el.
Geobacillus thermonitrificans	Alkane hydroxylase	LadA 	Lu Feng et al.
Serratia Marcescens	Lipases	Extracellular LipA 	Yi Huang et al.
Plant Species			
Panicum Virgatum	Root Exudates	Dihydroflavanol 4-reductase 	Patrick Macintosh et al.

Physical and biological factors affecting biodegradation:

The bioremediation of petroleum hydrocarbons is influenced by a combination of physical and biological factors that collectively dictate the success and efficiency of the remediation process. The flowchart (*Figure 3*) adapted from Teklit et al. Shows a list of various physical and biological factors that determines the pace of biodegradation under unaltered natural conditions.

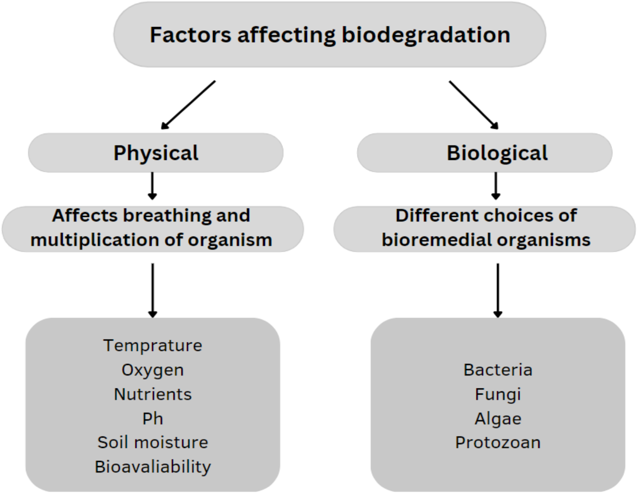


Figure 3- Physical factors and bioremedial choices that affects the biodegradation of petroleum hydrocarbons adapted from (Teklit et al.)

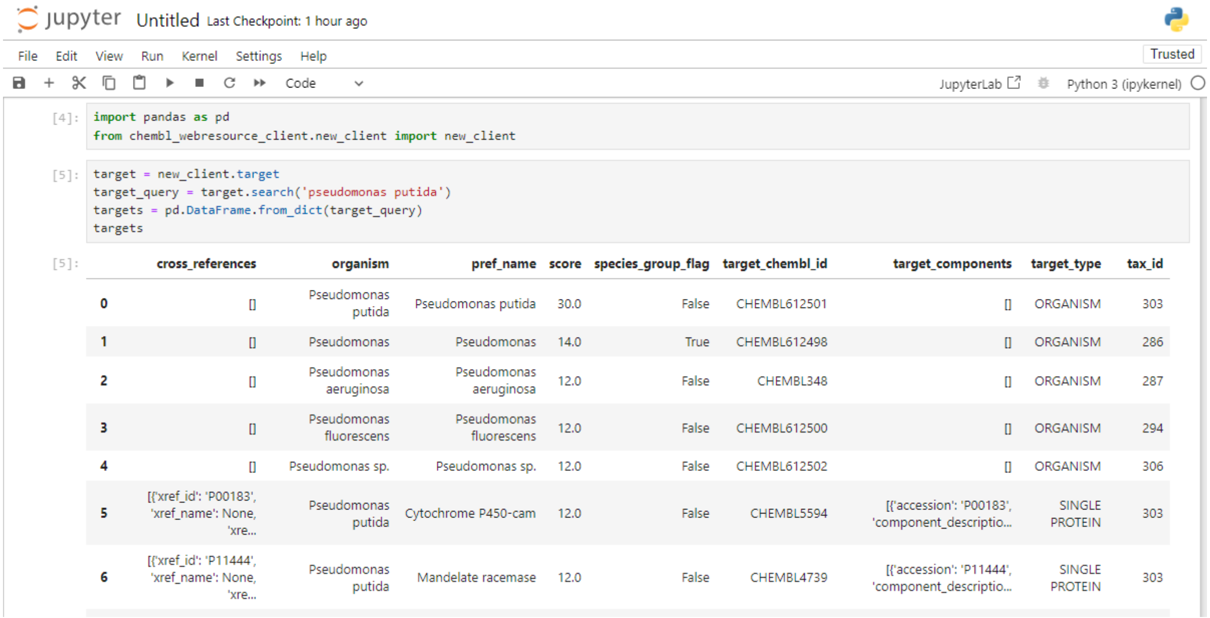
One of the diverse affecting factors of petroleum hydrocarbons involve the oxygen management for better breathing of the microbial culture in the contaminated environment. Techniques like aeration can help us to make oxygen available to the microbes. Aeration systems can be used in wastewater treatment plants or bioremediation systems to maintain aerobic conditions. While in other cases creating anaerobic conditions by limiting oxygen availability can promote the growth of anaerobic microorganisms that are effective in biodegrading certain pollutants. These conditions are regulated depending on the microbes that are to be enhanced for better biodegradation.

In addition to the oxygen management, pH control is another important factor to be considered. pH control is a critical aspect of biostimulation and various other processes in microbiology, chemistry, and environmental science. It involves the regulation and maintenance of the acidity or alkalinity of a solution or environment to create optimal conditions for desired biological or chemical reactions. pH significantly impacts the activity of microorganisms. Different microorganisms thrive in specific pH ranges, so controlling pH is crucial for creating an environment where desired microorganisms can flourish. Many chemical reactions are pH-dependent. Altering pH can affect reaction rates, solubility, and the availability of ions and molecules. In bioremediation, for example, adjusting pH can influence the rate of contaminant degradation. This adjustment helps in biostimulation but are dependent on the species of interest. Several methods have been employed to manage the pH of the contaminated environments including acid/base addition. The most common method for pH control involves adding acids or bases. Common acids include sulfuric acid and hydrochloric acid, while sodium hydroxide and calcium carbonate are typical bases used for pH adjustment. Another method to manipulate the pH is the use of the buffer systems. Buffer solutions consist of weak acids and their corresponding conjugate bases, which resist changes in pH when small amounts of acid or base are added. Some indirect methods of pH regulation include CO2 control as the concentration of carbon dioxide (CO2) in the environment can influence pH.

Temperature control is also a critical aspect of many scientific, industrial, and biological processes. It involves regulating and maintaining the temperature of a system or environment within a specific range to achieve desired outcomes. Temperature profoundly influences biological processes, including enzyme activity, microbial growth, and metabolic rates. Many organisms have specific temperature optima at which they function most efficiently. Temperature affects the rate of chemical reactions. In most cases, higher temperatures increase reaction rates. Temperature control systems typically involve heating and cooling elements. Heating is achieved through electrical or gas heaters, while cooling can be accomplished using refrigeration systems, cooling coils, or heat exchangers. Temperature control devices, such as thermostats and temperature controllers, monitor the current temperature and activate heating or cooling mechanisms to maintain the desired set-point. Many systems also make use of feedback control loops to adjust heating or cooling in response to temperature fluctuations. Proportional-Integral-Derivative (PID) controllers are commonly used for this purpose. Managing all the factors to be in Favor of the degradation of the contaminant helps in fastening the process of biodegradation and getting the natural balance of the contaminated zones back.

CHAPTER III
METHODOLOGY

Cytochrome P450 enzymes play a crucial role in the metabolism of various compounds, including hydrocarbons. Inhibition of specific cytochrome P450 enzymes can alter metabolic pathways, potentially leading to improved bioremediation of hydrocarbons. The procedure makes use of a popular database ChEMBL and uses python to find the best possible ligand for the blocking of Cytochrome P450. ChEMBL is a database of bioactive molecules with drug-like properties, maintained through the European Bioinformatics Institute (EMBL-EBI). It carries designated data at the residences and activities of over 2 million compounds, inclusive of their organic targets, pharmacological activities, and medicinal chemistry facts. ChEMBL is extensively utilized by researchers in drug discovery, pharmacology, and related fields to get admission to comprehensive and wonderful information on bioactive molecules.



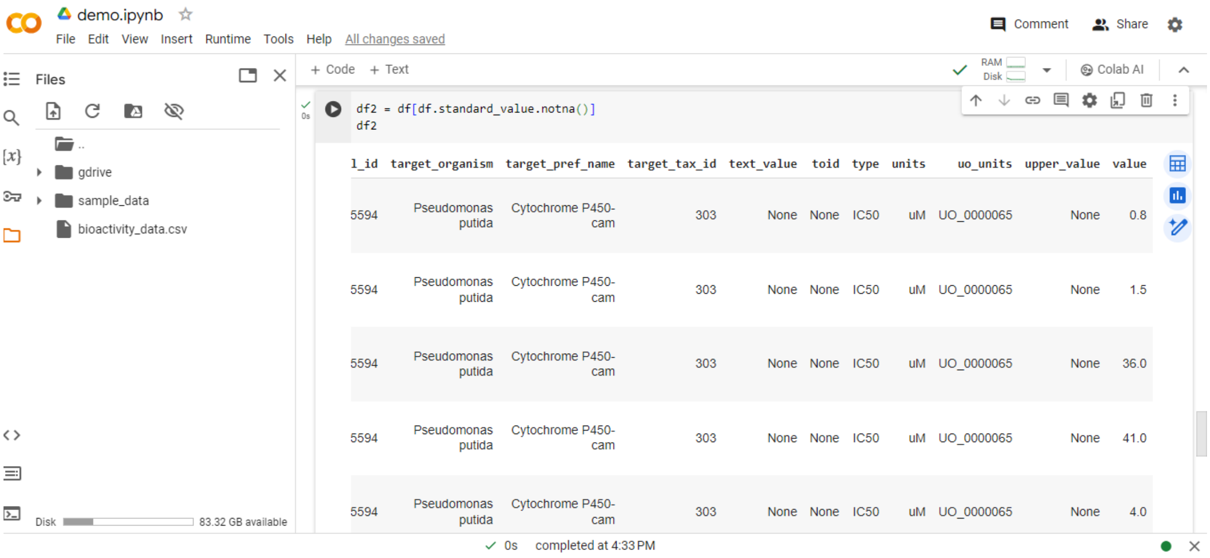
```
import pandas as pd
from chembl_webresource_client.new_client import new_client
```

```
target = new_client.target
target_query = target.search('pseudomonas putida')
targets = pd.DataFrame.from_dict(target_query)
targets
```

	cross_references	organism	pref_name	score	species_group_flag	target_chembl_id	target_components	target_type	tax_id
0	[]	Pseudomonas putida	Pseudomonas putida	30.0	False	CHEMBL612501	[]	ORGANISM	303
1	[]	Pseudomonas	Pseudomonas	14.0	True	CHEMBL612498	[]	ORGANISM	286
2	[]	Pseudomonas aeruginosa	Pseudomonas aeruginosa	12.0	False	CHEMBL348	[]	ORGANISM	287
3	[]	Pseudomonas fluorescens	Pseudomonas fluorescens	12.0	False	CHEMBL612500	[]	ORGANISM	294
4	[]	Pseudomonas sp.	Pseudomonas sp.	12.0	False	CHEMBL612502	[]	ORGANISM	306
5	[{'xref_id': 'P00183', 'xref_name': None, 'xre...	Pseudomonas putida	Cytochrome P450-cam	12.0	False	CHEMBL5594	[{'accession': 'P00183', 'component_descriptio...	SINGLE PROTEIN	303
6	[{'xref_id': 'P11444', 'xref_name': None, 'xre...	Pseudomonas putida	Mandelate racemase	12.0	False	CHEMBL4739	[{'accession': 'P11444', 'component_descriptio...	SINGLE PROTEIN	303

Figure 4- List of all the Molecules whose effects have been studied on Pseudomonas putida and are available on PubChem obtained using Jupyter Notebook.

ChEMBL affords exact information on chemical substances, inclusive of their chemical systems, synonyms, and physicochemical houses. This statistic is important for knowledge the properties and activities of the compounds. ChEMBL incorporates statistics on the biological targets of compounds, which includes proteins, enzymes, receptors, and different biomolecules. It provides information at the binding affinity and potency of compounds for their goals, that's crucial for understanding their mechanism of movement. ChEMBL consists of information at the pharmacological sports of compounds, which includes their efficacy, potency, and selectivity. This fact allows researchers check the therapeutic ability of compounds and prioritize them for similarly improvement. ChEMBL contains information on the chemical properties and shape-activity relationships (SAR) of compounds. This fact is precious for medicinal chemists in designing and optimizing new drug applicants. HEMBL provides facts on drug goals and their related indicators, helping researchers pick out capacity drug targets for specific sicknesses. ChEMBL is freely on hand on line and gives equipment for searching, browsing, and analyzing the data. It additionally offers programmatic get right of entry to via APIs, permitting researchers to integrate ChEMBL statistics into their own workflows and applications. ChEMBL is an increasingly common instrument in drug discovery that helps in finding and ranking lead compounds, to improve their qualities, and comprehend how they work. In pharmacological research, ChEMBL is used to investigate novel drug targets, evaluate drug safety and efficacy, and examine interactions between pharmaceuticals and their targets. Chemoinformatics uses ChEMBL data to create computer models that forecast the characteristics and actions of novel compounds [9].



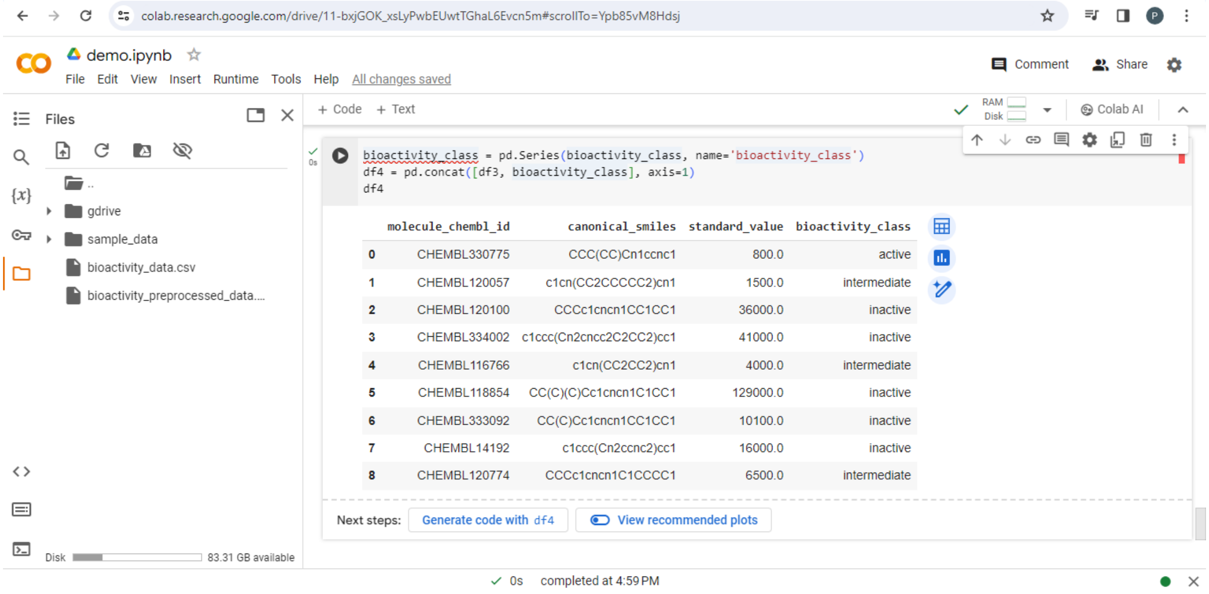
```
df2 = df[df.standard_value.notna()]
df2
```

l_id	target_organism	target_pref_name	target_tax_id	text_value	toid	type	units	uo_units	upper_value	value
5594	Pseudomonas putida	Cytochrome P450-cam	303	None	None	IC50	uM	UO_0000065	None	0.8
5594	Pseudomonas putida	Cytochrome P450-cam	303	None	None	IC50	uM	UO_0000065	None	1.5
5594	Pseudomonas putida	Cytochrome P450-cam	303	None	None	IC50	uM	UO_0000065	None	36.0
5594	Pseudomonas putida	Cytochrome P450-cam	303	None	None	IC50	uM	UO_0000065	None	41.0
5594	Pseudomonas putida	Cytochrome P450-cam	303	None	None	IC50	uM	UO_0000065	None	4.0

Figure 5- Specific molecules that show affinity mainly towards Cytochrome P450 CAM were separated using Google Colab.

For the search of the lead molecule, we used ChEMBL web services library. The ChEMBL Web Services Library is a Python library that provides programmatic get right of entry to the ChEMBL database thru its net services as shown in Figure 4. The ChEMBL Web Services Library permits customers to query the ChEMBL database and retrieve various kinds of statistics, consisting of chemical structures, biological targets, pharmacological sports, and assay consequences. This makes it easier for researchers, pharmacologists, and drug builders to get entry to and analyze ChEMBL data programmatically, the usage of Python code. Some not unusual obligations that can be performed the use of the ChEMBL Web Services Library encompass searching for compounds based on various standards, which includes chemical structure, hobby in opposition to a specific goal, or bioactivity facts, Retrieving exact information approximately compounds, together with their chemical systems, synonyms, and physicochemical residences, and accessing information on organic goals, including proteins, enzymes, and receptors, and their associated bioactivity information. It can also be used to retrieve assay records which include information of experimental assays, such as the assay kind, target, and hobby consequences.

The ChEMBL Webresource was installed using Jupyter Notebook. Jupyter Notebook is an open-source internet application that permits you to create and percentage documents containing stay code, equations, visualizations, and narrative textual content. It supports a couple of programming languages, together with Python, R, and Julia, making it a famous tool among information scientists, researchers, and educators for interactive computing and records analysis. Jupyter Notebook affords an interactive computing environment in which you could write and execute code in character cells. This lets in you to see the effects of your code at once, making it best for prototyping, records exploration, and iterative improvement. In addition to code execution, Jupyter Notebook supports the show of rich output, inclusive of plots, pix, motion pictures, and interactive widgets. This permits you to create interactive visualizations and rich multimedia content immediately in your notebooks. Jupyter Notebook integrates seamlessly with famous information technology libraries and frameworks, which include NumPy, pandas, matplotlib, and scikit-study, allowing you to effortlessly import, manage, and visualize information inside your notebooks. Jupyter Notebook lets in you to proportion your notebooks with others through exporting them to diverse formats, such as HTML, PDF, or slideshows. You also can proportion your notebooks online using services like GitHub or Jupyter's personal website hosting platform, JupyterHub. After assessing the ChEMBL webresource using Jupyter Notebook the search target was provided as '*Pseudomonas Putida'* to obtain all the ligands that binds to the bacteria and the specific sites or proteins they bind to. Here we can also observe the ChEMBL id which would further be used as reference [10]. The search was further narrowed down to the molecules that are specifically found to bind to the enzyme of interest i.e. Cytochrome P450 CAM. As is depicted in the *Figure 5*.



```
bioactivity_class = pd.Series(bioactivity_class, name='bioactivity_class')
df4 = pd.concat([df3, bioactivity_class], axis=1)
df4
```

	molecule_chembl_id	canonical_smiles	standard_value	bioactivity_class
0	CHEMBL330775	CCC(CC)Cn1ccnc1	800.0	active
1	CHEMBL120057	c1cn(CC2CCCCC2)cn1	1500.0	intermediate
2	CHEMBL120100	CCCc1cncn1CC1CC1	36000.0	inactive
3	CHEMBL334002	c1ccc(Cn2cncc2C2CC2)cc1	41000.0	inactive
4	CHEMBL116766	c1cn(CC2CC2)cn1	4000.0	intermediate
5	CHEMBL118854	CC(C)(C)Cc1cncn1C1CC1	129000.0	inactive
6	CHEMBL333092	CC(C)Cc1cncn1CC1CC1	10100.0	inactive
7	CHEMBL14192	c1ccc(Cn2ccnc2)cc1	16000.0	inactive
8	CHEMBL120774	CCCc1cncn1C1CCCC1	6500.0	intermediate

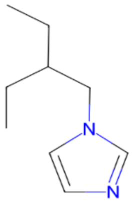
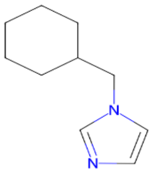
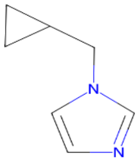
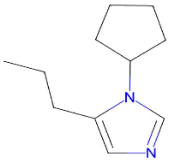
Figure 6- The molecules were further arranged on the basis of their IC_50 value, which was labeled as Standard_Type..

After obtaining the results, we created a new column named 'Standard_type' which arranged the molecules on the basis of their IC50 value in an ascending order as shown in *Figure 6*. The IC50 (half maximal inhibitory attention) is a measure of the efficiency of a substance in inhibiting a selected biological or biochemical feature, normally within the context of pharmacology and biochemistry. It represents the concentration of a substance (which include a drug or inhibitor) required to inhibit a organic system, such as enzyme pastime or cellular increase, by using 50%. The IC50 value lets in for the evaluation of the potency of various materials in inhibiting a biological system. A lower IC50 fee indicates better potency, as it signifies that a lower attention of the substance is required to gain the identical stage of inhibition. IC50 values are utilized in drug improvement to evaluate the efficacy of capacity drug applicants. Compounds with decrease IC50 values are considered greater promising candidates for similarly development, as they're stronger and might require decrease doses to gain healing outcomes. IC50 values can also provide insights into the protection profile of a substance. High IC50 values suggest decrease efficiency and might propose that better concentrations of the substance are required to provide negative consequences, which can be critical for determining secure dosages. The IC50 value can provide data approximately the mechanism of action of a substance. The structure of the top search for the lead molecule has been shown in ***Table 4***. The data offers insights into the molecular characteristics and inhibitory activities of these compounds, aiding in the identification and development of novel enzyme binders.

The data from jupyter notebook was imported to Google Colab as it provides free access to GPUs and TPUs, which can be beneficial for computationally intensive tasks such as machine learning and data analysis. If your local machine does not have access to these resources, using Colab can speed up your computations. It also allows for easy sharing and collaboration on notebooks. You can share your Colab notebook with others, allowing them to view and edit the notebook in real-time, similar to Google Docs.

The data was further cleaned of any missing data for more consistent results. The obtained data was used to create a new column depending on the standard_value. The value of IC50 was kept to the minimum I.e. 1000 and all the molecules below the value is deemed to be active while rest are determined to be inactive.

Table 4- Table depicts ChEMBL IDs, molecular structures, and IC50 statuses (active or intermediate) of lead compounds, highlighting their potential as candidates for Cytochrome P450.

CHEMBL *Id*	Structure	IC_50 Status
CHEMBL330775	*C*yclohexylamine 	Active
CHEMBL120057	1,2,3,4-tetrahydronaphthalene 	Intermediate
CHEMBL116766	1H- indozole 	Intermediate
CHEMBL120774	undecahydro-*1H*-pyrrolo[1,2-a] imidazole 	Intermediate

3.2 Docking of Cyclohexylamine to Cytochrome P450CAM

A computational method called "molecular docking" is used in structural biology and drug discovery to forecast the binding mode and affinity of a small ligand to a target protein. The method involves examining many ligand orientations and conformations within the protein binding region in order to identify the most energetically beneficial binding posture. This knowledge is essential for developing new therapies and comprehending the molecular underpinnings of ligand-protein interactions. In this study, we examined the binding of cyclohexylamine with cytochrome P450 CAM using AutoDock Vina, a popular docking programme.

The Protein Data Bank provided the crystal structure of cytochrome P450 CAM. PyMOL was used to prepare and visualise the protein structure. After removing heteroatoms and water molecules, the protein structure was preserved in PDB format.

219,140 Structures from the PDB
1,068,577 Computed Structure Models (CSM)

6WPL

Structure of Cytochrome P450tcu

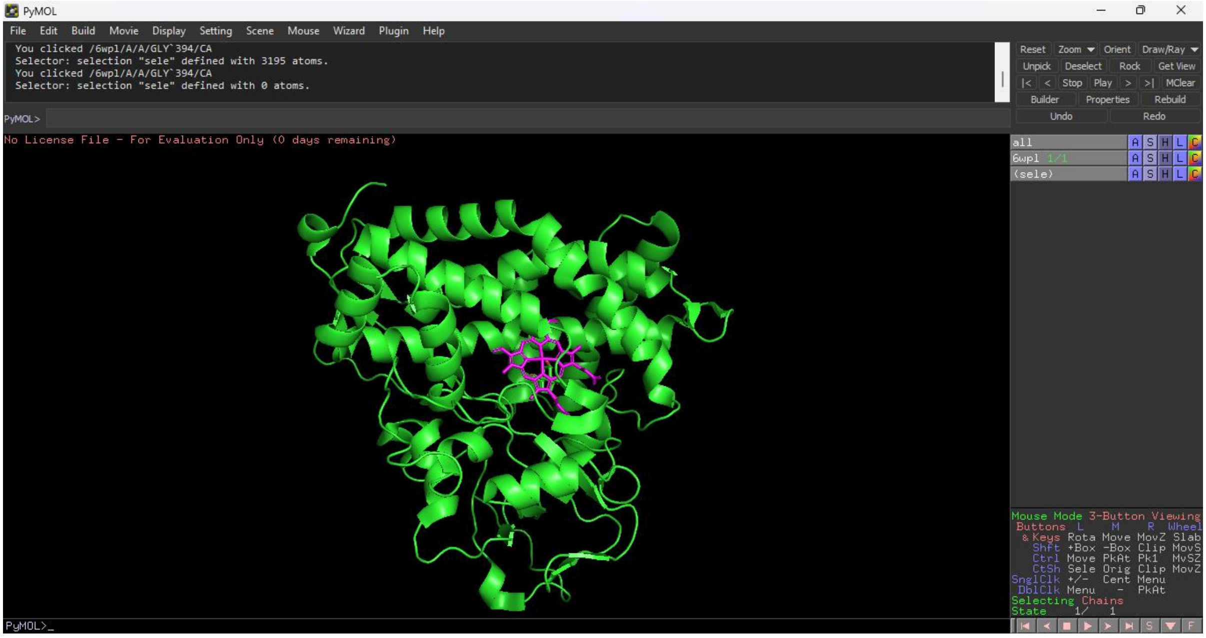
PDB DOI: https://doi.org/10.2210/pdb6WPL/pdb

Classification: OXIDOREDUCTASE
Organism(s): Pseudomonas sp. TCU-HL1
Expression System: Escherichia coli BL21(DE3)
Mutation(s): No

Deposited: 2020-04-27 **Released:** 2020-07-01
Deposition Author(s): Murarka, V.C., Batabyal, D., Amaya, J.A., Sevrioukova, I.F., Poulos, T.L.
Funding Organization(s): National Institutes of Health/Office of the Director

Figure 7- The structure of Cytochrome P450 was obtained from PDB database with the id of 6WPL.

We retrieved the crystal structure of cytochrome P450 CAM from the Protein Data Bank (PDB) which can be observed in *Figure 7*. PyMOL is a molecular visualisation programme that is used to analyse and visualise protein structures. PyMOL makes the three-dimensional structure of the protein visible and aids in locating the binding site and any residues that might obstruct ligand binding. The protein's binding site is cleared of water molecules and other heteroatoms, or non-protein atoms (*Figure 8*).



```
You clicked /6wpl/A/A/GLY`394/CA
Selector: selection "sele" defined with 3195 atoms.
You clicked /6wpl/A/A/GLY`394/CA
Selector: selection "sele" defined with 0 atoms.
PyMOL>
No License File - For Evaluation Only (0 days remaining)
```

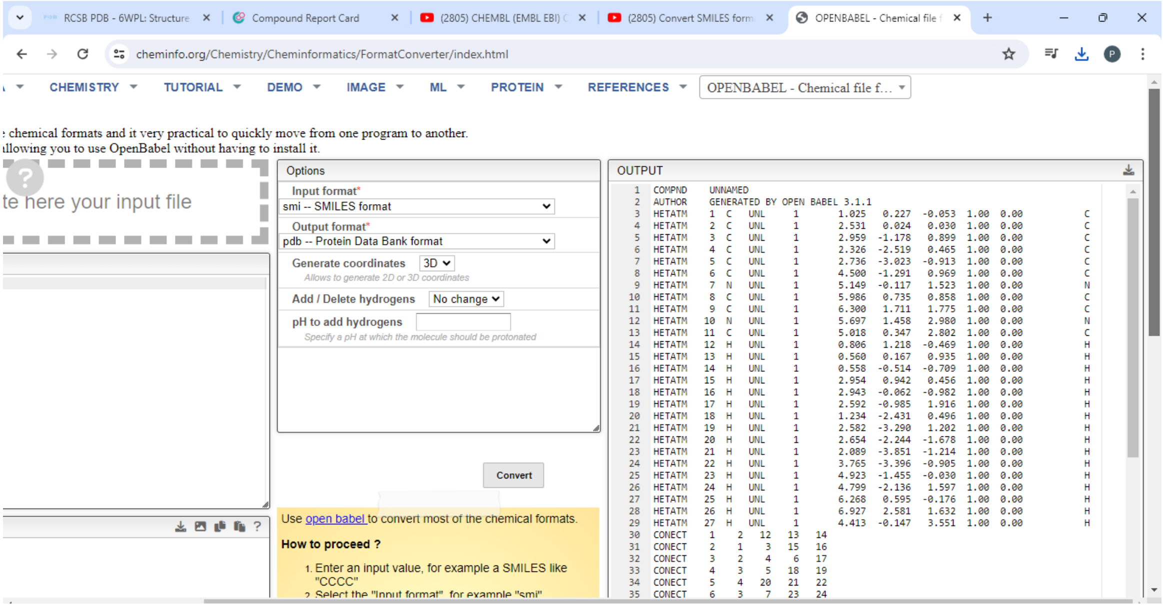
```
Mouse Mode 3-Button Viewing
Buttons  L    M    R  Wheel
 & Keys Rota Move MovZ Slab
   Shft +Box -Box Clip MovS
   Ctrl Move PkAt Pk1  MvSZ
   CtSh Sele Orig Clip MovZ
SnglClk +/-  Cent Menu
DblClk Menu  -    PkAt
Selecting Chains
State    1/    1
```

Figure 8- The structure of the protein visualized with the help of PyMol and cleaned of any additional molecules like water.

This phase guarantees that only the atoms pertinent to the docking simulation are present in the clean protein structure. PDB format, a common file format for storing the atomic coordinates of proteins and other biomolecules, is used to store the cleaned protein structure. The ligand of interest, cyclohexylamine, is synthesised and depicted using the molecular graphics programme PyMol. Molecular structures can be created and modified thanks to PyMol In order to maintain its chemical characteristics and three-dimensional dimensions, the produced ligand structure is saved in PDB format. PDB to PDBQT format conversion of the ligand structure is accomplished using AutoDock Tools, which is a component of the AutoDock software suite. OpenBable (*Figure 9*) was used to create a 3D structure from the smiles structure of lead molecule was obtained from ChEMBL. In this phase, the ligand atoms are somewhat charged in order to get them ready for docking with AutoDock Vina.

Parameters of docking were set as follows:
Total grid points per map: 1629029
Number of points in x-dimension: 100
Number of points in y-dimension:126
Number of points in z-dimension:126
Spacing (angstrom): 0.375
Center grid box:
X center: -9.33
Y center: -7.892
Z center: 29.515

The created protein and ligand structures are checked to make sure they are accurately prepared and prepared for docking before docking is carried out. This could entail examining the charges, atom kinds, and general molecular structure. The prepared protein and ligand structures, along with any necessary files (such as parameter files for AutoDock Vina), are organized in a suitable directory for the docking simulation.



: chemical formats and it very practical to quickly move from one program to another.
llowing you to use OpenBabel without having to install it.

Input format*: smi -- SMILES format

Output format*: pdb -- Protein Data Bank format

Generate coordinates: 3D
Allows to generate 2D or 3D coordinates

Add / Delete hydrogens: No change

pH to add hydrogens:
Specify a pH at which the molecule should be protonated

Use open babel to convert most of the chemical formats.

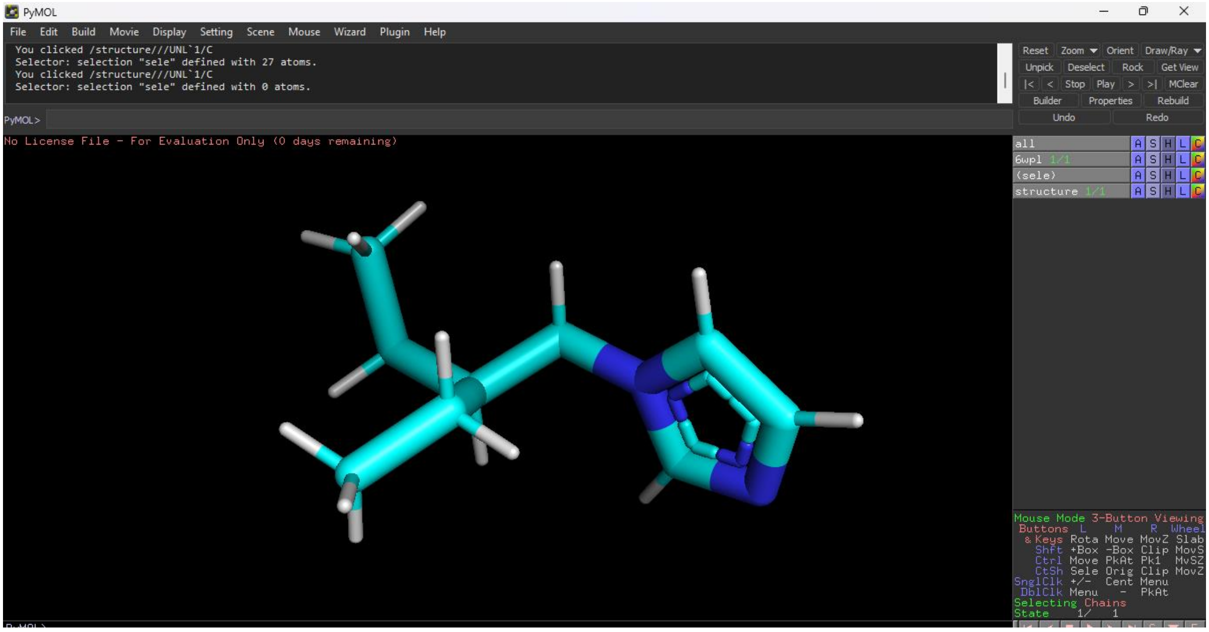
How to proceed ?

1. Enter an input value, for example a SMILES like "CCCC"
2. Select the "Input format", for example "smi"

```
COMPND    UNNAMED
AUTHOR    GENERATED BY OPEN BABEL 3.1.1
HETATM    1  C   UNL     1       1.025   0.227  -0.053  1.00  0.00           C
HETATM    2  C   UNL     1       2.531   0.024   0.030  1.00  0.00           C
HETATM    3  C   UNL     1       2.959  -1.178   0.899  1.00  0.00           C
HETATM    4  C   UNL     1       2.326  -2.519   0.465  1.00  0.00           C
HETATM    5  C   UNL     1       2.736  -3.023  -0.913  1.00  0.00           C
HETATM    6  C   UNL     1       4.500  -1.291   0.969  1.00  0.00           C
HETATM    7  N   UNL     1       5.149  -0.117   1.523  1.00  0.00           N
HETATM    8  C   UNL     1       5.986   0.735   0.858  1.00  0.00           C
HETATM    9  C   UNL     1       6.300   1.711   1.775  1.00  0.00           C
HETATM   10  N   UNL     1       5.697   1.458   2.980  1.00  0.00           N
HETATM   11  C   UNL     1       5.018   0.347   2.802  1.00  0.00           C
HETATM   12  H   UNL     1       0.806   1.218  -0.469  1.00  0.00           H
HETATM   13  H   UNL     1       0.560   0.167   0.935  1.00  0.00           H
HETATM   14  H   UNL     1       0.558  -0.514  -0.709  1.00  0.00           H
HETATM   15  H   UNL     1       2.954   0.942   0.456  1.00  0.00           H
HETATM   16  H   UNL     1       2.943  -0.062  -0.982  1.00  0.00           H
HETATM   17  H   UNL     1       2.592  -0.985   1.916  1.00  0.00           H
HETATM   18  H   UNL     1       1.234  -2.431   0.496  1.00  0.00           H
HETATM   19  H   UNL     1       2.582  -3.290   1.202  1.00  0.00           H
HETATM   20  H   UNL     1       2.654  -2.244  -1.678  1.00  0.00           H
HETATM   21  H   UNL     1       2.089  -3.851  -1.214  1.00  0.00           H
HETATM   22  H   UNL     1       3.765  -3.396  -0.905  1.00  0.00           H
HETATM   23  H   UNL     1       4.923  -1.455  -0.030  1.00  0.00           H
HETATM   24  H   UNL     1       4.799  -2.136   1.597  1.00  0.00           H
HETATM   25  H   UNL     1       6.268   0.595  -0.176  1.00  0.00           H
HETATM   26  H   UNL     1       6.927   2.581   1.632  1.00  0.00           H
HETATM   27  H   UNL     1       4.413  -0.147   3.551  1.00  0.00           H
CONECT    1    2   12   13   14
CONECT    2    1    3   15   16
CONECT    3    2    4    6   17
CONECT    4    3    5   18   19
CONECT    5    4   20   21   22
CONECT    6    3    7   23   24
```

Figure 9- OpenBabel helps you convert and process chemical data formats easily when performing any pharmaceutical study. Here it was used to convert the smiles structure of the ligand to a 3D structure.

The ligand is introduced into the protein's binding site during the docking simulation, and its many conformations and orientations are methodically investigated using a scoring function to gauge the binding affinity.



```
You clicked /structure///UNL`1/C
Selector: selection "sele" defined with 27 atoms.
You clicked /structure///UNL`1/C
Selector: selection "sele" defined with 0 atoms.
```

```
No License File - For Evaluation Only (0 days remaining)
```

Figure 10- The prepared ligand or the molecule of interest (CHEMBL330775) in a 3D structure observed using PyMol.

To determine the binding energy of each posture, the scoring function takes into account many aspects including hydrogen bonding, van der Waals interactions, electrostatic interactions, and desolvation energy. Based on their estimated binding energies, the docking software produces a variety of binding positions. Based on the scoring function, the optimal pose—which represents the most energetically advantageous binding mode—is chosen. An estimation of the strength of the ligand-protein interaction can be obtained from the binding affinity of the optimal pose.

3.3 Alternate lead molecule analysis and comparison with cyclohexylamine to study its in-silico effects

The other molecules were also obtained in the similar way as the lead molecule under study i.e. cyclohexylamine. The structure and IC 50 status of these molecules are shown in the *Table 4.* The structure of the molecules was obtained from the CHEMBL and the smiles format of the structure was converted into PDB format using OpenBable. Several reasons support the use of PDB format to view protein in Pymol. SMILES provides a flat representation of a molecule's structure, which does not contain spatial information. In contrast, a PDB file contains 3D coordinates of the atoms, which allow them to be visualized in PyMOL in three dimensions. This conversion ensures the right format of the file to be correctly opened, viewed and manipulated in PyMOL. PDB files also provide information about the exact position of every atom of a molecule, and they can contain multiple models that show different conformations of a molecule, which is invaluable for geometrical analysis and to determine possible interactions and conformations of a molecule. Also, in the context of molecular docking studies, a 3D structure is required to prepare ligands. *Figure 11* has the structures that were obtained from the CHEMBL database as SMILES structure which was then converted to PDB using OpenBable and visualized using PyMol.

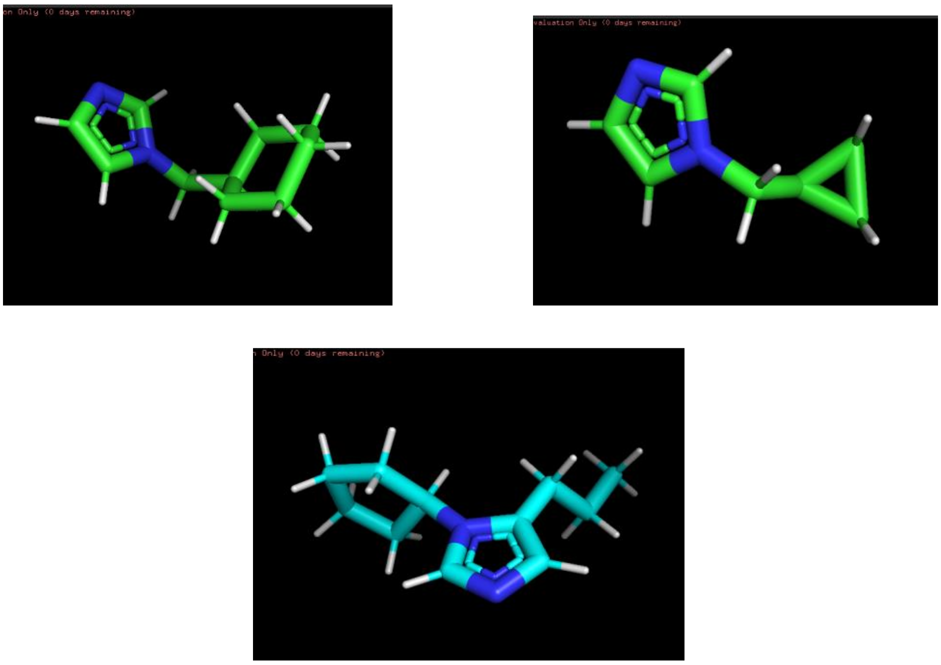


Figure 11- Visualized structures of the obtained molecules with CHEMBL id as CHEMBL120057, CHEMBL 116766 and CHEMBL120774 respwctively.

The protein molecule was prepared using Autodock Vina. The water molecules were removed from the protein. Removing water molecules from the protein structure before performing molecular docking is a common practice as it makes the job easier for the binding site, as it removes numerous positions for water molecules, allowing the docking algorithm to disregard all possible interactions between a protein and a gold level. Secondly, water molecules significantly expand the number of atoms to be included in simulation minimizing them makes the docking job less computationally intense. Furthermore, most water molecules surrounding ligands and proteins in crystal structures are a byproduct of crystallization and hold no physiological importance. Therefore, upon elimination of these waters, one can get the picture closer to the physiological reality. The protein was further modified by removing the already bound “Heme” ligand. The step upholds its importance as the presence of the original ligand can obstruct the binding site, preventing the new ligand from properly accessing and interacting with the protein. This obstruction can lead to inaccurate docking results and misinterpretation of the binding affinity and pose of the new ligand. The bound ligands are also seen to create steric hindrance and alter chemical structure of the protein Removing the existing ligand ensures that the scoring functions accurately reflect the interactions of the new ligand with the protein.

CHAPTER IV
RESULT

Numerous microbiological mechanisms involved in bioremediation of petroleum hydrocarbons were identified by literature review. Aerobic degradation is well-known mechanism. In this process bacteria use oxygen to break down hydrocarbons into carbon dioxide and water. It is frequently effective. When there is enough oxygen and nutrition available, this process results in total breakdown. Anaerobic degradation which takes place in absence of oxygen, is another significant process. Hydrocarbons can be broken down by anaerobic bacteria by fermentation. Also through methanogenesis and sulphate reduction. Anaerobic degradation can occur in conditions with low oxygen supply. This makes it relevant for bioremediation in some contexts even if it is slower than aerobic degradation.

4.1 Comparison of Studied Bacteria

The literature review included comparison of different microorganisms that have been investigated for their potential in bioremediation. Because they can break down variety of hydrocarbons. Bacteria including Pseudomonas Bacillus have been the subject of a great deal of research. For instance, Pseudomonas is well-known for its adaptable metabolism and capacity to break down a wide range of hydrocarbons. This includes aromatic compounds.

On the other hand, some bacteria such as Cycloclasticus and Alcanivorax, are specialised in breaking down particular kinds of hydrocarbons. For example, species of Cycloclasticus are specialised in breaking down polycyclic aromatic hydrocarbons (PAHs) Whereas species of Alcanivorax are known for their capacity to break down alkanes. These particular microorganisms are essential to bioremediation of certain hydrocarbon pollutants. The thesis describes number of bioremediation strategies for hydrocarbon pollution. Each of which shows differing levels of efficacy and suitability for various pollutants and environmental circumstances. Bacillus methylotrophicus and Rhodococcus species bioaugmentation stand out for their great efficacy in treating TPH. The former exhibits environmental adaptability and can remove up to 80.24% of aged TPH, while the latter can remove a substantial amount of aged TPH. Biostimulation with Sophorolipids and an indigenous consortium demonstrates fast TPH reduction, reaching 100% degradation in six days, highlighting the method's potential. Combining surfactants with Athrobacter globiformis results in effective biostimulation for the breakdown of DDT and PAH, with degradation rates of 29.3% and 60.7% higher than control. In terms of bioaugmentation for particular hydrocarbons, Pseudomonas brassicacearum MPDS and a mix of Seretia marcescenes, Bacillus megaterium, and Aeromonas hydrophilia show promise; the former removes 31.1% napthlene and increases PAH degradation, while the latter achieves 98.92% fluorine degradation and 98.16% acenephthalene degradation. Panicum virgatum in conjunction with bacterial strains proves effective in phytoremediation, particularly in anaerobic environments, increasing degradation of TPH. Acetinomycetales combined with Melilotus officinalis demonstrate significant TPH and PAH degradation, increasing TPH degradation by 32% and the degradation of other hydrocarbons. The comparison underscores the importance of selecting the appropriate bioremediation strategy based on the contaminant, environmental conditions, and desired outcomes.

4.2 Factors Influencing Bioremediation Efficiency

The study emphasizes the potential of various bacteria, including Bacillus methylotrophicus, Arthrobacter globiformis, and Seretia marcescenes, in microbial bioremediation of hydrocarbons. Such bacteria have been well reported in the degradation of many hydrocarbons from total petroleum hydrocarbons to polycyclic aromatic hydrocarbons, thereby indicating their versatility and effectiveness in different contexts. However, the efficiency of microbial bioremediation is not solely reliant on the choice of such bacteria but on various environmental factors. The growth and activity of hydrocarbon-degrading bacteria are temperature, pH, and nutrient availability controlled. Since optima for growth and enzymatic activity are different for every species of bacteria, a comprehension and control of these factors are required in the bioremediation process.

Also, in a polluted environment, the composition and concentration of hydrocarbons sometimes become critical stress factors in the process of bioremediation. There could be differences in the efficiency of bacteria toward a certain type of hydrocarbon, while being ineffective in their working ability toward other compounds. Thus, detailed knowledge of the hydrocarbon composition would be necessary in the selection of most appropriate bacterial strains and in the optimization of the bioremediation strategies. Besides, the activity of microorganisms might be affected by the presence of co-contaminants in the environment, which further affects the efficacy of bioremediation. In such a way, hydrocarbons interact with the present contaminants, thus creating competitive or inhibiting action on bacteria growth and biological metabolism, and by this fact, on bioremediation.

4.3 Various proteins produced by microbes which have been proven effective in controlling petroleum contamination

The species of *Rhodococcus* are fairly established to present a wide range of strong capacity in hydrocarbons degradation through the monooxygenase enzyme Cytochrome P450. This system enzyme is capable of catalysis of a wide range of hydroxylation reactions on hydrocarbon substrates in wide varieties of ways to promote the process of degradation. In contrast, *B. methylotrophicus* uses rhamnolipids, which are glycolipid biosurfactants, to create compounds for promoting the solubility of hydrophobic compounds like. The surfactant activity of these bacteria reduces the interfacial tension between hydrocarbons and aqueous phases, thus making it easy for hydrocarbons to be dispended and hence degraded by microbes. Alkane degradation by *Geobacillus thermonitrificans* is mediated through alkane hydroxylase LadA, whereby the first step in the breakdown of alkanes is induced to more polar forms that are chemically labile to further degradation. Secretion of extracellular LipA by Serratia marcescens is an enzyme referred to as a lipase, which catalyzes hydrolysis of hydrocarbon ester bonds to facilitate its more degradation. Additionally, Panicum virgatum's root exudates, including Dihydroflavanol 4-reductase, stimulate microbial activity in the rhizosphere, indirectly promoting hydrocarbon degradation through enhanced microbial diversity and activity. These diverse enzymatic and biochemical mechanisms underline the potential for tailored and synergistic approaches in hydrocarbon bioremediation strategies.

4.4 Blocker molecule for Cytochrome P450 produced by *pseudomonas putida*

The study with the help of ChEMBL database and some python libraries like ChEMBL WebResource and Pandas observed that the molecule with ChEMBL ID “CHEMBL 330775” was found to be the best suited ligand for binding with the enzyme Cytochrome P450 cam. A library of 1536 compounds was screened for their ability to inhibit the activity of Cytochrome P450CAM in Pseudomonas putida. The outputs of the screening assay are depicted below. The highest inhibitory potency is displayed by cyclohexylamine with an IC50 value of 800. The lead compound is a molecule with a CHEMBL ID of CHEMBL330775 for which the figure below shows the inhibition of Cytochrome P450CAM activity in a dose-dependent manner. This means that Cyclohexylamine has enough affinity for the enzyme and is capable of obstructing the active site of Cytochrome P450CAM by preventing it from binding with the petroleum hydrocarbons and therefore inhibiting its catalytic activity. The comparison of various molecules on different factors is depicted in *Figure 12*. The decision to prioritise Cyclohexylamine as a next compound to test was due to the compound’s superior efficacy of inhibition compared with the other compounds in the compound screening library. Additional studies are needed to determine the mechanism of inhibition and the use of nonane-2,6-diamine as a potential blocker of Cytochrome P450CAM for petroleum hydrocarbon bioremediation.

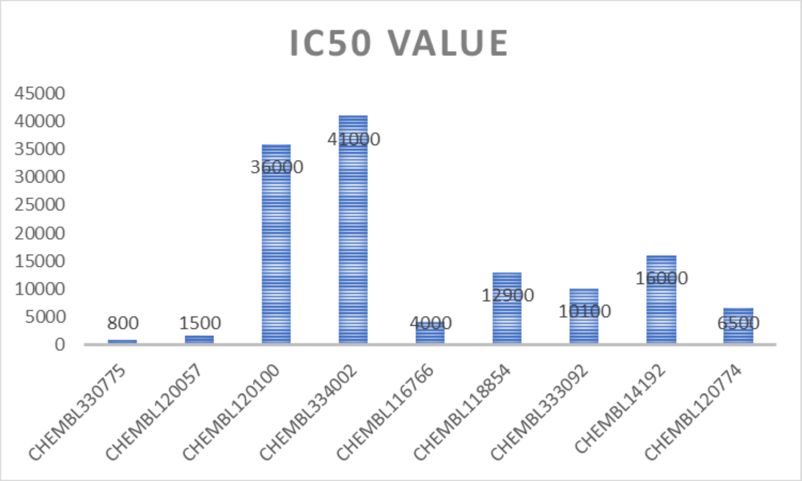


Figure 12 Most potential molecules depicted as their ChEMBL id. The IC50 value is a measure of the inhibitory potency of a compound, representing the concentration required to inhibit 50% of the enzymatic activity or biological response under study, thus indicating the compound's effectiveness as an inhibitor.

4.5 Molecular docking between cyclohexylamine and Cytochrome P450 CAM

Molecular docking simulations were carried out using Autodock Vina, a popular programme for forecasting ligand binding affinities and modes with target proteins, Further information about the approach and applications of the programme can be found on its website, https://vina.scripps.edu. **Table 1** provides an overview of the docking simulation findings, including the affinity values, distances from the optimal mode, and RMSD values for each mode. Lower affinity values indicate stronger binding between the molecules. Affinity values are a measure of this binding strength. The deviations of each mode from the most energetically advantageous binding arrangement are indicated by the distances from the optimum mode. The structural variation between a reference structure and the docked conformations is measured by RMSD values (both lower and upper bounds). More negative values indicate stronger binding, while the affinity values indicate favourable binding interactions. Better agreement between the docked conformations and the reference structure is shown by lower RMSD values. The most energetically advantageous binding configuration is probably represented by the mode with the highest affinity and lowest RMSD.

The software generates various modes or poses of the ligand within the receptor's binding site during a molecular docking simulation. With varying orientations and ligand-receptor interactions, each mode depicts a possible binding configuration. Based on a scoring formula that assesses the ligand's fit and binding affinity to the receptor's binding site, the software assigns a ranking to these modes. The most favourable or "best mode" is usually regarded as the one that indicates the strongest binding contact between the ligand and the receptor and has the highest score, which is normally the most negative number for binding affinity. In the study there were 9 modes generated with varying affinity. The molecular docking study revealed that cyclohexylamine exhibited a maximum binding affinity of -4.00 kcal/mol towards cytochrome P450 CAM. This affinity suggests a moderate to strong interaction between cyclohexylamine and the receptor, indicating a potentially favourable binding interaction. The docking simulation also identified a mode with a binding affinity of -3.3 kcal/mol for cyclohexylamine with cytochrome P450 CAM. Although slightly lower than the maximum affinity observed, this value still suggests a favourable interaction between cyclohexylamine and the receptor, indicating a potential binding configuration.

The term "distance from RMSD l.b." often denotes the departure of a given ligand's conformation or docking posture from the reference structure, which is typically an experimental or known crystal structure. A docking pose that is more comparable to the reference structure has a lower RMSD value, which suggests a more precise prediction of the ligand's binding mode. This metric measures how closely the predicted binding conformation fits the theoretically expected structure or the structure found in experiments, which aids in evaluating the quality of the docking data. The results of the molecular docking simulations for cytochrome P450 CAM and cyclohexylamine showed a range of RMSD l.b. values, with the lowest being 0.00 and the greatest being 35.467. A larger docking pose divergence from the reference structure is indicated by a higher RMSD value, which implies a less precise binding conformation prediction. Also, for some conformation, the RMSD number is smaller—i.e. 0.00—it means that the docking stance precisely matches the reference structure.

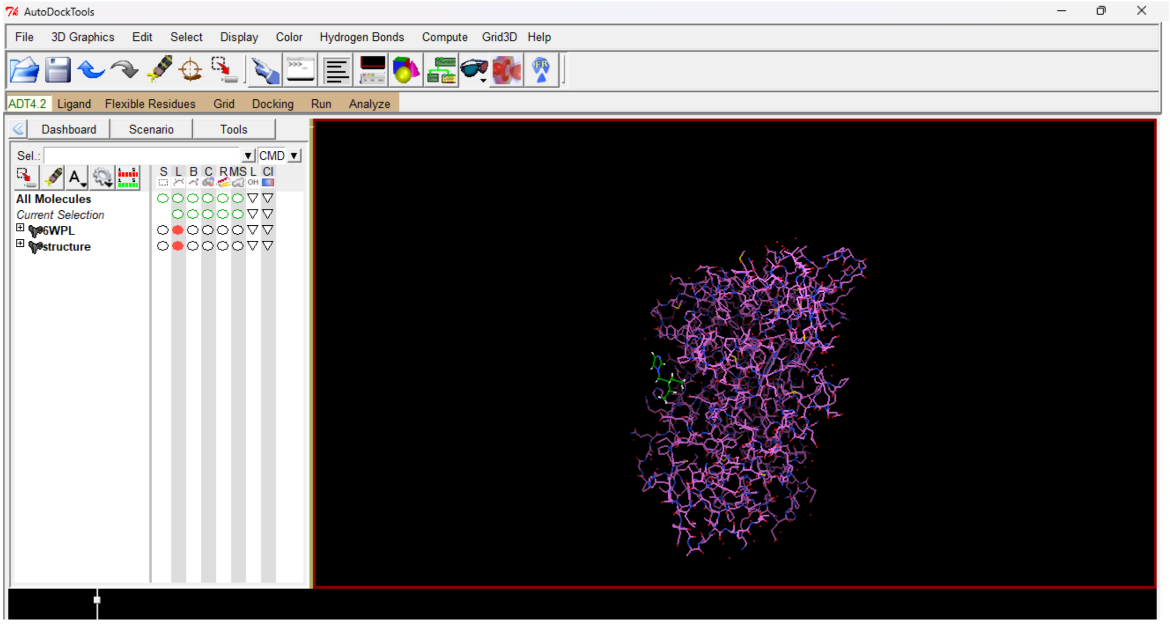
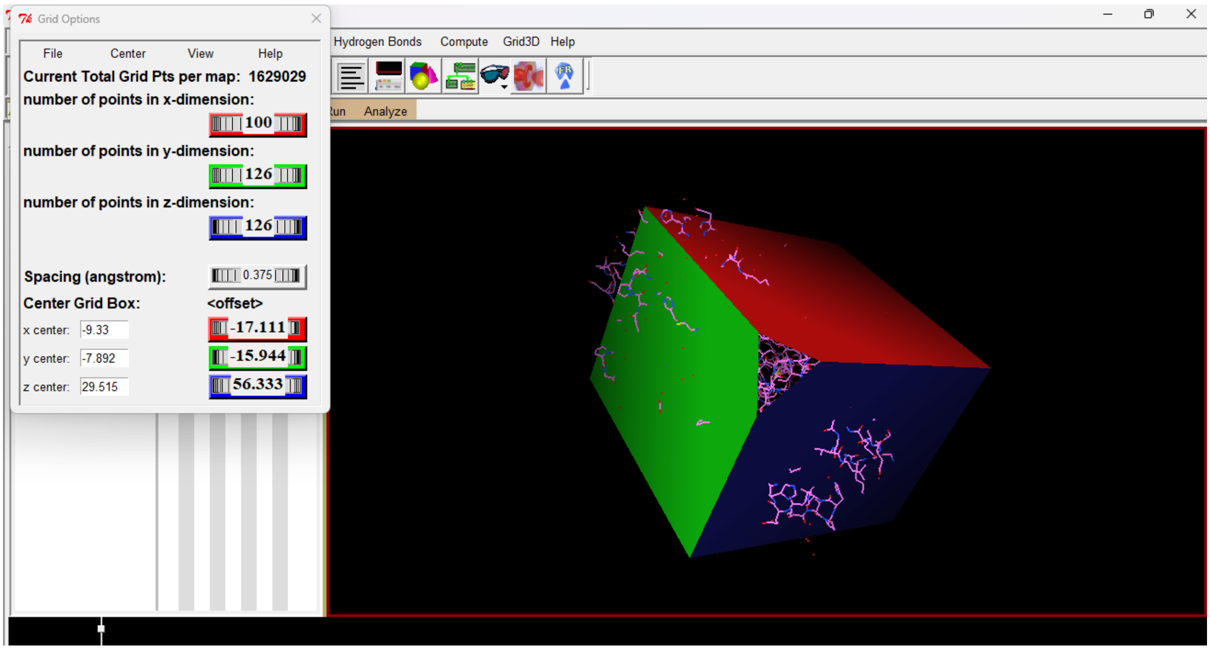


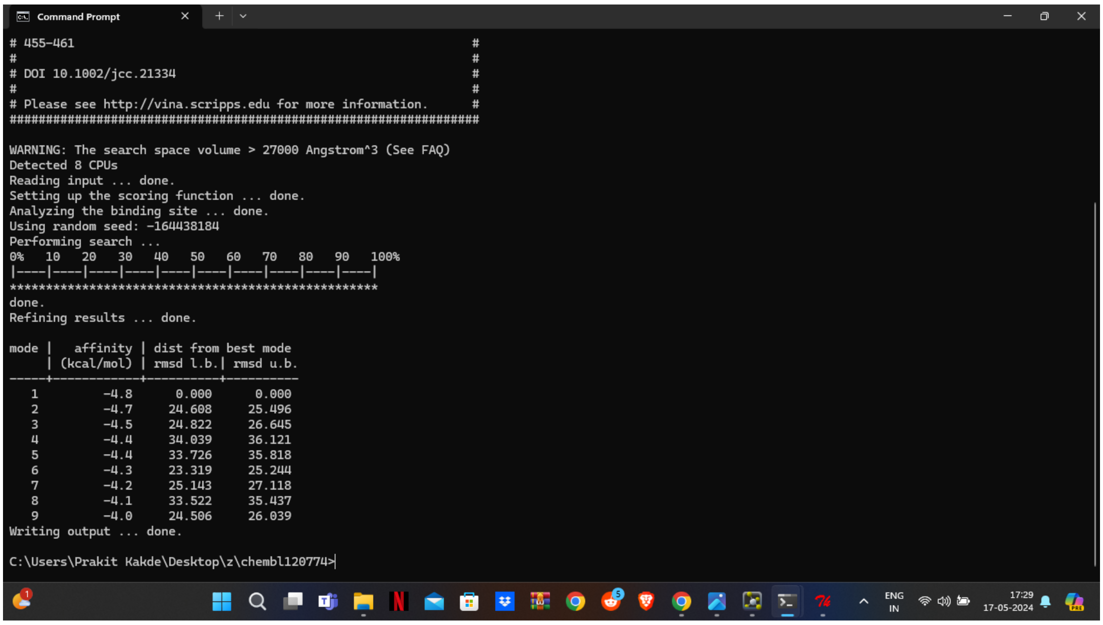
Figure 13- Visualization of completely cleaned and transformed protein and ligand in Autodock.



Current Total Grid Pts per map: 1629029
number of points in x-dimension: 100
number of points in y-dimension: 126
number of points in z-dimension: 126
Spacing (angstrom): 0.375
Center Grid Box: <offset>
x center: -9.33 -17.111
y center: -7.892 -15.944
z center: 29.515 56.333

Figure 14- The grid box dimensions were observed as this are ncessary for the molecular docking programme.

These findings suggest on the surface that cyclohexylamine can exist in a variety of conformations within the cytochrome P450 CAM binding region, with some positions substantially deviating from the reference structure and others closely approximating it. There is still thorough wet lab validation is required.



```
# 455-461                                                       #
#                                                               #
# DOI 10.1002/jcc.21334                                         #
#                                                               #
# Please see http://vina.scripps.edu for more information.      #
#################################################################

WARNING: The search space volume > 27000 Angstrom^3 (See FAQ)
Detected 8 CPUs
Reading input ... done.
Setting up the scoring function ... done.
Analyzing the binding site ... done.
Using random seed: -164438184
Performing search ...
0%   10   20   30   40   50   60   70   80   90   100%
|----|----|----|----|----|----|----|----|----|----|
***************************************************
done.
Refining results ... done.

mode |   affinity | dist from best mode
     | (kcal/mol) | rmsd l.b.| rmsd u.b.
-----+------------+----------+----------
   1         -4.8      0.000      0.000
   2         -4.7     24.608     25.496
   3         -4.5     24.822     26.645
   4         -4.4     34.039     36.121
   5         -4.4     33.726     35.818
   6         -4.3     23.319     25.244
   7         -4.2     25.143     27.118
   8         -4.1     33.522     35.437
   9         -4.0     24.506     26.039
Writing output ... done.

C:\Users\Prakit Kakde\Desktop\z\chembl120774>
```

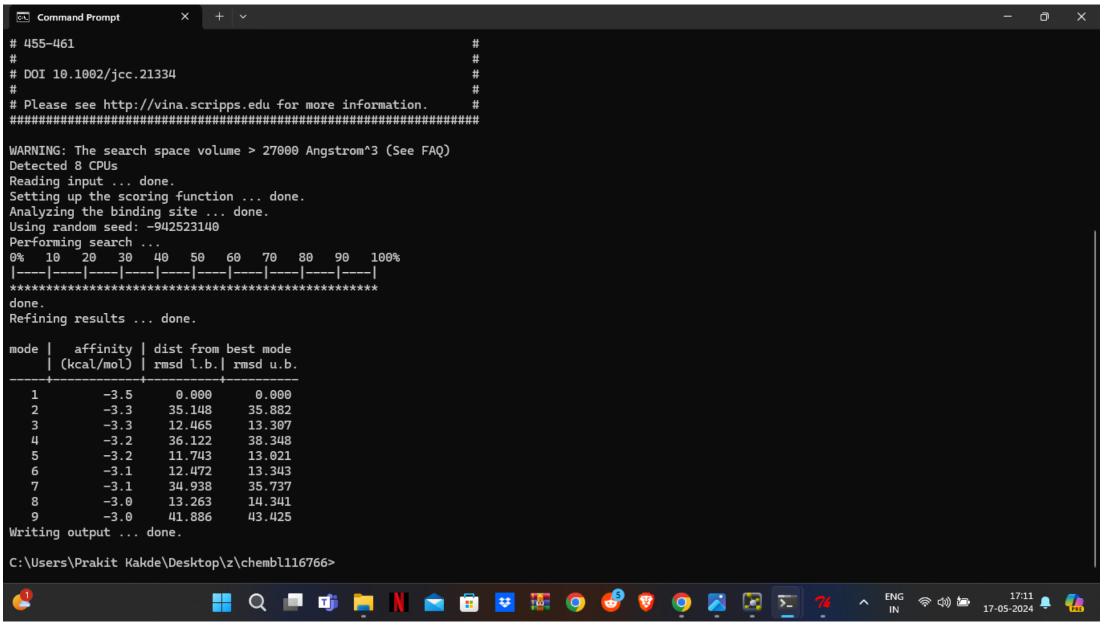
Figure 15- The result of Molecular Docking performed with the help of Auodock Vina to determine the binding affinity of Cyclohexylamine with respect to Cytochrome P450 .

The term "best mode RMSD (u.b.)" in molecular docking describes the upper bound of the Root Mean Square Deviation of the optimal docking pose from the reference structure. The difference in structure between the reference structure and the most energetically advantageous binding conformation (best mode) is represented by this value. A less optimal mode A tighter match between the projected binding pose and the reference structure is indicated by the RMSD (u.b.) value, which suggests a more precise prediction of the ligand's binding mode. This metric provides information about the structural similarity between the experimental and anticipated binding conformations, which aids in evaluating the quality of the docking data.

The most preferred conformation found while docking of Cyclohexylamine with Cytochrome P450 enzyme was found to be mode 1 with an affinity score of -4.8. Mode 1 has the most negative affinity score, making it the best among the poses considered in this docking run. With rmsd l.b. and rmsd u.b. both being 0.000, Mode 1 is the reference pose. All other modes are compared against this pose. This means Mode 1 represents the strongest binding interaction among the docked poses. Its binding affinity suggests a moderate affinity towards the protein molecule. The molecule with different conformational formations were tested to study the altered binding affinity While the affinity seems to decrease as the deviation from the main conformation i.e. mode 1. This suggests that the change in the conformation of the Lead molecule would adversely affect its binding with the target protein. As the rmsd u.b. value increases, the molecule can be said to be farther more deviated from the original structure of the ligand. Even if -4.8 kcal/mol is not very strong, a compound with this binding affinity can be a good starting point for optimization through medicinal chemistry. This is just one part of the overall picture. Factors like binding site specificity, ligand efficiency, pharmacokinetics, and ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties also play crucial roles in determining the potential of a compound.

4.6 Docking results of other searched molecules and their comparison with the molecule under study

The docking of molecules with CHEMBL ids 120057, 116766, 120774 were performed with the target molecule set to be Cytochrome P450 protein molecule that was obtained from PDB database. The specific molecules were selected on the basis of their IC50 values as well as their LD50 value for further molecular docking procedure.
The first molecule which was compared with Cyclohexylamine had a CHEMBL ID of CHEMBL116766 and according to nomenclature, is named 1-(cyclopropylmethyl)-1H-imidazole. The molecular weight as observed on CHEMBL is 122.17 g/mol. It is a cyclic compound with two nitrogen atoms and four carbon atoms arranged in a ring structure. The specific properties and uses of this compound may vary depending on its exact conformation and context within a larger system or study. This compound has been screened in various bioactivity assays to determine its effects on different biological targets. Specific bioactivity results, including binding affinities and IC50 values and EC50 values, was found while querying the ChEMBL database.

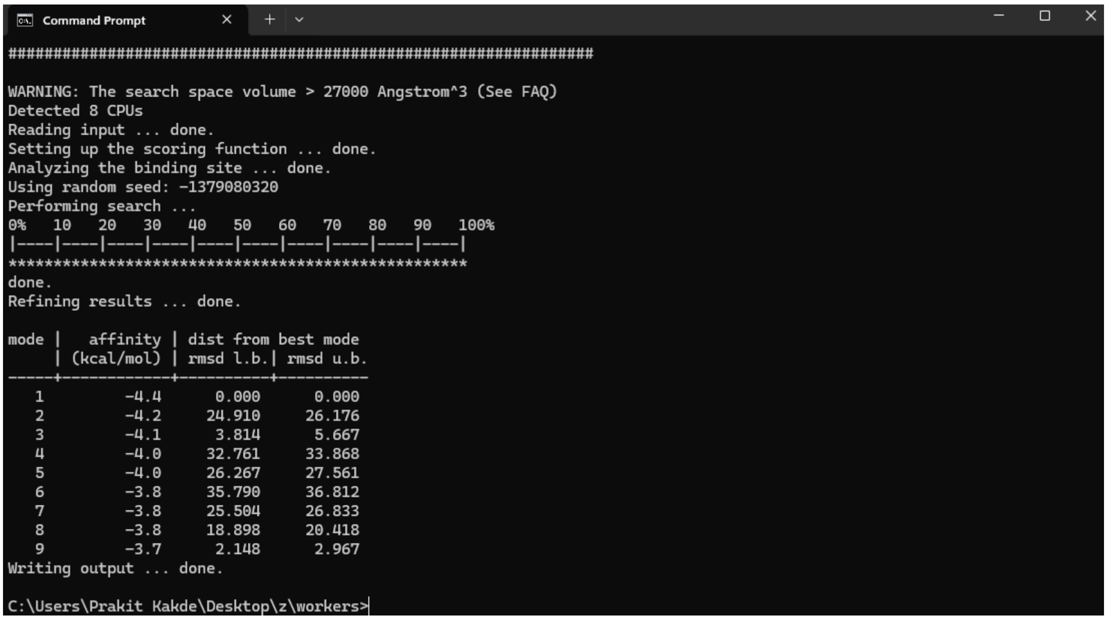


```
# 455-461                                                      #
#                                                              #
# DOI 10.1002/jcc.21334                                        #
#                                                              #
# Please see http://vina.scripps.edu for more information.     #
################################################################

WARNING: The search space volume > 27000 Angstrom^3 (See FAQ)
Detected 8 CPUs
Reading input ... done.
Setting up the scoring function ... done.
Analyzing the binding site ... done.
Using random seed: -942523140
Performing search ...
0%   10   20   30   40   50   60   70   80   90   100%
|----|----|----|----|----|----|----|----|----|----|
***************************************************
done.
Refining results ... done.

mode |   affinity | dist from best mode
     | (kcal/mol) | rmsd l.b.| rmsd u.b.
-----+------------+----------+----------
   1         -3.5      0.000      0.000
   2         -3.3     35.148     35.882
   3         -3.3     12.465     13.307
   4         -3.2     36.122     38.348
   5         -3.2     11.743     13.021
   6         -3.1     12.472     13.343
   7         -3.1     34.938     35.737
   8         -3.0     13.263     14.341
   9         -3.0     41.886     43.425
Writing output ... done.

C:\Users\Prakit Kakde\Desktop\z\chembl116766>
```

Figure 16- Docking result of molecule named N-methyl-2-pyridylamine and was obtained from CHEMBL.

The affinity was found to be 3.5 for the most stable i.e. a non-deviated conformation of the molecule. This affinity value is mostly considered a weaker interaction between the target and the ligand until the requirements suggests otherwise.

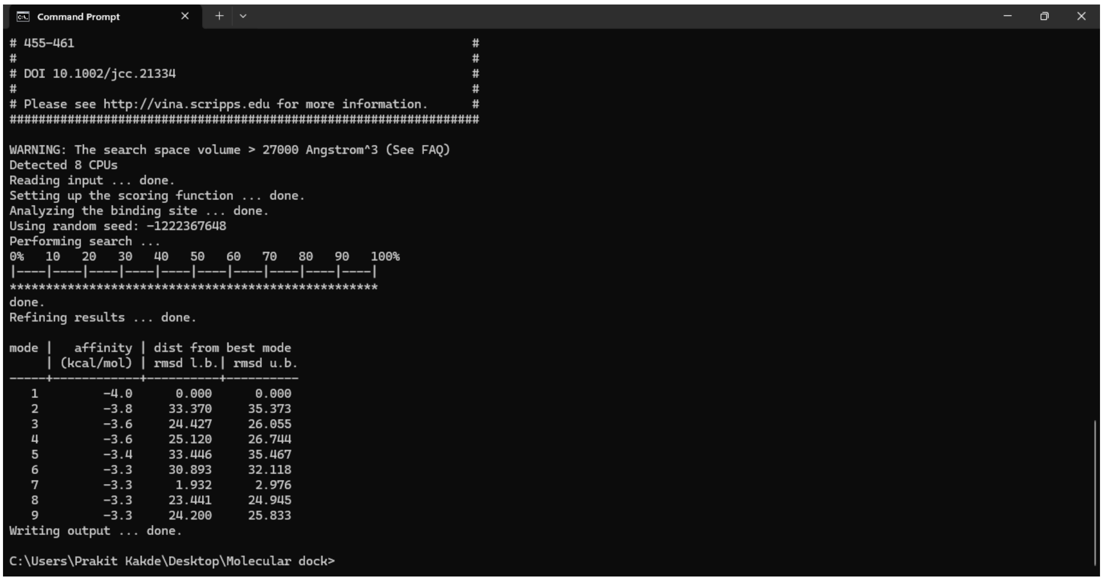
The other molecule with a certain amount of potential for being the lead molecules is 1-(Cyclohexylmethyl)-1H- imidazole. The molecule was found to have a CHEMBL Id of CHEMBL120057. The structure is made up of a six-membered ring called a pyridine ring, which has one nitrogen atom, connected to a piperidine ring, which has six members and one nitrogen atom along with five carbon atoms, by a methylene bridge and the molecular weight was found to be 164.25 KDa. Similarly, as with Cyclohexylamine The mode with least deviation from the core conformation had he strongest binding affinity which was found to be 4.4 which is considered to be a moderate interaction strength. The affinity of the best mode of this molecule is still lesser than the molecule under study which suggests that the binding of this molecule named 1-(Cyclohexylmethyl)-1H- imidazole will be weaker with the target protein when compared to the lead molecule whereas the toxicity that it can cause is predicted to be more. The molecule is indicative of moderate hydrophobicity, affecting its solubility and membrane permeability. It is Likely to have moderate solubility in polar solvents like water and good solubility in organic solvents. Because this kind of compound has the piperidine ring, which is present in many pharmacological molecules, it can be employed in medicinal chemistry as an intermediate for the manufacture of pharmaceuticals, especially those targeting neurological circuits.



```
##################################################################

WARNING: The search space volume > 27000 Angstrom^3 (See FAQ)
Detected 8 CPUs
Reading input ... done.
Setting up the scoring function ... done.
Analyzing the binding site ... done.
Using random seed: -1379080320
Performing search ...
0%   10   20   30   40   50   60   70   80   90   100%
|----|----|----|----|----|----|----|----|----|----|
***************************************************
done.
Refining results ... done.

mode |   affinity | dist from best mode
     | (kcal/mol) | rmsd l.b.| rmsd u.b.
-----+------------+----------+----------
   1         -4.4      0.000      0.000
   2         -4.2     24.910     26.176
   3         -4.1      3.814      5.667
   4         -4.0     32.761     33.868
   5         -4.0     26.267     27.561
   6         -3.8     35.790     36.812
   7         -3.8     25.504     26.833
   8         -3.8     18.898     20.418
   9         -3.7      2.148      2.967
Writing output ... done.

C:\Users\Prakit Kakde\Desktop\z\workers>
```

Figure 17- Molecular docking result of 1-(Cyclohexylmethyl)-1H- imidazole (CHEMBL120057)

Another important molecule that was found to be related to binding with Cytochrome P450 is CHEMBL120774. The name of the molecule according to the nomenclature would be 2-(4-ethylpiperazin-1-yl) pyrimidine. It is a small molecule with molecular weight of 178.28. With the chemical formula $C_{11}H_{16}N_2$, the molecule 2-(4-ethylpiperazin-1-yl) pyrimidine has a heterocyclic structure made up of a pyrimidine ring fused to a piperazine ring. An ethyl group is joined to the piperazine ring's nitrogen atom in this configuration.



```
# 455-461                                                       #
#                                                               #
# DOI 10.1002/jcc.21334                                         #
#                                                               #
# Please see http://vina.scripps.edu for more information.      #
#################################################################

WARNING: The search space volume > 27000 Angstrom^3 (See FAQ)
Detected 8 CPUs
Reading input ... done.
Setting up the scoring function ... done.
Analyzing the binding site ... done.
Using random seed: -1222367648
Performing search ...
0%   10   20   30   40   50   60   70   80   90   100%
|----|----|----|----|----|----|----|----|----|----|
***************************************************
done.
Refining results ... done.

mode |   affinity | dist from best mode
     | (kcal/mol) | rmsd l.b.| rmsd u.b.
-----+------------+----------+----------
   1         -4.0      0.000      0.000
   2         -3.8     33.370     35.373
   3         -3.6     24.427     26.055
   4         -3.6     25.120     26.744
   5         -3.4     33.446     35.467
   6         -3.3     30.893     32.118
   7         -3.3      1.932      2.976
   8         -3.3     23.441     24.945
   9         -3.3     24.200     25.833
Writing output ... done.

C:\Users\Prakit Kakde\Desktop\Molecular dock>
```

Figure 18- The molecule with Id as CHEMBL120774 was found to be named 2-(4-ethylpiperazin-1-yl)pyrimidine and the docking results seems to determine its affinity to be moderate with the protein.

This molecule has a polar surface area of 23.47 Å² and a molecular weight of 178.27 g/mol, meeting Lipinski's Rule of Five, indicating that it may have good oral bioavailability. It has two rings, two rotatable bonds, and a 193-complexity score, which is rather low. The compound's structure and characteristics make it a promising candidate for a number of medicinal chemistry and drug development applications, necessitating more research into its pharmacological actions and possible therapeutic applications. The ligand in its best conformation i.e. without deviation shows a value of 4.0 for the affinity. The value seems to be moderate providing us with a molecule that can bind to the protein but will not have strong enough interaction. Further tests are required to determine the usability of all the molecules according to the requirements in bioremediation. Overall, the ligands were compared on the basis of the data available on CHEMBL and literature to determine which one suits the best as the blocker molecule for the Protein Cytochrome P450. The molecule that best fits the description would be CHEMBL330775 also known as Cyclohexylamine. The graph below determines the comparative analysis of all the docking results in the form of the graph.

CHAPTER V
FUTURE PROSPECT

In the evolving landscape of microbial bioremediation for petroleum hydrocarbon-contaminated soil, future research endeavors could delve into the development and optimization of innovative strategies that capitalize on the synergy between various remediation techniques. Exploring the potential of synthetic biology and genetic engineering to design microbial consortia with enhanced hydrocarbon-degrading capabilities could revolutionize bioremediation approaches. Engineered microbes could be tailored to exhibit increased metabolic efficiency. Broader substrate specificity and resilience to environmental stressors. They could thus, elevate overall efficacy of remediation efforts. Additionally, there is growing need for more nuanced understanding of complex interactions within microbial communities during bioremediation processes. Metagenomic and metatranscriptomic analyses have potential to shed light on dynamics of microbial consortia. Especially in response to varying hydrocarbon compositions and environmental conditions. This information can guide development of strategies to manipulate microbial diversity for optimal remediation outcomes. Additionally, provide insights into adaptations at community level. As climate change continues to alter global ecosystems future research may also look at how changing environmental conditions affect efficacy of microbial bioremediation. It may also search for microbial strains that can thrive in various climatic scenarios This comprehensive approach that incorporates genetic engineering. Advanced molecular analyses. Climate resilience studies has potential to advance field of microbial bioremediation. It opens the door for development of more affordable and ecologically friendly soil remediation techniques in the future

CHAPTER VI

CONCLUSION

In a nutshell the wide-ranging research on microbial bioremediation methods for petroleum hydrocarbon-contaminated soils provides a comprehensive understanding of the intricate problems associated with different types of petroleum pollution and their wide-ranging effects on ecosystem health and human welfare. Bioaugmentation approaches have demonstrated the ability to accelerate the bio-degradation of compounds resistant to degradation by introducing specialised microbial communities that can metabolise a broad spectrum of hydrocarbons. In addition, the review has shown how biostimulation strategies can sustain native microbial populations by giving them the nutrients they require, making use of the soil microbiome's inherent ability to naturally attenuate.

This new fusion of phytoremediation with bioaugmentation draws strongly from mutualistic associations between plants and their associated microbial community in the remediation of sites contaminated with petroleum hydrocarbons. However, it is these long-term effects and ecological implications that need a very good consideration. This again calls for the need for studies to determine the sustainability and effectiveness of these strategies in diverse environmental circumstances. The present study shows the critical need for powerful inhibitors of Cytochrome P_450_CAM that are produced by P. putida and are used during the bioremediation process of petroleum hydrocarbons. Leading this line was 2,6-nonane-diamine (CHEMBL330775), having an IC_50 value of 800, which is good inhibitory potential. This compound should act by blocking the active site of the enzyme, thus allowing less hydrocarbon oxidation and providing more time for the bacterial consortia to break down byproducts before they accumulate. Such strategies, for example, may bring about significant changes in enhancing the sustainability and effectiveness of bioremediation operations, minimizing toxicity due to byproducts and cell death by inhibition via nonane-2,6-diamine. The inhibition mechanism would call for more formulations but facilitate proper interpretations and a gradation of whether nonane-2,6-diamine can be fit under field conditions for long-term bioremediation of petroleum hydrocarbons. The molecular docking studies with cytochrome P450 CAM have shown that the range's binding affinities range from -3.3 kcal/mol to -4.00 kcal/mol. These results give from moderate to good binding of the receptor to cyclohexylamine.

In this regard, the study has developed some details involving structural conformations and binding interactions of cyclohexylamine with cytochrome P450 CAM. Other research efforts, in addition to a study on the physiological effect of such a binding interaction and ways of increasing the accuracy of the predictions with respect to this work, could also be an effort to improve the docking parameters mentioned above. The found lead molecule still has to go through various validations and experimentation before it can be used as a viable option. Some of the further studies that can help the analysis and use of the lead molecule nonane-2,6-diamine include Structure-Activity Relationship (SAR) analysis and toxicity analysis to guess its accessibility in real world scenarios. And in vitro experimentation would give a detailed view over the function of the lead compound over the enzyme.

Going forward, further research should be done to better understand the mechanisms underlying microbial interactions in intricate soil conditions. This will help with the development of microbial consortia that are more adaptable and effective against certain hydrocarbon compositions. Furthermore, it is essential to take a comprehensive approach that takes into account the socioeconomic factors and public acceptance of microbial bioremediation techniques. In the effort to lessen the negative effects of petroleum contamination on the environment, sustainable and environmentally friendly restoration techniques ought to take centre stage. As the area develops, the incorporation of state-of-the-art technologies—such as omics-based analyses—will clarify the dynamics of microbial communities and provide guidance for customized and successful remediation procedures. In essence, the evolving landscape of microbial bioremediation holds immense potential for mitigating the complex challenges posed by petroleum hydrocarbon contamination, requiring ongoing interdisciplinary collaboration and a commitment to sustainable environmental stewardship.

REFERENCES

1. Chaaomeng Dai et. al.; Review on the contamination and remediation of polycyclic aromatic hydrocarbons (PAHs) in coastal soil and sediments; Environ res; 2022 Apr; 1:205:112423; https://doi.org/10.1016/j.envres.2021.112423.

2. Sumathi K & Rameshpathi Manian; Bioremediation of polycyclic aromatic hydrocarbons contaminated soils: recent progress, perspectives and challenges ; Environ Monit Assess 2023:195-1441 Volume 195; https://doi.org/10.1007/s10661-023-12042-7.

3. Qianwei Li, Jicheng Liu and Geoffrey Michael Gadd; Fungal bioremediation of soil co-contaminated with petroleum hydrocarbons and toxic metals; Appl Microbiol Biotechnol. 2020 Nov;104(21):8999-9008.; DOI:10.1007/s00253-020-10854-y

4. Mallavarapu Megharaj, Ravi Naidu; Soil and brownfield bioremediation; Microb Biotechnol. 2017 Sep;10(5):1244-1249.; https://doi.org/10.1111/1751-7915.12840.

5. Sandra Curiel-Alegre et al.; Evaluation of biostimulation, bioaugmentation, and organic amendments application on the bioremediation of recalcitrant hydrocarbons of soil; Chemosphere . 2022 Nov;307(Pt 1):135638; https://doi.org/10.1016/j.chemosphere.2022.135638.

6. Siti Rozaimah Sheikh Abdullah, Israa Abdulwahab Al-Baldawi, Asia Fadhile Almansoory, Ipung Fitri Purwanti, Nadya Hussin Al-Sbani, Siti Shilatul Najwa Sharuddin; Plant-assisted remediation of hydrocarbons in water and soil: Application, mechanisms, challenges and opportunities; institut teknologi sepuluh; 16-01-2023; DOI: 10.1016/j.chemosphere.2020.125932.

7. Sofia Nobili, Carolina Elisabet Masin, Cristina Susana Zalazar, Maia Raquel Lescano; Bioremediation of hydrocarbon contaminated soil using local organic materials and earthworms; Environ Pollut. 2022 Dec 1:314:120169.; https://doi.org/10.1016/j.envpol.2022.120169.

8. Yang, B., Zhou, M., Meng, Y. *et al.* Hydrocarbons removal and microbial community succession in petroleum-contaminated soil under hydrogen peroxide treatment. *Environ Sci Pollut Res* 30, 27081–27091 (2023).

https://doi.org/10.1007/s11356-022-23875-z

9. Meng Zhang, Bo Wu, Penghong Guo, Sa Wang, Shuhai Guo; Bioremediation of polycyclic aromatic hydrocarbons contaminated soil under the superimposed electric field condition; Chemosphere; 2021 Jun:273:128723; https://doi.org/10.1016/j.chemosphere.2020.128723.

10. TeklitGebregiorgis Ambaye, Alif Chebbi, Francesca Formicola, Shiv Prasad, Franco Hernan Gomez, Andrea Franzetti, Mentore Vaccari ; Remediation of soil polluted with petroleum hydrocarbons and its reuse for agriculture: Recent progress, challenges, and perspectives ;Chemosphere ;2022 Apr: 293:133572; https://doi.org/10.1016/j.chemosphere.2022.133572.

11. Xin Chen, Guangbo Shan, Jiamin Shen, Feng Zhang, Yongdi Liu, Changzheng Cui; In situ bioremediation of petroleum hydrocarbon-contaminated soil: isolation and application of a Rhodococcus strain; International Microbiology;Volume 26, pages 411–421; 09-12-2022.

12. Xiaoxu Wang, Lina Sun, Hui Wang, Hao Wu, Su Chen, Xuehao Zheng; Surfactant-enhanced bioremediation of DDTs and PAHs in contaminated farmland soil; Environ Technol; 2018 Jul; 39(13):1733-1744; https://doi.org/10.1080/09593330.2017.1337235

13. Zhengshi Chen , Haiyang Hu , Ping Xu, Hongzhi Tang; Soil bioremediation by Pseudomonas brassicacearum MPDS and its enzyme involved in degrading PAHs; Sci Total Environ; 2022 Mar 20:813:15252; https://doi.org/10.1016/j.scitotenv.2021.152522.

14. Judia Magthalin Christopher, Rajalakshmi Sridharan, Swarnalatha Somasundaram, Sekaran Ganesan ; Bioremediation of aromatic hydrocarbons contaminated soil from industrial site using surface modified amino acid enhanced biosurfactant; Environmental pollution; Volume 289, 15 November 2021, 117917; https://doi.org/10.1016/j.envpol.2021.117917

15. Chongshu Li, Changzheng Cui, Jie Zhang, Jing Shen, Baoyan He, Yan Long, Jinshao Ye; Biodegradation of petroleum hydrocarbons based pollutants in contaminated soil by exogenous effective microorganisms and indigenous microbiome; Ecotoxicology and Environmental safety; Volume 253, 15 March 2023, 114673; https://doi.org/10.1016/j.ecoenv.2023.114673.

16. Eman Koshlaf, Andrew S Ball ; Soil bioremediation approaches for petroleum hydrocarbon polluted environments; AIMS Microbiol. 2017; 3(1): 25–49.; DOI: 10.3934/microbiol.2017.1.25.

17. Shafiul Haque et al.; Functional microbiome strategies for the bioremediation of petroleum-hydrocarbon and heavy metal contaminated soils: A review; Science of The Total Environment; Volume 833, 10 August 2022, 155222; https://doi.org/10.1016/j.scitotenv.2022.155222.

18. L. Paikhomba Singha, Piyush Pandey; Rhizosphere assisted bioengineering approaches for the mitigation of petroleum hydrocarbons contamination in soil; 2021 Aug;41(5):749-766; https://doi.org/10.1080/07388551.2021.1888066.

19. Patrick McIntosh, Cristian P Schulthess, Yulia A Kuzovkina, Karl Guillard; Bioremediation and phytoremediation of total petroleum hydrocarbons (TPH) under various conditions; int. J Phytoremediation; 2017 Aug 3;19(8):755-764; DOI: 10.1080/15226514.2017.1284753.

20. Sunita J. Varjani; Microbial degradation of petroleum hydrocarbons; Am J Biomed Sci & Res. 2022 - 16(1). AJBSR.MS.ID.002182; DOI: 10.34297/AJBSR.2022.16.002182.

21. Luge Rong, Xuehao Zheng, Belay Tafa Oba, Chenbo Shen, Xiaoxu Wang, Hui Wang, Qing Luo, Lina Sun; Activating soil microbial community using bacillus and rhamnolipid to remediate TPH contaminated soil; Chemosphere; 2021 Jul:275:130062; https://doi.org/10.1016/j.chemosphere.2021.130062.

22. Jennifer L. Kirk, John N. Klironomos, Hung Lee, Jack T. Trevors; The effects of perennial ryegrass and alfalfa on microbial abundance and diversity in petroleum contaminated soil; Environ Pollut; 2005 Feb;133(3):455-65.; https://doi.org/10.1016/j.envpol.2004.06.002.

23. Leiyu Feng, Xiupeng Jiang, Yanning Huang, Dongdong Wen, Tianyu Fu, Rongbing Fu; Petroleum hydrocarbon-contaminated soil bioremediation assisted by isolated bacterial consortium and sophorolipid; Environ Pollut; 2021 Jan 11:273:116476; https://doi.org/10.1016/j.envpol.2021.116476.

24. Farzaneh Fanaei, Gholamreza Moussavi, Sakine Shekoohiyan; Enhanced bioremediation of oil-contaminated soil in a slurry bioreactor by H2O2-stimulation of oil-degrading/biosurfactant-generating bacteria: performance optimization and bacterial metagenomics; Biodegradation;2023 Feb; 34(1):83-101; 2023; Volume 34, pages 83–101.

25. Teresa Steliga, Dorota Kluk; Assessment of the Suitability of Melilotus officinalis for Phytoremediation of Soil Contaminated with Petroleum Hydrocarbons (TPH and PAH), Zn, Pb and Cd Based on Toxicological Tests; Toxics;2021 Jun 25;9(7):148; https://doi.org/10.3390/toxics9070148.

26. Huu-Tuan Tran, Chitsan Lin, Xuan-Thanh Bui, Huu-Hao Ngo, Nicholas Kiprotich Cheruiyot, Hong-Giang Hoang, Chi-Thanh Vu; Aerobic composting remediation of petroleum hydrocarbon-contaminated soil. Current and future perspectives; Sci Total Environ ; 2021 Jan 20:753:142250; https://doi.org/10.1016/j.scitotenv.2020.142250.

27. Nandita Das, Bhrigu Bhuyan, Piyush Pandey; Correlation of soil microbiome with crude oil contamination drives detection of hydrocarbon degrading genes which are independent to quantity and type of contaminants; Environ Res; 2022 Dec;215 (Pt 1); https://doi.org/10.1016/j.envres.2022.114185.

28. Yun-Yeong Lee, Soo Yeon Lee, Kyung-Suk Cho; Long-term comparison of the performance of biostimulation and phytoextraction in soil contaminated with diesel and heavy metals; Chemosphere;2023 Oct: 337:139332. doi: 10.1016/j.chemosphere; https://doi.org/10.1016/j.chemosphere.2023.139332.

29. M Megharaj 1, I Singleton, N C McClure, R Naidu; Influence of petroleum hydrocarbon contamination on microalgae and microbial activities in a long-term contaminated soil; Arch Environ Contam Toxicol; 2000 May;38(4):439-45; https://doi.org/10.1007/s002449910058.

30. Katarzyna Wojtowicz, Teresa Steliga, Piotr Kapusta, Joanna Brzeszcz; Oil-Contaminated Soil Remediation with Biodegradation by Autochthonous Microorganisms and Phytoremediation by Maize (Zea mays); Molecules; 2023 Aug 17;28(16):6104; https://doi.org/10.3390/molecules28166104.

31. Colin S. Chen, Chien-Jung Tien; Factors affecting in situ analysis of total petroleum hydrocarbons in contaminated soils by using a mid-infrared diffuse reflectance spectroscopy; Chemosphere; 2020 Dec: 261:127751. https://doi.org/10.1016/j.chemosphere.2020.127751.

32. Mohamed Hashem, Saad A Alamri, Sharefah S A A Al-Zomyh, Sulaiman A Alrumma; Biodegradation and detoxification of aliphatic and aromatic hydrocarbons by new yeast strains; Ecotoxicol Environ Saf; 2018 Apr 30:151:28-34; https://doi.org/10.1016/j.ecoenv.2017.12.064.

33. Zhilin Xing, Xia Su, Xiaoping Zhang, Lijie Zhang, Tiantao Zhao; Direct aerobic oxidation (DAO) of chlorinated aliphatic hydrocarbons: A review of key DAO bacteria, biometabolic pathways and in-situ bioremediation potential; Environ Int; 2022 Apr:162:107165; https://doi.org/10.1016/j.envint.2022.107165.

34. Miho Yoshikawa, Ming Zhang, Koki Toyota; Biodegradation of Volatile Organic Compounds and Their Effects on Biodegradability under Co-Existing Conditions; Microbes Environ; 2017 Sep 27;32(3):188-200; https://doi.org/10.1264/jsme2.ME16188.

35. Mukhtiar Ali, Xin Song, Da Ding, Qing Wang, Zhuanxia Zhang, Zhiwen Tang; Bioremediation of PAHs and heavy metals co-contaminated soils: Challenges and enhancement strategies; Environ Pollut; 2022 Feb 15:295:118686; https://doi.org/10.1016/j.envpol.2021.118686.

36. João Jesus, Dario Frascari, Tatiana Pozdniakova, Anthony S Danko; Kinetics of aerobic cometabolic biodegradation of chlorinated and brominated aliphatic hydrocarbons: A review; J Hazard Matter; 2016 May 15:309:37-52; https://doi.org/10.1016/j.jhazmat.2016.01.065.

37. Yaling Gou et al.; Polycyclic aromatic hydrocarbon removal from subsurface soil mediated by bacteria and archaea under methanogenic conditions: Performance and mechanisms; Environ Pollut; 2022 Nov:15:313:120023; https://doi.org/10.1016/j.envpol.2022.120023.

38. Avani Bharatkumar Patel, Shabnam Shaikh, Kunal R Jain, Chirayu Desai , Datta Madamwar; Polycyclic Aromatic Hydrocarbons: Sources, Toxicity, and Remediation Approaches; Front Microbiol; 2020 Nov 5:11:562813; https://doi.org/10.3389/fmicb.2020.562813.

39. Nagalakshmi Haleyur et al.; Influence of bioaugmentation and biostimulation on PAH degradation in aged contaminated soils: Response and dynamics of the bacterial community; Journal of Environmental Management; Volume 238, 15 May 2019, Pages 49-58; https://doi.org/10.1016/j.jenvman.2019.02.115

40. Oluwadara Oluwaseun Alegbeleye, Beatrice Olutoyin Opeolu, Vanessa Jackson; Bioremediation of polycyclic aromatic hydrocarbon (PAH) compounds: (acenaphthene and fluorene) in water using indigenous bacterial species isolated from the Diep and Plankenburg rivers, Western Cape, South Africa; BJM;Volume 48, Issue 2, April–June 2017, Pages 314-325; https://doi.org/10.1016/j.bjm.2016.07.027.

41. Reid A Simmer, Jerald L Schnoor; Phytoremediation, Bioaugmentation, and the Plant Microbiome; Environ Sci and Tech; 2022, 56, 23, 16602–16610;https://doi.org/10.1021/acs.est.2c05970

42. Asmara Ahmad et. al; Improvements in Bioremediation Agents and Their Modified Strains in Mediating Environmental Pollution; Curr Microbiol; 2023 May 11;80(6):208; https://link.springer.com/article/10.1007/s00284-023-03316-x.

43. Yoong Kit Leong, Jo-Shu Chang; Bioremediation of heavy metals using microalgae: Recent advances and mechanisms; Bioresour Technol; 2020 May:303:122886; https://doi.org/10.1016/j.biortech.2020.122886

44. Alexis Nzila A, Razzak SA, Zhu J. Bioaugmentation: An Emerging Strategy of Industrial Wastewater Treatment for Reuse and Discharge. Int J Environ Res Public Health. 2016 Aug 25;13(9):846. doi: 10.3390/ijerph13090846. PMID: 27571089; PMCID: PMC5036679.

45. Thaís Strieder Machado at el. Effects of homemade biosurfactant from Bacillus methylotrophicus on bioremediation efficiency of a clay soil contaminated with diesel oil. Ecotoxicol Environ Saf. 2020 Sep 15:201:110798. https://doi.org/10.1016/j.ecoenv.2020.110798

46. Isolation and characterization of biosurfactant-producing Serratia marcescens

ZCF25 from oil sludge and application to bioremediation. Environ Sci Pollut Res Int. 2020 Aug;27(22):27762-27772. https://doi.org/10.1007/s11356-020-09006-6

47. Lu Feng et al. Genome and proteome of long-chain alkane degrading Geobacillus thermodenitrificans NG80-2 isolated from a deep-subsurface oil reservoir. Proc Natl Acad Sci U S A. 2007 Mar 27;104(13):5602-7. 10.1073/pnas.0609650104.

PUBLICATIONS

1. The publication of the paper titled “ADVANCES IN MICROBIAL BIOREMEDIATION OF PETROLEUM HYDROCARBONS AND BLOCKER SEARCH TO OPTIMIZE CYTOCHROME P450 IN CONTAMINATION CLEANUP” is under process and the acceptance letter is awaited.

PAPER NAME

plag check trial.docx

WORD COUNT

14621 Words

CHARACTER COUNT

88557 Characters

PAGE COUNT

38 Pages

FILE SIZE

3.2MB

SUBMISSION DATE

May 29, 2024 7:28 PM GMT+5:30

REPORT DATE

May 29, 2024 7:29 PM GMT+5:30

● 3% Overall Similarity

The combined total of all matches, including overlapping sources, for each database.

- 2% Internet database
- 2% Publications database
- Crossref database
- Crossref Posted Content database
- 1% Submitted Works database

● Excluded from Similarity Report

- Bibliographic material
- Quoted material
- Cited material
- Small Matches (Less then 8 words)

plag check trial.docx

My Files

My Files

Delhi Technological University

Document Details

Submission ID

trn:oid:::27535:60270332

Submission Date

May 29, 2024, 7:28 PM GMT+5:30

Download Date

May 29, 2024, 7:32 PM GMT+5:30

File Name

plag check trial.docx

File Size

3.2 MB

38 Pages

14,621 Words

88,557 Characters

How much of this submission has been generated by AI?

***5%**

of qualifying text in this submission has been determined to be generated by AI.

* Low scores have a higher likelihood of false positives.

Caution: Percentage may not indicate academic misconduct. Review required.

It is essential to understand the limitations of AI detection before making decisions about a student's work. We encourage you to learn more about Turnitin's AI detection capabilities before using the tool.

Frequently Asked Questions

What does the percentage mean?
The percentage shown in the AI writing detection indicator and in the AI writing report is the amount of qualifying text within the submission that Turnitin's AI writing detection model determines was generated by AI.

Our testing has found that there is a higher incidence of false positives when the percentage is less than 20. In order to reduce the likelihood of misinterpretation, the AI indicator will display an asterisk for percentages less than 20 to call attention to the fact that the score is less reliable.

However, the final decision on whether any misconduct has occurred rests with the reviewer/instructor. They should use the percentage as a means to start a formative conversation with their student and/or use it to examine the submitted assignment in greater detail according to their school's policies.

How does Turnitin's indicator address false positives?
Our model only processes qualifying text in the form of long-form writing. Long-form writing means individual sentences contained in paragraphs that make up a longer piece of written work, such as an essay, a dissertation, or an article, etc. Qualifying text that has been determined to be AI-generated will be highlighted blue on the submission text.

Non-qualifying text, such as bullet points, annotated bibliographies, etc., will not be processed and can create disparity between the submission highlights and the percentage shown.

What does 'qualifying text' mean?
Sometimes false positives (incorrectly flagging human-written text as AI-generated), can include lists without a lot of structural variation, text that literally repeats itself, or text that has been paraphrased without developing new ideas. If our indicator shows a higher amount of AI writing in such text, we advise you to take that into consideration when looking at the percentage indicated.

In a longer document with a mix of authentic writing and AI generated text, it can be difficult to exactly determine where the AI writing begins and original writing ends, but our model should give you a reliable guide to start conversations with the submitting student.