CLONING, EXPRESSION, PURIFICATION AND BIOINFORMATICS ANALYSIS OF ARSENITE OXIDASE (homologue) FROM *Comamonas testosteroni KF1*

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I, **Tushar Agarwal, Roll No. 2K21/IBT/15,** student of M.Tech. (Industrial Biotechnology), hereby declare that the dissertation report titled "**Cloning, expression, purification and bioinformatics analysis of arsenite oxidase (homologue) from *Comamonas testosteroni KF1***" which is submitted by me to the Department of Biotechnology, Delhi Technological University, Delhi in partial fulfillment of the requirement for the award of the degree of Master of Technology, is original and contain information with proper citation. This work has not previously formed the basis for the award of any Degree, Diploma Associateship, Fellowship, or other similar title or recognition.

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CERTIFICATE-I

I, hereby certify that the Dissertation titled "**Cloning, expression, purification and bioinformatics analysis of arsenite oxidase (homologue) from *Comamonas testosteroni KF1***" which is submitted by **Tushar Agarwal, Roll No. 2K21/IBT/15** (Industrial Biotechnology), Delhi Technological University, Delhi in partial fulfillment of the requirement for the award of the degree of **Master of Technology (Industrial Biotechnology)**, is a record of the project work carried out by the student under my guidance and supervision. To the best of my knowledge this work has not been submitted in part or full for any Degree or Diploma to this University or elsewhere.

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TO WHOMSOEVER IT MAY CONCERN

This is to certify that Mr. Tushar Agarwal, an M.tech. (Industrial Biotechnology) student from Delhi Technological University (Formerly DCE), completed his M.tech. dissertation under my guidance from 26th December to 29th May, at the Macromolecular Crystallographic Unit, Institute Instrumentation Centre, IIT Roorkee. He learned basic molecular biology techniquessuch as cloning, protein expression and purification. He has completed the project entitled: **Cloning, expression, purification, and bioinformatic analysis of Arsenite Oxidase (homologue) from *Comamonas testosterone KF1*.**

I wish him all the best for his future endeavors.

PKumar

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ABSTRACT

Arsenic pollution in water is a major problem that has been observed in many underdeveloped nations across the world. Arsenic (As) contamination has recently drawn major attention to the environmental destiny and behavior of As in Southeast Asian nations including Bangladesh, India, China, Chile, and Japan. Water contamination with As is brought on by a number of natural and anthropogenic processes, both direct and indirect. Because it causes As poisoning in humans, arsenic-contaminated water poses a serious hazard to humanity. Therefore, it is essential to handle polluted water as such and provide clean water to the public at this time. Different approaches have been suggested for treating As-contaminated water. Bioremediation is one such strategy to reduce environmental contamination. Despite the fact that heavy metals kill microorganisms, some bacteria, such as arsenic-tolerant bacteria, are resistant to heavy metals. Bacteria limit the toxicity of arsenite and arsenate by either reducing ingestion, actively exporting the arsenicals, or by chemically altering them to produce versions that are relatively less dangerous. As (V) is a hundred times less poisonous than As (III), as is well known. As a result, the arsenic bioremediation techniques involve a two-step process: first, As (III) is biologically oxidized by arsenite oxidizing bacterial strains, and subsequently the As (V) that results is eliminated. It has been documented that a broad variety of bacteria isolated from diverse polluted environments had the capacity to manufacture Arsenite oxidase, that then catalyzes different enzymatic oxidations of As3+ to As5+. It has been discovered that one of the Alcaligenes faecalis bacterial strains has the enzyme Arsenite Oxidase. This Arsenite Oxidase enzyme underwent sequence alignment analysis as a result of which As3+ to As5+ enzymatic oxidation has been described. It has been discovered that one of the Alcaligenes faecalis bacterial strains has the enzyme Arsenite Oxidase. We used Uniprot to perform sequence alignment analysis on the Arsenite

Oxidase enzyme sequence of A. faecalis against the bacterial strains present in the Macromolecular Characterization Unit Laboratory, Dept. of Biosciences and Bioengineering, IIT Roorkee, and we discovered homologous protein sequences in the various strains. In the project a gene which was found to be homologous to arsenite oxidase was amplified from *Comamonas testosteroni KF1* bacterial strains and clones into cloning vectors E. coli DH5α. Successful cloning was obtained in E. coli DH5α host cells. However, protein of 104 kDa could not be confirmed by SDS PAGE analysis and hence, the protein may not have purified due to various reasons. 3D structure of arsenite oxidase was predicted from AlphaFol2 and analyzed on Pymol

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LIST OF SYMBOLS AND ABBREVIATION

Symbols	Abbreviations
As	Arsenic
Cd	Cadmium
Zn	Zinc
Pb	Lead
Hg	Mercury
Ag	Silver
Fe	Iron
Mg	Magnesium
Ni	Nickel
HCl	Hydro chloric acid
DNA	Deoxyribonucleic acid
gDNA	Genomic deoxyribonucleic acid
RNA	Ribonucleic acid
RBCs	Red Blood Cells
KDa	Kilo-Dalton
HiPIP	High Potential Iron Protein
$AsO_3H_2^-$	Arsentite
Mo	Molybdenum
pH	Potential of Hydrogen
NCBI	National Center for Biotechnology Information
BLAST	Basic Local Alignment Search Tool
FASTA	Fast A where A stands for All

UniProtKB	The UniProt Knowledgebase
EDTA	Ethylenediamine tetraacetic acid
SDS	Sodium Dodecyl Sulfate
mL	Milliliter
Rpm	Revolutions per minute
µL	Microliter
mM	Millimeter
µM	Micrometer
nM	Nanometer
mg	Milligram
Sec/Min	Second/Minute
^{o}C	Degree Celsius
β	Beta
ATL	A tissue lysis
MCT	Medium-Chain Triglyceride
PCR	Polymerase Chain Reaction
DNase	Deoxyribonuclease
RNase	Ribonuclease
GC Content	Guanine-Cytosine content
NEB	National Enforcement Bodies
dNTP	Deoxynucleoside triphosphate
DMSO	Dimethyl sulfoxide
EtBr	Ethidium bromide
EEO	Electroendosmosis
UV	Ultraviolet
V/cm	Volt/centimeter

Kbp	Kilo-base pair
$CaCl_2$	Calcium Chloride
LB	Lysogeny broth
cDNA	complementary DNA
IPTG	Isopropyl β- d-1-thiogalactopyranoside
NaCl	Sodium Chloride
DTT	Dithiothreitol
SDS PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
Ni-NTA	Nickel-Nitrilotriacetic acid
APS	Ammonium persulphate
TEMED	Tetramethylethylenediamine
SCED	Single-Crystal Electron Diffraction

CHAPTER 1
INTRODUCTION

Water is the most prevalent element on earth. It is essential for the survival of species in all kingdoms. Humans depend heavily on water, and problems with water directly affect society and quality of life. Water contamination may be caused by both natural and man-made factors, such as industrial and agricultural operations, poor waste management, and urbanization. Water pollution may have a variety of negative consequences for both individuals and the environment. Contaminated drinking water, for example, can cause health problems such as diarrhea, cholera, and typhoid fever. Polluted water is also harmful to aquatic plants and animals, resulting in population and biodiversity losses. Water pollutants come in a variety of forms, including chemical pollutants (such as pesticides, heavy metals, and therapeutic purposes), biological pollutants (like viruses and bacteria), and physical pollutants (such as plastic debris) [1].

Water is essential for both our survival and day-to-day operations. It covers the majority of our planet, yet not all of it is suitable for human consumption. Therefore, it is essential for humans to utilize this transparent chemical substance. On the basis of the origin of the water level it is identified into two types: Ground water and Surface water. "Ground water" is underground liquid that forms when all of the pores in soil or other materials including rock are saturated. "Surface water" refers to bodies of water like seas, streams, lakes and rivers. The drinking water of most of the communities and municipalities is obtained from the surface sources only. Also, the large amount of waste water from domestic sources, industries, agriculture as well as from sewage treatment plants of municipality are received by rivers and lakes [2]. This water, which contains substances which cannot be identified, get typically mixed in drinking water. Drinking water of a high standard can be consumed without endangering one's health. Such water is referred to as "potable" when it is free of both inorganic and organic materials, aesthetically pleasing, and free from offensive colors, tastes, odor and turbidity. A measurement of water quality compares the condition of the water to the requirements of one or more human use and/or any biotic species or purpose.

Water quality criteria should be agreed upon in connection with water quality standards issued by the government board and representing legal requirements. There are three types of criteria in general: in-stream, drinking water, and wastewater discharge. Each kind has its own set of requirements, which are all measured in the same method. The World Health Organization (WHO) has established minimum drinking water standards that all countries should claim toward. Water quality is crucial for human health, social and economic growth, and the environment. As populations grow and natural habitats deteriorate, securing enough and secure water sources to everyone becomes extremely challenging. The term "pollution" frequently refers to human activities as the source of contamination, despite the reality that natural processes also have an impact on water quality. The goal is to reduce pollution and enhance wastewater management. Water that has been tainted by domestic, industrial, and commercial activity is called wastewater. Because of this, it is impossible to define the composition of any wastewater because it is constantly shifting and are very variable [3][4]

If wastewater is not adequately handled, it could have negative effects on the environment and people's health. These effects may include harm to animal and fish populations, reduction in oxygen levels, closure of beaches and other restrictions on recreational water use as well as there will be limitations on the harvesting of shellfish and fish, and also it will cause contamination of drinking water. Domestic wastewater is formed by activities such as toilet use, bathing, food preparation, and washing [5].

Water pollution is a critical global problem that requires ongoing observation. A body of water, such as an ocean, ponds, lake, river, stream or even the water below the ground, becomes contaminated and polluted when sewage leaks, agricultural runoff, or chemical spills contaminate it. Water that has been contaminated makes it unhealthy for humans to consume since it includes harmful or poisonous compounds as well as germs and organisms that can cause health problems. Growing populations and growing enterprises strain water supplies while introducing nutrients and physical contaminants (such as plastic waste products) into water sources [6].

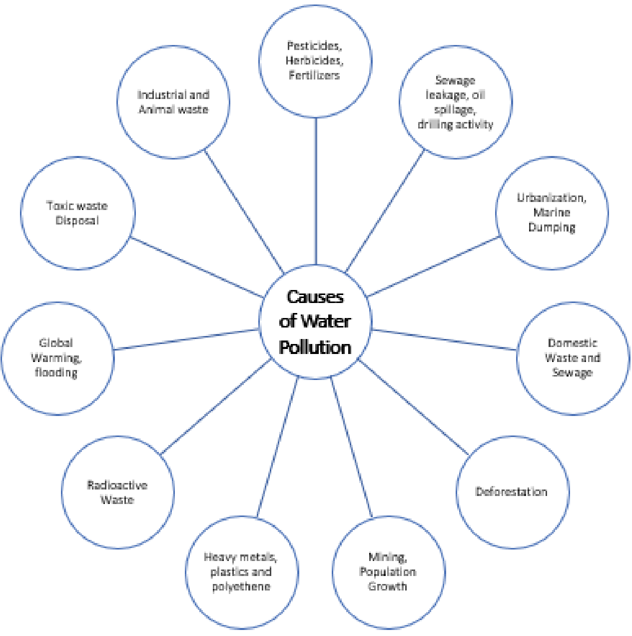


Figure 1.1: Causes of water pollution

Groundwater contamination leads to a decrease in water supply as a result of the growing worldwide population and water demand. The primary source of drinking water is groundwater, which may be found below the earth's surface in rock crevices and between soil granules. Metals and groundwater can have a complicated connection. Heavy metal contamination in water can have severe impacts. In India, groundwater provides 85% of the country's drinking water and around 60% of its agricultural needs [7].

It is well recognized that heavy metals can interfere with normal bodily biochemistry and metabolic functions. When consumed, they get changed to their stable states of oxidation (Cd^{2+}, Zn^{2+}, As^{2+}, Pb^{2+}, Hg^{2+}, $As^{3+}$and Ag^{+}) in acidic conditions of the stomach, where they mix with the biomolecules of a human body – for example proteins and enzymes - for establishing stable and strong chemical interactions [8].

The heavy metals are a class of metallic elements having high atomic weights, such as lead, mercury, cadmium, arsenic, and chromium. Excessive levels of some metals in groundwater, on the other hand, can be harmful to human health. Similar to high lead levels, contaminants in drinking water, including mercury, cadmium, and arsenic, can have a variety of negative health effects. To prevent and reduce heavy metal pollution in water, it is important to implement regulations and best practices to limit the release of heavy metals into water bodies. This entails keeping an eye on and assessing the quality

of the water, cutting back on or doing away with the use of heavy metals in industrial operations, and appropriately disposing of hazardous waste. Efforts can also be made to remediate areas that have been affected by heavy metal pollution, such as cleaning up contaminated sediment and restoring degraded ecosystems. Various health issues are brought on by the environmental toxicity of heavy metals. Reactive oxygen species are produced when heavy metals stress bacteria physiologically [9].

Bioremediation has become a crucial strategy for pollution avoidance due to the growing negative consequences of pollution nowadays. By using biosystems of naturally occurring or artificially created microbes, contaminants are either removed from the environment, destroyed, or converted into less hazardous forms. Even though the presence of heavy metals harms microorganisms, certain toxic metal variations have genetic resistance factors that enable them to withstand larger concentrations of the toxic chemicals. Numerous bacterial resistance mechanisms to poisonous metals have been thoroughly investigated at the molecular level.

As is a metalloid that is present in the environment naturally as well as through human activity. Being toxic and carcinogenic, it can be fatal to human health at even minute quantities. More than 150 million people globally are at risk due to arsenic contamination of drinking water. As a result, it is crucial to remediate As-contaminated water. An overview of chemistry, distribution, and toxicity is given at the outset of the current investigation. These three concepts are crucial to comprehend in order to develop remediation strategies. We review the advantages, drawbacks, and most recent developments of the most popular As removal techniques (chemical precipitation, adsorption, ion exchange, membrane filtering, phytoremediation, and electrocoagulation) [10][11].

Arsenic is a widely distributed hazardous metalloid with a high solubility potential. Arsenic's toxicity has long been recognized, and arsenic salts have long been used as poisons. It is a common element found in many minerals, primarily as Nickel, Copper and Iron salts or as Arsenic Sulphide or Oxide. The most prevalent forms of arsenic in water are arsenate As (V), Arsenite As (III) and methylated arsenic compounds produced by biological activity. “The toxicity of arsenic depends heavily on its oxidation state; trivalent arsenicals are at least 100 times more toxic than pentavalent derivatives”. Both versions have the capacity to cause different kinds of cellular harm, which accounts for

their extreme lethargicness.

Arsenic-resistant microorganisms resist the harmful effects of arsenite and arsenate by reducing ingestion, forcefully exporting the arsenicals, or chemically modifying them to fewer toxic forms. Arsenite oxidation via the arsenite oxidase enzyme is a possible detoxifying method. Arsenite oxidase is a protein that catalyzes the conversion of As^{3+} to As^{5+}. Because this enzyme converts arsenite to arsenate, which is less harmful than arsenite, this bacterial approach can be used as a biological solution to the problem of arsenic contamination. In Alcaligenes faecalis, arsenite oxidase enzymes have already been identified, purified, and characterized [12]. In this project we have amplified arsenite oxidase gene from the strain *Comamonas testosteroni KF1*.

CHAPTER 2
LITERARTURE OF REVIEW

2.1 ARSENIC TOXICITY

Arsenic mobilization in water sources endangers the health of 100 million people across the world. Many companies discharge heavy metal contaminants into river waterways. Mobility and toxicity in aquatic environments are highly influenced by its oxidation state. As^{5+} has a higher propensity for surface absorption than As^{3+}, which prefers to stay in the aqueous phase [4]. As is hazardous to life in both forms; however, As^{3+} is 100 times more toxic than As^{5+} [13]. Arsenate (As (V)) is a hazardous 357 analogue for inorganic phosphorylating activity, whereas Arsenite (As (III)) demonstrates toxicity through attaching to the protein sulfhydryl groups [4]. Ultimately, As^{3+} interacts with cysteine groups, glutathione, glutarodoxin, and theoredxin found on the active sites of several enzymes for controlling the equilibrium of intracellular redox, protein folding, Sulphur metabolism, synthesis and repair of DNA as well as for detoxification of xenobiotic. Arsine gas binds to RBCs, resulting in hemolysis and membrane damage [5]. There are four oxidation states of arsenic: As^{5+}, As^{3+}, As^{0} and As^{3-} and the two higher oxidation states are the most widespread in nature, whereas the two lowest are uncommon.

2.2 ARSENIC RESISTANT BACTERIA & THEIR MECHANISM

Bacteria indigenous to arsenic contaminated environment employ several different strategies to resist arsenic toxicity. Primary mechanism is the prevention of uptake. Different defense systems are initiated once arsenic enters the organism. Many research studies conducted in the last several years have revealed a plethora of As- transforming bacteria in a diversity of pollution environments [14] – [18].

Some examples of As resistant bacteria include Pseudomonas putida, Herminiimonas arsenicoxydans, Thiomonas arsenivorans and several strains of Bacillus, Agrobacterium, Pseudomonas, Acinetobacter, Crenarchae, Stenotrophomonas, Archromobacter, Citrobacter, Rhodobium, etc. They have been reported to show high tolerance level of Arsenicals [1].

Studies on Arsenic polluted environments have depicted that bacteria interact with A using a sequence of mechanisms. There are mainly 4 strategies that bacteria utilize to resist arsenic toxicity:

a) Efflux of Arsenicals or minimization of arsenic intake by increasing the phosphate uptake specificity [As(V)] and the glycerol transporter (aquaglyceroporin) [As (III)] [4] [19] [20] Faecalis.
b) Reduction of As (V) to As (III) by arsenate reductase enzymes (Azurin)
c) Methylation of Arsenite catalyzed by SAM (S-adenosyl methionine methyltransferases)
d) Oxidation of As (III) to As (V)

The following diagram shows several mechanisms of As metabolism of prokaryotes:

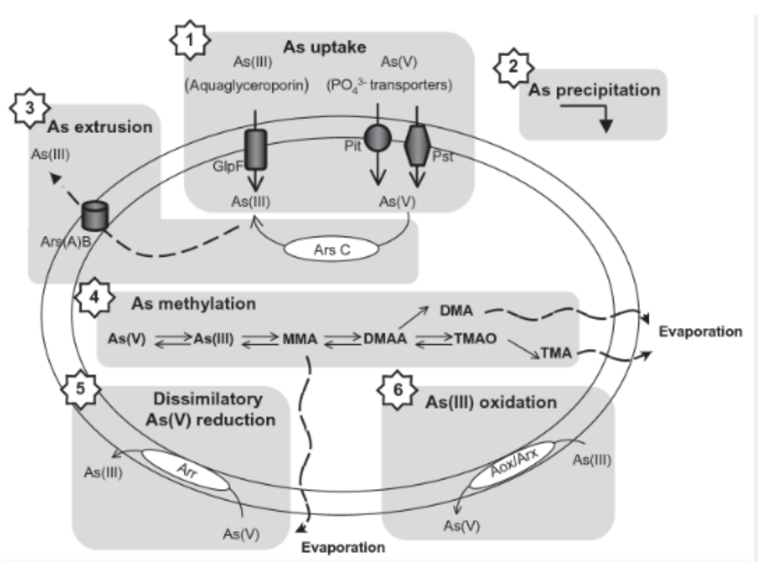


Figure 2.1: Mechanisms of Arsenic metabolism of Prokaryotes [39]

- Arsenate (As^{5+}) enters the cells through the phosphate, once it penetrates the cell, As^{5+} is reduced then to As^{3+} by the process of cytoplasmic As^{5+} Reductase. Subsequently, after reduction As^{3+} is disposed out from the cell by arsB efflux pump. In an anaerobic environment, As^{5+} is then utilized as a terminal acceptor of electrons during the process of respiration.
- The periplasmic AIO (Arsenite Oxidase) system can act to oxidase As^{3+} to act as an electron donor or a bacterial resistance mechanism. The aquaglyceroporin channel allows the entry of As^{3+} into the cell and is further expelled out directly from the cells with the help of another As^{3+} transporter known as acr3p.

- Additionally, methylated is used to modify inorganic As into organic species.

Several chemolithotrophic bacteria with a respiratory As^{3+} oxidation system may use arsenite as an energy source, however certain heterotrophic organisms can only use As^{3+} for detoxification without receiving any energy [21]-[23][13].

2.3 ARSENITE OXIDASE

The Aio (arsenate oxidase) System in bacteria represents a potential detoxification technique Arsenite Oxidase catalyzes the conversion of As^{3+} to As^{5+}. Since these enzymes has property of converting arsenite into less toxic arsenate form, this bacterial technique can be utilized as a biological way to evade the problem of Arsenic pollution. This arsenite oxidase enzymes has already been isolated, purified and characterized in strains of Alcaligenes Faecalis.

The enzyme is present on the outer surface of the inner membrane and possesses the ability to oxidase arsenite in the presence of azurin and cytochrome C as the acceptor of electron. The purified protein has the molecular weight of 85KDa and its further residues as the monomer with various metal centers, including most likely both [4 Fe- 4S] i.e., HiPIP (High Potential Iron Protein) as well as the Rieske- types [2 Fe-2S] centers. And the protein – molybdenum cofactor present is an arsenite-sensitive molybdenum- protein helps in proper functioning of this enzyme system.

2.4 STRUCTURE AND MECHANISM OF ARSENITE OXIDASE

The crystal structure of Arsenite Oxidase enzyme has been already revealed through X ray diffraction technique [12] and the genes for the two structural subunit polypeptides have also been sequenced. The structure of enzymes of arsenite oxidase depicts two sub-units: 1) a larger 88kDa polypeptide consisting the Mo-pterin cofactor and 2) a HiPIP 3Fe^4S center and a smaller 14kDa subunit having the Rieske2Fe^2S center. The Mo-pterin cofactor contain two pterin, aligned respectively up and down to each other in the protein.

A plain funnel shaped split is present on the large subunit that enables entrance of Arsenite (AsO3H2-) and after oxidation facilitates the release of arsenate (AsO3H2-) from the protein in reserve direction.

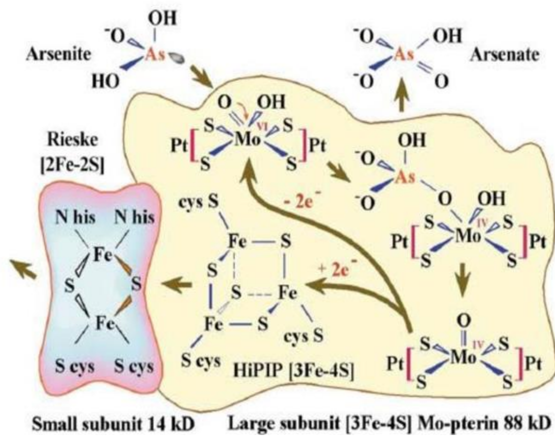


Figure 2.2: Structure and proposed reaction mechanism of Arsenite Oxidase [40]

The proposed reaction mechanism includes following steps:

a) First arsenite (ASo3H2-) binds to the enzyme by bonding closely with the oxidized co-factor of value Mo(VI), enabling a direct attach of the nucleophile and by transferring two electrons.
b) Two electrons are then transferred from arsenite to Mo, after which oxidizing As (III) to As(V) takes place and finally reducing the Mo (VI) to Mo (IV) occurs.
c) This results into release of arsenate, AsO4H2-
d) Simultaneously, two electrons are transferred from Mo (IV) to HiPIP 3Fe-4S center which is 14 Å away from the atom of Mo, therefore it requires an electron pathway that consists of several intermediates. This step is responsible for the regeneration of Mo (VI) center.
e) Subsequently, two electrons are then transferred from the center of HiPIP 3Fe-4S of the larger sub-unit to the Rieske center of 2Fe-2S of small sub-unit.
f) Finally, electrons are one of the most potent environment biocatalysts and can be used as an effective tool in bioremediation.

2.5 MOLYBDOPTERIN OXIDOREDUCTASE

The Molybdopterin Oxidoreductase enzyme from *Comamonas testosteroni KF1* has been shown to be a soluble, protein with a molecular weight of approximately 104 kDa. The enzyme requires molybdenum, iron-sulfur clusters, and heme for its activity, and it exhibits a broad pH range for optimal activity. *Comamonas testosteroni KF1* is a gram-negative bacterium that has been widely studied for its ability to assimilate nitrogen from

various sources. Molybdopterin Oxidoreductase from *Comamonas testosteroni KF1* has been cloned and characterized in several studies.

It has been studied for its potential use in bioremediation and biocatalysts applications. Overall, the Molybdopterin Oxidoreductase enzyme from *Comamonas testosteroni KF1* is a promising target for further research and development in the field of biotechnology and environmental science.

CHAPTER 3
MATERIALS AND METHODS

3.1 BIOINFORMATIC ANALYSIS

In addition to being a key tool for routine DNA analysis, bioinformatics is a crucial resource for molecular biology research. There are a massive number of sequences from several species, including the human genome, which is being stored in genetic databases. The NCBI BLAST is among the most valuable bioinformatics resources that allow comparing a DNA sequence with all the other sequences which are stored within a database of DNA for example, GenBank, EMBL, DDBJ, etc.

The comparative modelling, or homology modelling, aims to predict the structure of a protein by comparing it with structures of related or homologous proteins. A generalized process for homology modelling of a target protein including following steps:

a) Identification of related proteins having similar protein sequence using search tools such as BLAST and FASTA. The 3D structure of these related proteins serves as a template for model.

b) The sequence of target protein is aligned with the template protein sequences using multiple alignment tool such as CLUSTAL ω to look for regions of target that are highly conserve across all the templates and those which are less conserved.

c) By superimposing the template structures and identifying structurally conserved sections, a model structure is constructed. A core for the model is formed by aligning the template backbone with conserved fragments of structure.

d) Following the modelling of the core loops using various computational techniques, such as choosing the optimal loop from a database of known loop conformations, etc.

e) Following the creation of this core structure, the positions of the side chain atoms are established using various techniques, such as rotamer library search, etc.

f) Finally, the quality of structure constructed through this process is evaluated via different software.

In this project the structure prediction was done through AlphaFold2. Additionally, the Swiss-Model was used to examine the Ramachandran Plot's structural integrity.

3.1.1 Sequence Analysis

Sequence of Arsenite Oxidase enzymes of *A. faecalis* which has already been isolated, purified and characterized [24] was taken from UniprotKB database (a protein sequence database) with primary accession number (Q7SIF4). To find out homologous sequences for this protein sequence, protein BLAST was done against PDB database (a protein structure databases) for bacterial strains already available in the lab.

3.1.2 Structure Prediction by AlphaFold2

To search for the template in homology modelling for Arsenite Oxidase, protein BLAST was done and from the results of BLAST we found protein showing structural and functional similarity to Arsenite Oxidase from *A. faecalis* (PDB 1G8K). Thus, we close this protein as template for comparative modelling of Arsenite Oxidase. From AlphaFold2 the protein was modelled and then aligned with 1G8K template in PyMOL [25].

3.1.3 Multiple Sequence Alignment

The process of Multiple sequence alignment was done for sequences of Arsenite Oxidase from *A. faecalis* and Molybdopterin Oxidoreductase from *C. testosteroni KF1* by CLUSTAL OMEGA.

3.2. CLONING OF ARSENITE OXIDASE

A gene is isolated from an organism and placed into a vector for replication in a process known as molecular cloning. These molecular clones can be used to produce several copies of DNA for gene sequence analysis and/or to express the appropriate protein for study or use in understanding the function of the protein after being extracted. In this project we have attempted to produce molecular clones of an enzyme that was found to be homologous to Arsenite Oxidase [26]. Once cloned and expressed, further research on this protein can be done to find out its activity; structure through crystallography, and/or its mutants.

3.2.1 Isolation of genomic DNA from *C. testosteroni KF1* cells.

- **Principle:** The separation and purification of gDNA from cells marks the transition

from cell biology to molecular biology. In gDNA isolation the total DNA content is separated from the RNA, protein, lipids, etc. For this first the cell membranes are disrupted through SDS, after which Mg^{2+} (an important cofactor for nucleases) are removed by chelating with EDTA to protect DNA from degrading. To degrade the RNA and protein, RNase and proteases are added. Phenol and chloroform play role in denaturing and separation of the proteins from DNA. The denatured proteins are then eliminated by centrifugation. Finally, the DNA released from disrupted cells is precipitated cells is precipitated using cold ethanol.

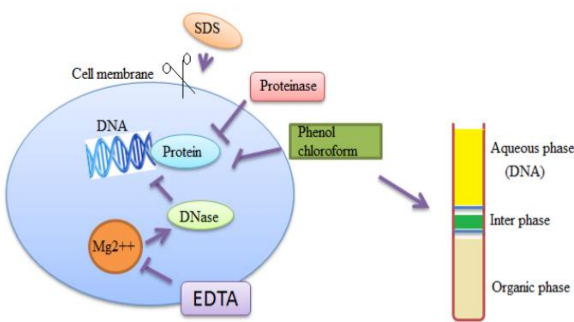


Figure3.1: Schematic representation showing principle of gDNA isolation [41]

- Requirements:
  a) Sample and Reagents: Cell culture for DNA isolation, Qiagen Genomic DNA extraction kit.
  b) Apparatus & Equipment's: Eppendorf Centrifuge 5810R and 5418, Vortex mixer, Micropipettes, micro tips, microcentrifuge tubes, etc.
- Procedure:
  a) Pipet out 1ml of bacterial culture into a microcentrifuge tube of 1.5 ml, and then centrifuge it at 12000 rpm for the duration of 5 min. Throw away the excess liquid.
  b) Now, add a buffer ATL to a pellet to a total volume of 200 µl.
  c) 20 µl proteinase K is added and is the mixed with the help of vortexing it is then incubated at a temperature of 56ºC until the tissue gets lysed completely. To enable efficient analysis, a swaying platform or a water bath should be used.
  d) Short spin the tube for removing the extra drops from the walls and lid.
  e) First add 20 µl 20mg/ml RNAse A, mix for 15 sec using the pulse-vortexing, and then incubate it at room temperature for 2 min. Further, you can short spin the tube (optional step)
  f) After this, 200 µl AL Buffer (lysis buffer) is added. Vortex the mix for 15 sec, and

incubate it at the temperature of 70ºC for 10 min. Then, shortly centrifuge the MCT tube.

g) To this sample add absolute ethanol of 200 µl and mix it for 15 sec by vortexing.
h) The sample from last step is then applied to the QIAamp Mini spin column without letting the rim get wet and then centrifuged for 1 min at 8000 rpm. The column if transferred to new collection tube and then the process of centrifugation is performed again at around 6000 x g (8000 rpm) for reducing the noise.
i) Now, add to the QIAamp Mini spin column 500 µl buffer AW1 and centrifuge it for 1 min at 6000 x g (8000 rpm). With the help of the filtrate discard the collection tube.
j) After this, carefully open the QIAamp Mini spin column and pour 500 µl buffer AW2 into it without dampening the rim followed by the process to centrifuge at full speed (14,000 rpm) for 3 min. Discard the collection tube.
k) Place the column in a new 2 ml collection tube. To eliminate the buffer AW2 carry over, briefly spin the tube at full speed for 1 min and discard the collection tube containing the filtrate.
l) The spin column is then placed into a clean1.5 ml MCT. Cautiously open the column and then add 200 µl Buffer AE (Elution buffer) to it. Further incubate the solution for 5 min at room temperature, and then centrifuge it at 8000 rpm for 1 min.
m) Repeat step (k).

- **Precautions:**

a) Use plastic containers and reagents devoid of DNase.
b) Use of cut tips is advised to prevent mechanical damage to the DNA.
c) You shouldn't elute more than 200 L of elute into a 1.5 mL microcentrifuge tube because the spin column will come into touch with the elute and cause the centrifugation to produce aerosols.

3.2.2 Primer Design for PCR

For selective amplification of the desired genes in PCR, the primer designing is the most crucial factor. The DNA Sequence of the target gene is used to generate two primers called amplimers that are specific to the flanking sequences of the target DNA sequence [27].

To design primers, it is essential to consider certain factors:

a) Primer Length: When the primers are short in length they may produce unwanted amplification products, while longer primers produce higher specificity for a particular target. However, every longer primer promotes higher specificity for a particular target. However, very long primers (more than 18-20) nucleotides are also not ideal since very long primers (more than 18-20) nucleotides are also not ideal since very long primers interferes with the rate at which the primer hybridized to the template DNA.
b) Nature of sequences: The two primers should not have any self complementarily or complementarity to each other to prevent dimer or hair pin structure formation.
c) Tm Values: The melting point of primers, or Tm, is the temperature at which half of the DNA strands are double-stranded.
d) Base Composition: The GC content should lie between 40 to 60%.

Based on the above aspects Primer Designing was done with help of NEB cutter and Oligo Analyzer Primer Quest Tool For this tBLASTn was done to find out the nucleotide sequence for our protein and using NEB cutter zero cutters were identified. After this we took around 10 nucleotides to design forward primer and similarly 10 nucleotides from the last of its reverse complement to design reverse primer through Oligo Analyzer. Oligo Analyzer was also used for further evaluation and designing of Primers.

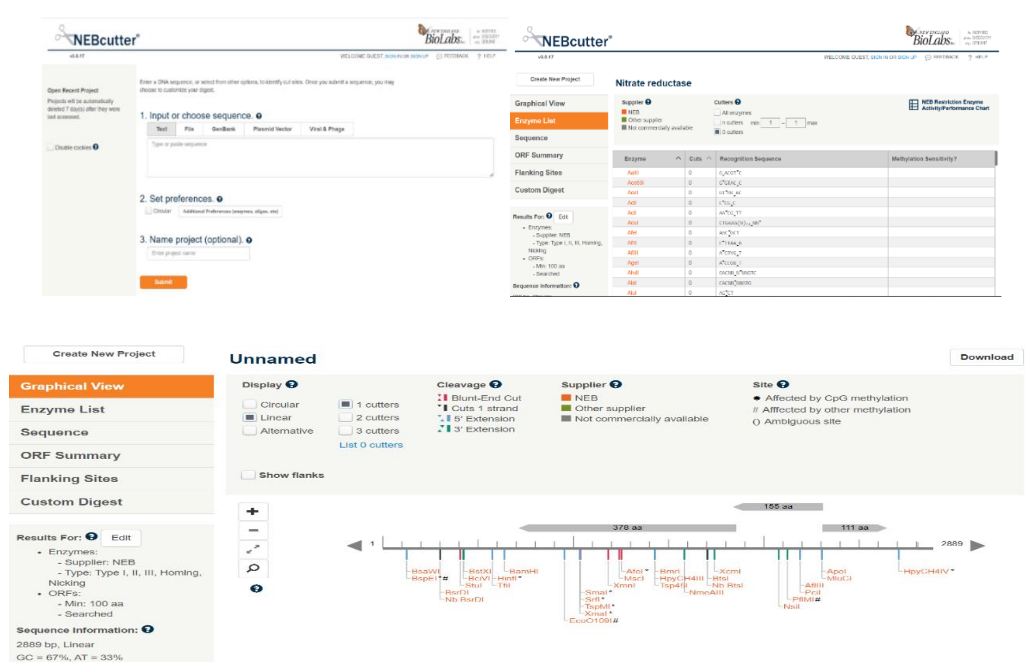


Figure3.2: NEB Cutter Webpage [42]

3.2.3 Gene Amplification by PCR

A specific DNA fragment (target DNA) is amplified using the Polymerase Chain Reaction (PCR) from the template DNA. The PCR is divided into three phases, each having a set of timings and temperatures:

a) Denaturation by heating the reaction to 90°C,
b) Annealing primers to each original strand in order to synthesize additional strands.
c) The new DNA strands created by the primers are extended.

The extra oligonucleotide primers can attach to their complementary sites flanking the target DNA after annealing, which aids in their ability to hybridize. Annealed oligonucleotides serve as primers for DNA polymerase because they provide it a 3' hydroxyl group [28].

Taq DNA Polymerase, Phusion, and other thermostable DNA polymerase enzymes carry out the "extension" stage of DNA synthesis. Once the new strands have been extended along and past the target DNA to be amplified, DNA synthesis continues from both primers. A region at the new strands' 3' end will be complimentary to the other primer. The amount of DNA created will therefore expand exponentially because the new strands will serve as templates alongside the old strands in a subsequent round.

Component	**Concentration**	**Volume (µl) (25 µl x 4)**
Nuclease free water (Mili Q)		66 µl
GC Buffer	5X	20 µl
dNTPs	10mM	2.5 µl
Template (gDNA C. testosterone KF1)	25- 50ng	2.5 µl
DMSO	100%	4.0 µl
Forward Primer	10 µM	2.0 µl
Reverse Primer	10 µM	2.0 µl
Phusion Enzyme	1 unit/ 50 µl	1.0 µl
TOTAL		100 µl

Table 3.1: PCR Formulation

Step	Temperature & Time	Cycle
Initial Denaturation	98° C, 1min.	1
Denaturation	98° C, 30 sec.	35
Annealing	72° C, 1:30 min.	35
Extension	72° C, 1:30 min.	35
Final Extension	72° C, 5 min.	1
Hold	4° C, ∞	

Table 3.2: PCR program for amplification

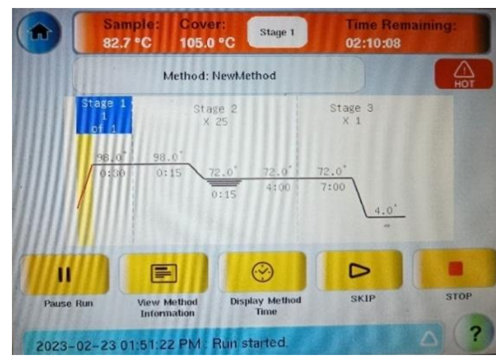


Figure 3.3: Thermo-scientific Thermal Cycler showing PCR program

3.2.4 Analysis of Gene Amplification by Gel Electrophoresis

A technique called gel electrophoresis is used to separate macromolecules like DNA, proteins, and other molecules, as well as their fragments, according to their molecular size and charge. Due to its phosphate backbone, DNA is negatively charged when an electric field is applied. Because DNA has a constant mass/size ratio, it separates in gel in a pattern where the larger the fragment, the shorter the distance traversed [29]. Therefore, the rate at which DNA moves on the gel depends upon following factors:

a) size of DNA
b) concentration of Agarose (determines pore size and molecular sieving properties of gel)
c) voltage applied
d) presence of EtBr, etc.

- **Requirements (for 1% Agarose Gel):**
  1x TAE (40 mM Tris-acetate, 1 mM EDTA), EtBr 0.5µg/ml, Agarose low EEO, loading dye, DNA ladder.

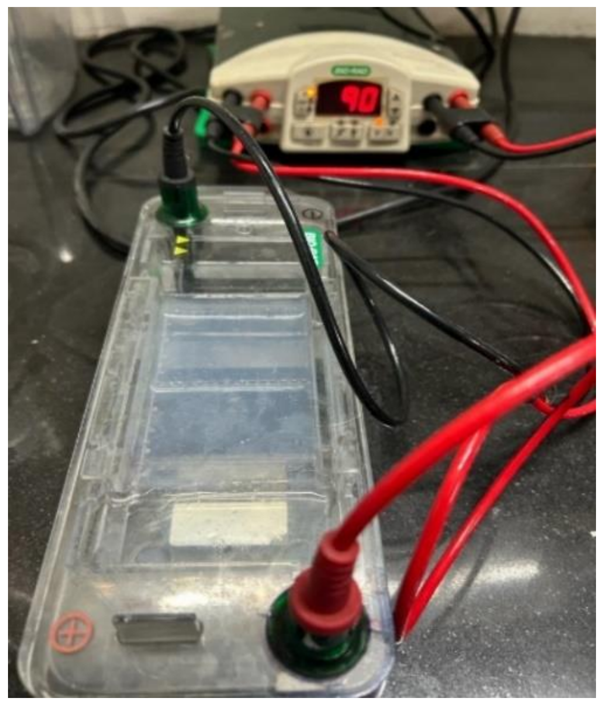


Figure 3.4: Agarose Gel Electrophoresis apparatus

- **Protocol:**
    a) Take 0.4g Agarose EEO and put into an Erlenmeyer flask. Now add 40 ml of the running buffer TAE into the flask containing Agarose.
    b) Microwave the agarose for intervals of 30 to 40 seconds to melt it. Stir it, then let it to cool.
    c) Now add a little drop EtBr (0.5µg/ml) to the mix
    d) Insert the gel tray into the casting device with the proper combs. To make molds, pour the melted agarose right now.
    e) Permit the gel to cool and solidify. Put the tray in the gel box after removing the combs.
    f) Include loading dye in the DNA sample preparation process. Load the samples in wells.
    g) Set the desired voltage (1-5V/cm between electrodes) on the power supply.
    h) Cover the gel with buffer and connect the gel box's leads to the power source. Turn on the power supply. Run the gel until dye reaches appropriate distance.
    i) After electrophoresis is completed, take out gel from the box by removing excess buffer.
    j) On the UV transilluminator, apply the gel. Orange bands would represent DNA bands. Take a photo for later research.

3.2.5 PCR Purification

PCR Purification is a critical procedure. Since nucleotides, enzymes, buffer and primers components must be removed before using the DNA, purification is thought to be important for downstream applications.

A reaction mixture including DNA and binding buffer, a chaotropic agent that can break up hydrogen bonds, denature proteins, and promote DNA binding to the column's silica membrane, is put into a purification column. A purification column is filled with a reaction mixture containing DNA and binding buffer [30]. For optimum DNA binding, the binding buffer also features a color indication that makes it straightforward to monitor the pH of the solution. Impurities are eliminated using a straightforward wash method. The pure DNA is subsequently removed from the column using the elution buffer. The DNA that's been retrieved is now suitable to be used in downstream applications.

- **Requirements:**
  - Thermo Scientific™ Gene JET Gel Extraction and DNA Cleanup Micro Kit containing
    a) Binding Buffer
    b) Extraction Buffer
    c) Prewash Buffer (conc.)
    d) Wash Buffer (conc.)
    e) Elution Buffer
    f) DNA Purification Micro Column & Collection Tube
  - PCR samples.
- **Protocol:**
  - Add 1:1 volume of binding buffer to the PCR mixture to be purified.
  - Now add 1:2 volume of neutralizing buffer (100% Isopropanol) to the combined mixture. (i.e., for 100µl PCR mix add 100 µl Binding Buffer and 100 µl of Isopropanol)
  - Transfer up to 800 µl of the above solution into the Gene JET Purification Column. Centrifuge for 1.5 min at 12000 rpm. Discard the flow-through after using it.

- Now add 700 µl of Wash Buffer (Ethanol) to the column and centrifuge again at 12000 rpm for 1.5 min.
- Throw away the flow-through after using it. To get rid of any remaining buffer, centrifuge the empty column for one minute. Discard the flow-through. Give it a minute to dry.
- Add roughly 30-35 µl of warm elution buffer and the Gene Jet filter to a fresh, clean microcentrifuge tube. Keep it for 3-4 min.
- Now Centrifuge for 1min. Throw away the column, then keep the DNA at -20°C.

3.2.6 Plasmid Isolation

Plasmids are remarkably useful tools in molecular biology and genetics, especially in the field of genetic engineering. Plasmids are circular extrachromosomal DNA molecules that contain genes for conjugation, antibiotic resistance, and the metabolism of 'abnormal' substrates. Plasmids that can be extensively reproduced by bacteria like E. coli are excellent vectors. Several artificial plasmids have been designed over the years for cloning purposes. These plasmids have several restriction sites at which they can be cleaved or digested and ligate gene of our choice having same sticky ends (digested by same restriction enzymes) [32]. The ligated DNA or rDNA can then be transformed into competent cells of E. coli. Plasmid isolation involves removal of cell lysate including proteins, lipids, genomic DNA, RNA, etc. Genomic DNA isolation uses strong lysis, such as the enzymatic or mechanical breakdown of cell membranes, to release genomic DNA into the solution, whereas plasmid DNA isolation uses mild alkaline lysis to release plasmid DNA into the solution alongside genomic DNA. This is the main distinction between genomic DNA isolation and plasmid DNA isolation. Furthermore, whereas plasmid DNA separation uses neutralization with potassium acetate to separate plasmid DNA from genomic DNA, cell lysis allows for the isolation of genomic DNA from lipid membranes and proteins.

- **Requirements:**
  - Thermo Scientific Gene JET Plasmid Miniprep Kit containing Resuspension Solution, Lysis Solution, Neutralization Buffer, Wash Buffer, RNase A, Elution Buffer (10 mM Tris-HCl, pH 8.5), Gene JET Spin Columns and 2ml

Collection tubes.

- Secondary culture of pET28a plasmid containing E. coli cells.

- **Procedure:**
  a) Pellet down 5ml culture in an Eppendorf tube by centrifugation at 10,000 rpm for 15 mi. Discard the supernatant and collect the pellet.
  b) Resuspend the pellet in 250 µl resuspension buffer and vortex for 15 sec or resuspend by pipetting up and down.
  c) Pour 250 µL of the Lysis Solution and mix properly by inverting the tube 4 -6 times till the solution turns thick and somewhat transparent. Note: To prevent shearing of gDNA do not vortex and don't incubate beyond 5 min to avoid denaturation of supercoiled plasmid DNA.
  d) Now pour 350 µl of the Neutralization Solution and mix properly.
  e) Centrifuge at 10,000 rpm for 15 min. This will pellet down the cell debris and chromosomal DNA.
  f) Pipette out the supernatant in the plasmid isolation filter and spin for 2 min.
  g) Discard the filtrate. Put in 750 µl. Wash Buffer Solution and centrifuge again for 1 min.
  h) Discard the filtrate and spin the empty column again to remove residual wash buffer.
  i) Now take out the filter from the column and put in a new 1.5ml microcentrifuge tube. Open the cap of filter and let it dry for 5 min.
  j) Add 30-35µL warm Elution Buffer exactly on the filter. Incubate for 2-5 min at room temperature.
  k) Centrifuge the tube. Discard the column and store the purified plasmid DNA at -20°C.

3.2.7 Restriction Digestion of amplified gene and plasmid vector pET 28a

The amplified gene and vectors were then digested with the appropriate enzymes (Ndel and Xhol) in order to make their ends sticky for ligation after successful gene amplification and plasmid isolation. The digestion reaction carried out for 3 hours at 37°C. The restriction digestion protocol for Gene and pET28a (+) vector is tabulated below:

Components	Volume
CutSmart Buffer	3 μL
Enzyme (NdeI)	1.5 μL
Enzyme (XhoI)	1.5 μL
Template (Gene)	24 μL
Total	30 μL

(a)

Components	Volume
CutSmart Buffer	3 μL
Enzyme (NdeI)	1.5 μL
Enzyme (XhoI)	1.5 μL
Nuclease free water	4 μL
Template (plasmid)	20 μL
Total	30 μL

(b)

Table 3.3 (a) and (b) Restriction Digestion of gene and plasmid pET28a.

*The digested gene and vector (plasmid) were then subjected to Agarose Gel Electrophoresis and PCR purification (to remove restriction enzymes).

3.2.8 Ligation of digested gene and vector

After digestion, gene and vector having complementary sticky ends (digested by same restriction enzymes) are ligated. The ligation reaction was carried out for 20 hours. The protocol for Gene and pET28a vector ligation reaction is given below:

- **Requirements**
  a) Purified plasmid vector, Insert DNA, T4 DNA ligase, T4 DNA Buffer, Nuclease water.
- **Procedure**
  a) Thaw and resuspend T4 DNA ligase buffer and add it to reaction tube
  b) Add vector and insert DNA followed by nuclease free water.
  c) Add T4 DNA ligase enzyme.
  d) Mix the contents gently, make sure liquid doesn't stick to walls.
  e) Incubation is done overnight at 16°C with heat shock at 65°C.
  f) After ligation, ligation must be inactivated with heat shock at 65°C

Components	Volume
Gene	20 μl
Vector	6 μl
T4 DNA Buffer	3 μl
T4 DNA ligase	1μl
Total	30 μl

Table 3.4. Ligation reaction of pET28a and Gene

*After ligation the ligated DNA is transformed into competent cells.

3.2.9 Competent Cell Preparation of Cloning Vector *E. coli DH5α* Cells.

For transforming the rDNA or any foreign DNA into a cell it is important first to make them competent so that they can take up the foreign DNA from their surroundings. Calcium chloride heat shock transformation is a potent molecular biology technique which is used for generating competent cells to fulfil the purpose of introduction of foreign DNA or plasmid into a host cell. When $CaCl_2$ is added to a cell solution, the Ca ions create a cation bridge between the negatively charged phosphate backbone of DNA and the negatively charged phosphorylated lipid in lipopolysaccharide, which makes it easier for foreign DNA to enter the host cell. Upon heat shock treatment the cell membrane becomes porous, allowing the uptake of DNA into the cell. Thus, this step is very crucial for transformation of rDNA into the host [33].

- **Requirements for Competent Cell Preparation:**
  a) Equipment's: 37 °C Shaking incubator, 42 ºC water bath
  b) 1 ml of overnight *Ecoli DH5α* Cell culture.
     100 ml of 0.1 ssM Chilled $CaCl_2$
     200 ml of 0.1 M $CaCl_2$, with 15% glycerol solution
     100 ml fresh LB media.
- **Procedure:**
  a) Preparation of $CaCl_2$ Buffers:
     - 1M $CaCl_2$: Stock Solution: 11.1g Anhydrous $CaCl_2$ + 80ml of dH_2O + fill upto 100ml
     - 0.1M $CaCl_2$ Working Solution: 10ml Stock + 90ml dH_2O
     - 0.1 M $CaCl_2$ with 15% glycerol solution: 6 ml 1M $CaCl_2$ + 9 ml glycerol + 45 ml dH_2O.
  b) Culture of *E. coli DH5α* cells: (Primary Culture)
     - Inoculate 1ml of LB with *E. coli DH5α* cells.
     - Place in shaking incubator at 37°C and 200 rpm for overnight.
  c) Generation of Competent cells:
     - Transfer 500 µL cells from overnight culture to a fresh LB media tube.
     - Place in shaker incubator for approx. 3 hours at 37°C, 190 rpm so as to

achieve an OD 0.6 to 0.8 nm. (Secondary culture)

- Transfer cells from secondary culture tube to red cap tubes. Centrifuge for 7 min at 4000 rpm, 4°C and pellet down the cells.
- Keep them in ice bucket. Take to laminar hood, decant media and add 4ml 0.1M cold $CaCl_2$ and resuspend
- the cells. (Washing Step)
- Now again centrifuge the tubes at 4000 rpm, 4°C for 10 min. Take to laminar hood and decant the $CaCl_2$.
- Add fresh 4 ml $CaCl_2$ and resuspend. Repeat last two steps (centrifugation and resuspension) 4-5 times.
- Incubate in ice for 30 min.
- Centrifuge at 4000 rpm, 4°C for 10 min. Decant the supernatant. Competent Cells are pelleted down.

3.2.10 Transformation of pET28a plasmid with gene into *E. coli DH5α* host cells

In order to create several copies of a recombinant DNA molecule, bacterial transformation is an essential step in the molecular cloning process. Bacterial cells can acquire foreign DNA (also known as naked DNA) by transformation via horizontal gene transfer. Transformation can be used for various applications such as:

- for making multiple copies of the desired DNA
- in cloning procedures
- for expression of large amounts of proteins and enzymes
- to generate cDNA libraries
- in DNA linkage studies

In this experiment we wanted to make multiple copies of the DNA so we utilized genetically engineered *E. coli DH5a* cells to maximize transformation efficiency.

Transformation efficiency is calculated using the formula below:

$$Transformation\ efficiency = \frac{Number\ of\ colonies\ on\ plate}{Amount\ of\ DNA\ plated\ (\mu g)} \times 1000 \qquad \text{Eq (1)}$$

<u>Characteristics of *E. coli DH5a* cells:</u>

- This strain is a nonpathogenic strain engineered by American biologist Douglas Hanahan
- DH5α cell has three distinct mutations: recA1, endAl.
- recA1 is a point mutation in the recAl polypeptide that replaces glycine 160 with and an aspartic acid residue which makes recombinases disabled resulting into inactivation of homologous recombination that ensures a higher insert stability.
- The endA1 mutation turns an intracellular endonuclease inactive, preventing it from digesting the plasmid.

<u>Procedure for transformation</u>:

- Resuspend competent cells in 200µL. 0.1M $CaCl_2$
- Label the fresh red cap tubes as control and ligated and add 70ul. cells to each.
- Add 15 µL ligation product to the tube.
- Incubate in ice for 30 min (meanwhile turn on the water bath at 42°C.
- 1 min heat shock treatment.

<u>Recovery:</u>

- Add 1ml LB media (autoclaved) to each tube, and place in a beaker tightly.
- Incubate at 37 °C to allow the antibiotic resistance genes to start expression.
- After 2-3 hours, melt 200ml LB agar for 10 plates (autoclaved) in microwave. Let it cool and then add kanamycin.
- Pour the media in plates and allow them to cool down in Laminar.
- After 20-30 min, with the help of spreader proceed for plating. Add around 70 µL of cells to the plate and spread evenly using autoclaved spreader.
- Label the plates. Incubate at 37 °C.

3.2.11. Analysis of recombinant clones in *E. coli DH5a* by Colony PCR

Colony PCR is a method for quickly determining whether the desired gene is present in colonies of transformed bacterial cells that have grown up on selective medium [34]. The colony PCR reaction is essentially identical to the standard PCR reaction in that it uses colonies made up of cells that may or may not contain the plasmid containing the desired

gene as the template, along with primers, polymerase, and dNTPs.

Component	Volume (ul) (25 µL X 10 colonies)
Nuclease free water (MiliQ)	168 µl
GC Buffer	50 µL
dNTPs	5 µL
Forward Primer	5 µL
Reverse Primer	5 µL
Template (Colonies from plate)	5 µL
DMSO	15 µL
Phusion enzyme	2 µl
TOTAL	250 µl

Table 3.5: Colony PCR formulation for gene.

Step	Temperature & Time	Cycle
Initial Denaturation	98°C, 5 min	1
Denaturation	98°C, 30 sec	20
Annealing	60 °C, 1:30 min	20
Extension	72°C, 1:30 min	20
Final Extension	72°C, 5 min	1
Hold	4°C, infinite	-

Table 3.6: Colony PCR program used.

3.3 EXPRESSION OF ARSENITE OXIDASE.

Once the molecular clones are produced, we have multiple of copies of the gene required. But, for studying the characteristics of the resultant protein such as its structure, activity and for other genetic engineering purposes we need to first synthesize the protein encoded by the gene. Therefore, we transform our cloned gene into a host cell having gene expression system which is capable of active expression of a foreign gene.

3.3.1 Isolation of recombinant plasmid pET28a from *E. coli DH5α*

After successful cloning of the desired gene in *E. coli DH5α* cells the recombinant plasmid is isolated from the cells of colonies that showed positive results for transformation. For this purpose, cells from colony that showed positive result for transformation was taken and were inoculated in 10ml LB media in a tube [35]. This tube was incubated at 37°C and then recombinant plasmid was isolated from it by following the same procedure given below.

3.3.2 Competent cell preparation of expression vector *E. coli BL21(DE3)* strain.

Competent Cell preparation of *E. coli BL21 (DE3)* cells was done following the same procedure given below.

Characteristics of *E. coli BL21(DE3)* cells:

- BL21 DE3 is a derivative of BL21, that contain the λDE3 lysogen that carries the gene for T7 RNA polymerase under control of the lacUVS promoter.
- It is suitable for protein expression as it is deficient in LON protease and hence brings stability to the expressed proteins.
- OmpT (outer membrane protease) is also absent whose function is to degrade extracellular proteins.
- Due to the hsdSB mutation in these cells prevents foreign DNA methylation and disruption in the cells.
- Hence, for the above reasons BL21 DE3 is an excellent expression vector.

Mechanism of Expression of desired gene in *BL21 DE3* cells:

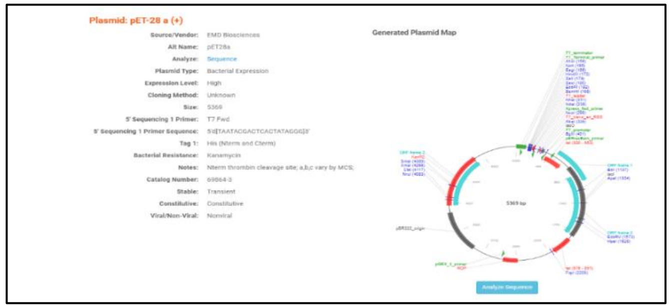


Figure 3.5: Plasmid map of pET28a showing restriction sites including Ndel and Xhol, and the T7 promoter and terminator sites [43].

As shown in the diagram above the pET28a plasmid vector contains the T7 RNA promoter gene so when this recombinant plasmid is inserted into the BL21 DE3 host cells that carries the T7 RNA polymerase (T7RNAP) gene under control of lacUV5 promoter, we can induce over expression of the insert gene by using this machinery with help of IPTG.

IPTG Induction Theory

Isopropyl β D-1 thiogalactopyranoside (IPTG) is a molecular biology reagent which is a molecular mimic of allolactose, a lactose metabolite that triggers transcription of the lac operon, and it is therefore used to induce expression of our gene of interest.

When IPTG induction is done, the T7RNAP produces, this protein binds to the T7 promoter in the plasmid leading to expression of the gene insert under the control of T7 RNA promoter.

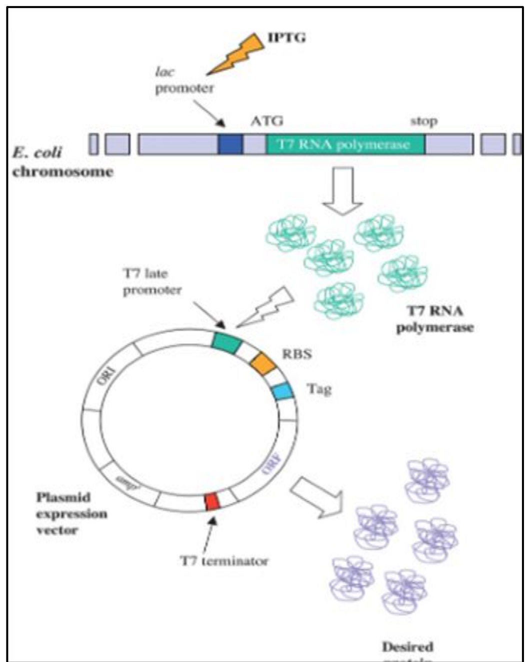


Figure 3.6. Schematic representation of recombinant protein expression [44].

3.3.3 Analysis of recombinant clones in *E.coli BL21*.

Colony PCR was done to analyse the expression of recombinant gene in the vector. Colonies were picked from the LB agar plate and was resuspended in the PCR reaction mixture.

Component	Volume (µl) (25 ul. X 10 colonies)
Nuclease free water (MiliQ)	168 µL
GC Buffer	50 µL
dNTPs	5 µL
Forward Primer	5 µL
Reverse Primer	5 µL
Template (colonies from plate)	2-3 µL
DMSO	15 µL
Phusion enzyme	2 µL
TOTAL	250 µL

Table 3.7: Colony PCR formulation for gene.

Step	Temperature & Time	Cycle
Initial Denaturation	98°C, 5 min	1
Denaturation	98°C, 30 sec	20
Annealing	60 °C, 1:30 min	20
Extension	72°C, 1:30 min	20
Final Extension	72°C, 5 min	1
Hold	4°C, infinite	-

Table 3.8: Colony PCR program used.

3.3.4 Expression of Arsenite Oxidase by induction with IPTG.

After overnight incubation, positive clones were selected and the culture was inoculated with positive clones into 100 ml of fresh medium including 500 µL of Kanamycin. The cells were allowed to grow at 37°C shaking at 200 rpm and growth monitored by measuring the absorbance at 600 nm (A600). At the log phase of the culture reaches -0.8, 1mM isopropyl-1- thio-β-D-galacto-pyranoside (IPTG) will be added to the culture and the culture will be incubated at 18°C for 24 hours, shaking 150 rpm to improve the expression level. Then, the cell will be harvested by centrifugation (8000 rpm, 20 min, and 4°C) and the pellet was stored at -80°C until purification [36].

3.4 PROTEIN PURIFICATION

For purification of protein first the pellet is resuspended in Binding Buffer (containing 50mM Tris HCL 10% glycerol, 200 mM NaC1 and 2mM DTT, rest water). The Cell disruption is then performed using Constant Systems CFI cell disruptor. The cell debris will be eliminated by centrifugation for 30 min at 15,000 rpm and crude extract will be used for protein purification.

Protein is purified by **Ni metal (Ni NTA) affinity chromatography**. The supernatant will be loaded onto 5ml Hi-Trap Chelating column charged with Ni. Protein is eluted with gradient (using Imidazole and Binding Buffer), and fractions containing purified protein are pooled and concentrated. The purified protein can be stored at −20 °C until used [37].

To check for successful purification of protein (arsenite oxidase) the purified product from chromatography is run through SDS PAGE electrophoresis.

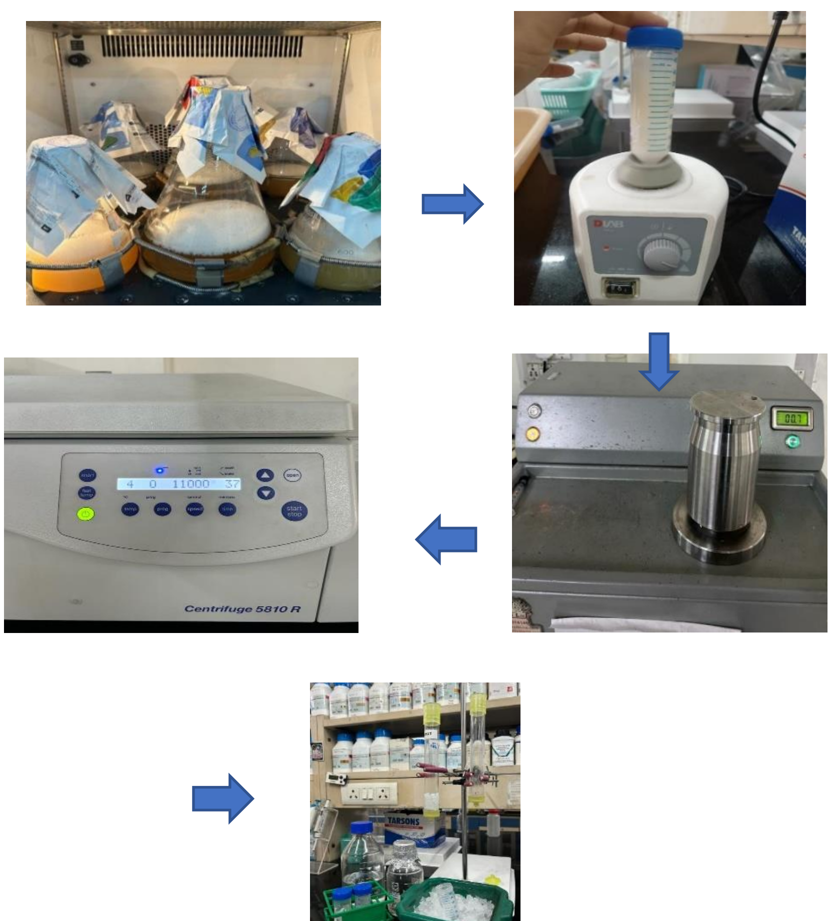


Figure 3.7: Different steps of Protein Purification (IPTG induced large culture, Cell Disruptor, Centrifugation, Purification)

3.4.1 Ni NTA Chromatography

- **Principle**

Ni NTA chromatography is a type of affinity chromatography through which recombinant proteins having His tag. Histidine residues in the His tag attached to our protein that is being purified, bind to the unoccupied sites in the coordination sphere of the immobilized nickel ions with high specificity and affinity. This leads to attachment of our protein to bind to the Ni containing beads bed, so when gradients of imidazole are passed through the column the loosely bound protein was away first leaving behind our protein. Then at the final elution step with imidazole our protein is usually purified separately without noise, which can be monitored through SDS PAGE [36].

- **Requirements**

Ni- NTA agarose, 5 ml coloum with luer lock on both ends, 10 ml luer lock syringe.

Buffer	Composition
Equilibration buffer	20 mM Tris/HCL 200 mM NaCl; pH7.5
Washing buffer	5 mM imidazole + buffer
Elation buffer 1	20 mM imidazole +buffer
Elution buffer 2	60 mM imidazole+ buffer
Elution buffer 3	250 mM imidazole + buffer

Table 3.9: Buffer Compositions for Protein Purification by Ni NTA Chromatography

- **Procedure:**
  1. First, using the Ni NTA Agarose a bed is created in the column.
  2. The column is equilibrated before use with Equilibration buffer.
  3. Sample is then applied to the column by gravity flow. (Flow through)
  4. Washing buffer is passed through the column once sample reaches the bed.
  5. Now the bound proteins are eluted with the help of elution buffers E1, E2 and E3.
  6. The progress of the purification is monitored by analyzing each fraction by SDS-PAGE

Figure 3.8: Ni NTA Chromatography Column

3.4.2 SDS Page

SDS PAGE is a technique given by Raymond & Weintraub which is used in separate proteins based upon the size and relative mol. wt of proteins SDS is an anionic detergent that linearizes the polypeptide chain β-mercaptoethanol acts by reducing all disulfide bends and assists in protein denaturation.

The stacking gel is more porous and have a lower pH (pH 6.8) than resolving gel (pH 8.8), this difference in ionic strength and pH between both get is a phenomenon called isotachophoresis. This leads to concentration of protein samples into a narrow band on the surface of resolving gel which on application of current separates proteins. The buffers in both gels have Glycine as a counter ion which at pH 6.8 remains in zwitterion form (neutral), and Cl ion from buffer moves actively toward the anode with current. So, the protein samples with dye migrate towards the anode faster than Glycine but slower than chloride ion making a thin band sandwich [37].

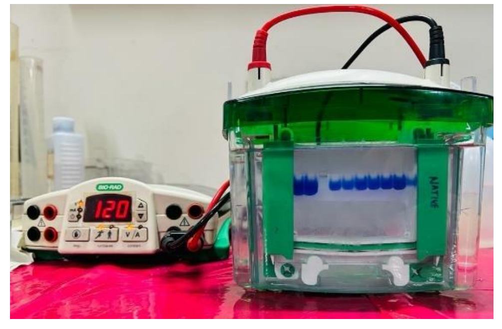


Figure 3.9: SDS PAGE Apparatus

- **Requirements:**
    - Protein samples
    - Protein mol. wt. markers
    - SDS Gel Loading Buffer (5x)

Reagent	Quantity (for 1ml)	Final Concentration
Tris-cl (1M, pH 6.8)	0.25 ml	250 mM
SDS (electrophoresis grade)	80mg	8%
Bromophenol Blue	1mg	0.1%
Glycerol (80% v/v)	0.5 ml	40% w/v
Dithiothreitol (DTT) 1M	0.1ml	100mM
Water	0.25 ml	

Table 3.10 SDS Gel Loading Buffer

- Protein Stain: 0.1% Coomassie brilliant blue R250 in 50% methanol, 10% GAA
- Destaining solution: (1:1) 10% methanol, 7% glacial acetic acid
- Resolving Gel (12%): (Table 3.11)

For 1 gel	12%
dH_2O	1.6 ml
1.5 M Tris pH 8.8	1.3 ml
30% Acrylamide	2.0 ml
10% SDS	50 µl
10% APS	50 µl
TEMED	10 µL

Table 3.11 Resolving Gel

- Stacking Gel (for 1 gel): (Table 3.12)

For 1 gel	12%
dH_2O	1.4 ml
1.5 M Tris pH 6.8	250 µl
30% Acrylamide	330 µL
10% SDS	20 µL
10% APS	20 µL
TEMED	10 µL

Table 3.12 Stacking Gel

- **Procedure:**
  - Stacking and Resolving gels are prepared in the caster using given formulations
  - Preparation of samples and running the gel:
    - Add 10 µl of protein sample and 5 µl of sample buffer and mix by vertexing
    - To denature the proteins heat for 5 min at 95-100°C, then keep on ice
    - Remove the Teflon comb from stacking gel and load up to 10µl the samples into the wells
    - Run the samples at 200V for 3 hours
  - Staining and De-staining:
    - The gel is taken out from glass tray, the stacking gel is discarded, and then the resolving gel is placed in stain solution for 1-3 hours at room temperature on a rocking platform.
    - The gel is soaked in the acetic acid: methanol solution for 6-8hours for de-staining.
  - Store the gels in water and 20% glycerol solutions.

Crystallography Technique

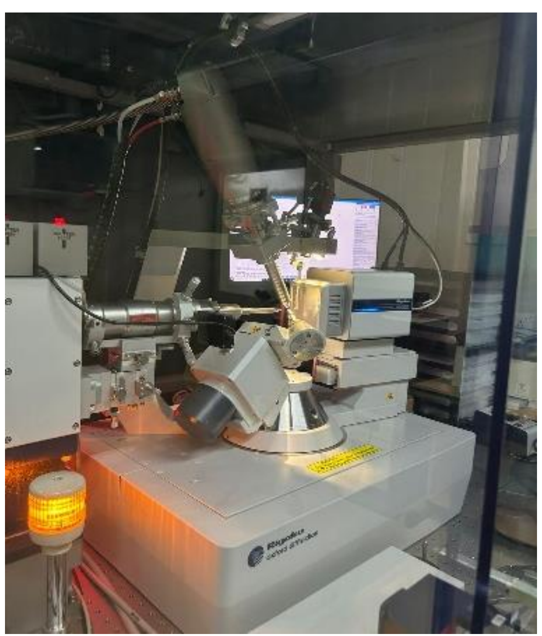
Crystallography is a scientific discipline focuses on understanding crystals and their atomic or molecular structure. It makes use of a number of approaches to figure out how atoms or molecules are arranged within a crystal lattice. These methods offer insightful information about the biological, chemical, and physical characteristics of materials [37].

Here are a few commonly used crystallographic methods:

X-ray Crystallography, Neutron Diffraction, Electron Crystallography, Synchrotron X-ray Crystallography, Powder Diffraction, Single-Crystal Electron Diffraction (SCED).

We are going to use the X-ray crystallography technique in our study.

The most used approach for finding crystal structures is X-ray crystallography. It entails subjecting a crystal to X-rays and examining the diffraction pattern that develops as a result of the X-rays' interactions with the crystal's lattice. The diffraction pattern provides information about the arrangement of atoms or molecules within the crystal [38].



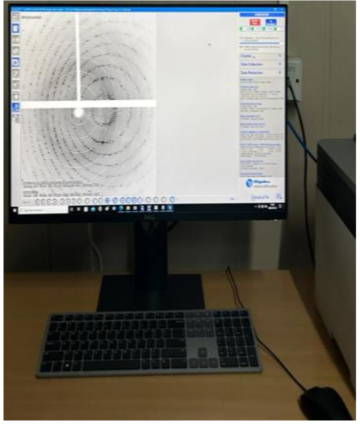


Figure 3.10: Crystallization plate for Vapor diffusion technique, X-ray Crystallography machine and setup.

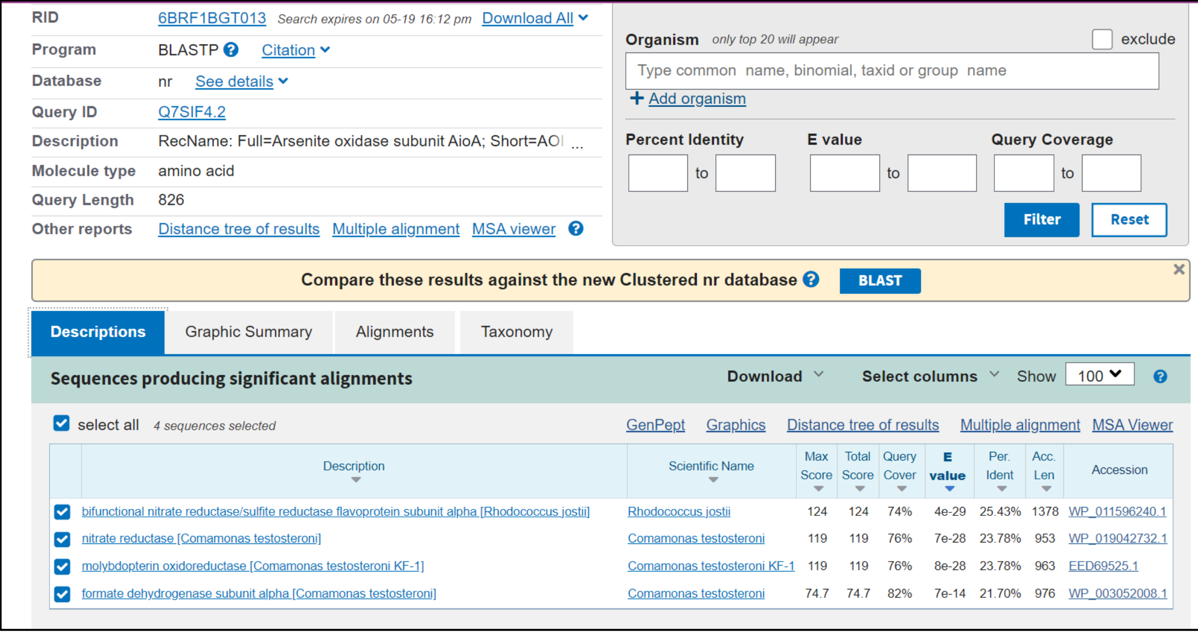
CHAPTER 4
RESULTS

4.1 BIOINFORMATICS ANALYSIS

4.1.1 NCBI Blast

Sequence of Arsenite Oxidase was taken from NCBI with accession number (UniProtKB Q7SIF4.2) (GenBank: EJC61961.1). Protparam Tool results showed that the protein length is of 826 amino acids, and molecular weight of this Arsenite oxidase enzyme/ protein is of 92kDa and Protein pI (isoelectric point) is 7.03.

Protein BLAST (blastp) was performed and the search was restricted to the sequences in the database that correspond to the organisms already available in the department laboratory. Following results were obtained from protein BLAST.



Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
bifunctional nitrate reductase/sulfite reductase flavoprotein subunit alpha [Rhodococcus jostii]	Rhodococcus jostii	124	124	74%	4e-29	25.43%	1378	WP_011596240.1
nitrate reductase [Comamonas testosteroni]	Comamonas testosteroni	119	119	76%	7e-28	23.78%	953	WP_019042732.1
molybdopterin oxidoreductase [Comamonas testosteroni KF-1]	Comamonas testosteroni KF-1	119	119	76%	8e-28	23.78%	963	EED69525.1
formate dehydrogenase subunit alpha [Comamonas testosteroni]	Comamonas testosteroni	74.7	74.7	82%	7e-14	21.70%	976	WP_003052008.1

Figure 4.1: BLAST sequence analysis results

Query coverage of 76% was observed in a protein from *Comamonas Testosteroni KF1* with sequence identity 23.78% respectively. This protein is non redundant and is similar to Arsenite Oxidase. In this project, this protein with accession number EED69525.1 was cloned, expressed and purified.

FASTA sequence for this protein with accession number EED69525.1 was found to be:

```
>EED69525.1 molybdopterin oxidoreductase [Comamonas testosteroni KF-1]
MSIPHLLGDTMKETKSTCPYCGVGCGVIVESDGLQITGVRGDPEHPANLGRLCTKGSTLHLTAAPAHAQQ
ARLLQPMRRLERTAVPQAVSWDLALDELAGRIARIHAAHGPDALGFYLSGQLLTEDYYVFNKLVKGLLGT
NNLDTNSRLCMSSAVAGYKATLGADAPPACYEDIDLAGCLFITGSNMAWAHPILFRRVEEARERNSQLRI
IVADPRRTETAELADLYLPLQPGSDVMLFHGLLHIMLWEGWTDSAFIAQHTSGFAALKELVREATPEKVA
ALCGLPVADLYRAARWMALGGAEEPADVRRPTLSLYCQGLNQSRSGTANNAALINLHLATGQIGRPGAGP
LSLTGQPNAMGGREVGGLSNLLSAHRDLSNAAHRAEVARLWGVESIPQQPGKSALEMFEAAADGQIKALW
IACTNPAQSMPEQAMVRRALERAELVVVQEAFAATETTAYADWLLPASTWGEKLGTVTNSERRISRVRAA
VVAPGAARHDWQIGVQLARRLEQHLRPSLPSLFPYDTANADAGAEAVWNEHRESTRGRDLDITGLSWAVL
EVKGPQQWPMPQGASQGRQRLYGDAVFATEDGRACFDTQPWQAPAVPRDARHPFSLNTGRLRDQWHGMTR
TGQLGRLFAHVSEPQLQVSPQDMQRLQLQDGDLVHASNRYGAIVLPVQSDPGLQPAQLYLPMHWGSMYLS
GMGSKGQRLAGVNALTTPERCPRSKQPELKHVAVRLLKAELPWTCLGMAWLDEQHVQSVRQALSELMAEF
DFASCVLFGRAVPLEKADQGRTGVQFRAAAYAQPGAEVLARLHGLLRLDGPQAMRYADARRQCSRAMAIG
RQTGEPRLDAFLLCGDASAGRWLGPVLRDEQSVQSYGRLLLSSGARPPAAMPARSPQICACMNVDEARIN
SALTACEGSADERLAQLKSSLGCGTRCGSCIPRIKQLVHATPAPQSAALPVAT
```

Number of amino acids: 963

Molecular weight: 104330.98

Theoretical pI: 7.80

Amino acid composition: CSV format

Amino acid	Count	%
Ala (A)	129	13.4%
Arg (R)	77	8.0%
Asn (N)	23	2.4%
Asp (D)	44	4.6%
Cys (C)	22	2.3%
Gln (Q)	57	5.9%
Glu (E)	50	5.2%
Gly (G)	82	8.5%
His (H)	25	2.6%
Ile (I)	26	2.7%
Leu (L)	114	11.8%
Lys (K)	19	2.0%
Met (M)	24	2.5%
Phe (F)	19	2.0%
Pro (P)	60	6.2%
Ser (S)	57	5.9%
Thr (T)	46	4.8%
Trp (W)	19	2.0%
Tyr (Y)	18	1.9%
Val (V)	52	5.4%
Pyl (O)	0	0.0%
Sec (U)	0	0.0%
(B)	0	0.0%
(Z)	0	0.0%
(X)	0	0.0%

Figure 4.2 FASTA sequence for this protein with accession number EED69525.1

Protparam Tool results for this homologous protein Molybdopterin oxidoreductase from the strain *Comamonas testosterone KF1* showed that the protein length is of 963 amino acids, and molecular weight of this enzyme is 104kDa and Protein pI is 7.80.

4.1.2 Structure Prediction by AlphaFold2 and Analysis on PyMOL.

Structure prediction was done on AlphaFold2 and then analyzed on PyMOL. Following structures shows results from analysis.

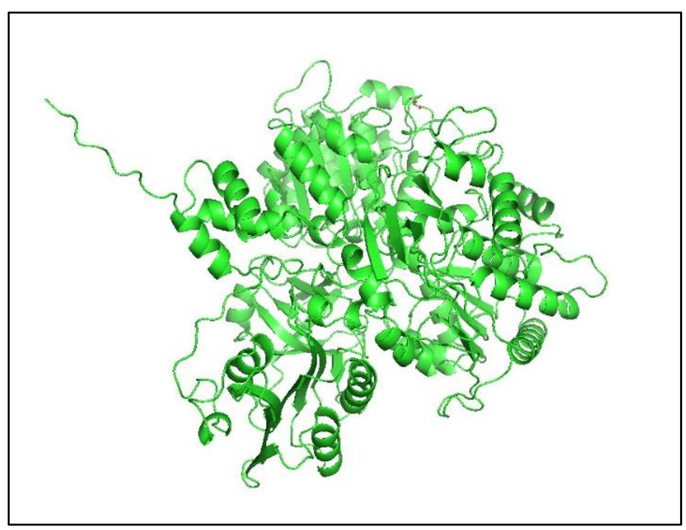
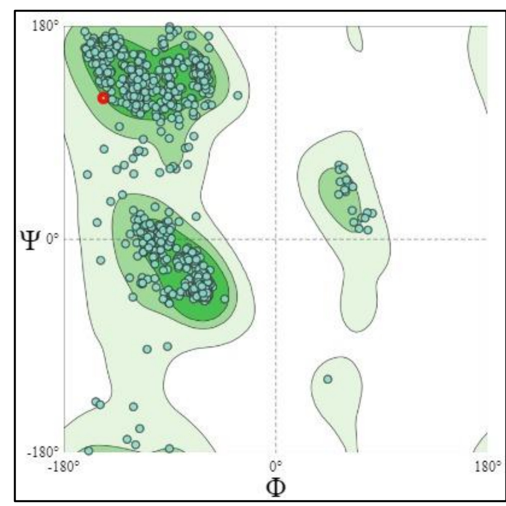


Figure 4.3: Structure of Molybdopterin Oxidoreductase from *Comamonas Testosteroni KF1* (predicted by AlphaFold2)



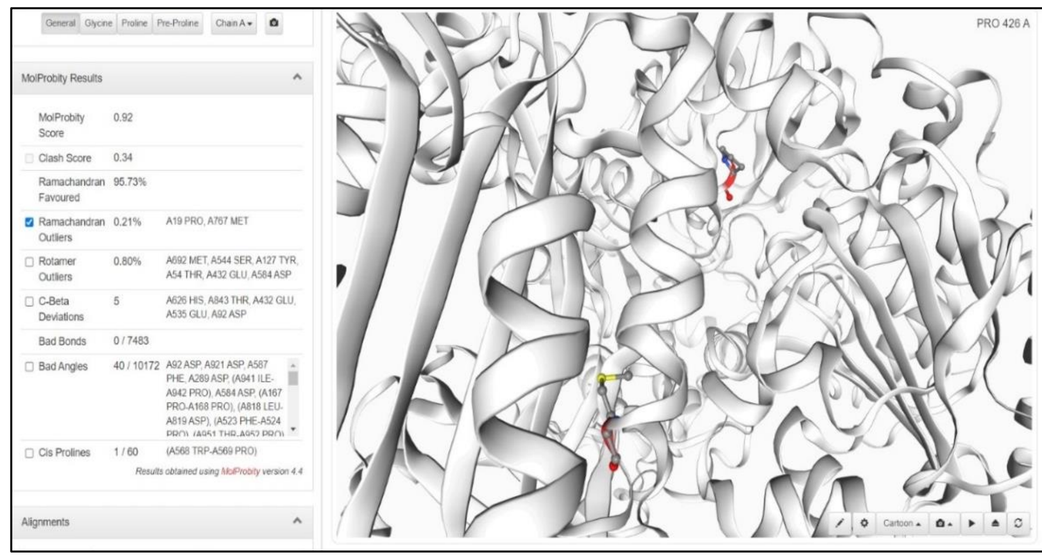


Figure 4.4: Ramachandran Plot for Molybdopterin Oxidoreductase from *Comamonas Testosteroni KF1*

4.1.3 Multiple Sequence Alignment

Multiple Sequence Alignment was successfully done using CLUSTAL OMEGA, and following results were obtained for sequence alignment.

```
CLUSTAL O(1.2.4) multiple sequence alignment


EED69525.1        MSIP----HLLGDTMKETKSTCPYCGVGCGVIVESDGLQITGVRGDPEHPANL-------    49
sp|Q7SIF4.2       MSRPNDRITLPPANAQRTNMTCHFCIVGCGYHVYKWPELQEGGRAPEQNALGLDFRKQLP    60
                  ** *      *    . :.*: ** :* ****  * .         * *.   ::   .*

EED69525.1        ----------------------------GRLCTKGSTLHL---------TAAPAHAQQAR    72
sp|Q7SIF4.2       PLAVTLTPAMTNVVTEHNGRRYNIMVVPDKACVVNSGLSSTRGGKMASYMYTPTGDGKQR    120
                                              .: *. .* *             :*:   : *

EED69525.1        LLQPMRRLERTAVPQAVSWDLALDELAGRIARIHAAHGPDALGFYLSGQL-----L----    123
sp|Q7SIF4.2       LKAP--RLYAADQWVDTTWDHAMALYAGLIKKTLDKDGPQGVFFSCFDHGGAGGGFENTW    178
                  *  *  **  :      .:** *:   ** * :    .**:.: *    .:        :

EED69525.1        TEDYYVFNKLVKGLLGTNNLDTNSRLCMSSAVAGYKATLGADAPPACYEDIDLAGCLFIT    183
sp|Q7SIF4.2       GTGKLMFSAIQTPMVRIHNRPAYNSECHA------TREMGIGELNNAYEDAQLADVIWSI    232
                    .  :*. : . ::  :*  : .  * :         .  :* .    .*** :**. ::

EED69525.1        GSNMAWAHPILF---------------RRVEEARERNSQLRIIVADPRRTETAEL-----    223
sp|Q7SIF4.2       GNNPYESQTNYFLNHNLPNLQGATTSKKKERFPNENFPQARIIFVDPRETPSVAIARHVA    292
                  *.*    ::    *                :: .  .*.  * ***..***.* :. :

EED69525.1        ---ADLYLPLQPGSDVMLFHGLLHIMLWEGWTDSAFIAQHTSGFAALKELVREATPEKVA    280
sp|Q7SIF4.2       GNDRVLHLAIEPGTDTALFNGLFTYVVEQGWIDKPFIEAHTKGFDDAVKTNRL-SLDECS    351
                        *:* ::**:*. **:**:  :: :** *. **  **.**    :  *  : :: :

EED69525.1        ALCGLPVADLYRAARWMALGGAEEPADVRRPTLSLYCQGLNQSRSGTANNAALINLHLAT    340
sp|Q7SIF4.2       NITGVPVDMLKRAAEWSYKPKASGQ---APRTMHAYEKGIIWGNDNYVIQSALLDLVIAT    408
                   : *:**  * ***.*      *.          *:  * :*:  .... . ::**::* :**

EED69525.1        GQIGRPGAGPLSLTGQPNAMGGREVGGLSNLLSAHRDLSNAAHRAEVARLWGVESIPQQP    400
sp|Q7SIF4.2       HNVGRRGTGCVRMGGHQEGYTRPPYPGDKKIYIDQ---ELIKGKGRIMTWWGCNNFQTSN    465
                   ::** *:* : : *: :.          * .::   :   .    :..:   ** :.:  .

EED69525.1        GKSALEMFEAAADGQIKALWIACTNPAQSMPEQAMVR---RALERAELVVVQEAFAATET    457
sp|Q7SIF4.2       NAQALREAILQRSAIVKQ----AMQKARGATTEEMVDVIYEATQNGGLFVTSINLYPTKL    521
                  . .**.        .. :*     . : *:.   : **    .* :.. *.*..   :  *:

EED69525.1        TAYADWLLPASTWGEKLGTVTNSERRISRVRAAVVAPGAARHDWQIGVQLARRLEQHLRP    517
sp|Q7SIF4.2       AEAAHLMLPAAHPGEMNLTSMNGERRIRLSEKFMDPPGTAMADCLIAARIANALRDMYQK    581
                  :  *. :***:  **    *  *.****    .  :  **:*  *  *..::*. *.:  :

EED69525.1        S----LPSL---FPY-------------------------DTANADAGAEAVWNEHRESTRGR    548
sp|Q7SIF4.2       DGKAEMAAQFEGFDWKTEEDAFNDGFRRAGQPGAPAIDSQGGSTGHLVTYDRLRKS-GNN    640
                  .    : :    * :                           *: ...:*  ..::. *:*  ..

EED69525.1        DLDITGLSNAVLEVKGPQQWPMPQGASQGRQRLYGDAVFATEDGRACFDTQPWQAPAVP-    607
sp|Q7SIF4.2       GVQLPVV-----------SWDESK-GLVGTEMLYTEGKFDTDDGKAHFKPAPWNGLPATV    688
                  .:::  :            .*   : .  * : ** :. * *:**:* *.  **:.  .

EED69525.1        --RDARHPFSLNTGRLRDQWHGMTRTGQLGRLFAHVSEPQLQVSPQDMQRLQLQDGDLVH    665
sp|Q7SIF4.2       QQQKDKYRFNLNNGRNNEVWQTAYHDQYNSLMQERYPMAYIEMNPDDCKQLDVTGGDIVE    748
                    :. :: * **.** .: *:   :    . :  :     :::.*:* ::*:: .**:*.

EED69525.1        ASNRYGAIVLPVQSDPGLQPAQLYLPMHWGSMYLSGMGSKGQRLAGVNALTTPERCPRSK    725
sp|Q7SIF4.2       VYNDFGSTFAMVYPVAEIKRGQTFMLFGY----VNGIQ--GD--------VTTDWTDRNI    794
                  . * :*: .  *       :: .* :: : :    :.*:   *:           .* :   *.

EED69525.1        QPELKHVAVRLLKAELPWTCLGMAWLDEQHVQSVRQALSELMAEFDFASCVLFGRAVPLE    785
sp|Q7SIF4.2       IPY-----------------YKGTWGDIRKVGSMEE----FKRTVSFK------------    821
                   *                  :* * ::* *:.:      :    ..*
```

Figure 4.5: Multiple Sequence Alignment using CLUSTAL OMEGA

4.2 CLONING

4.2.1 Genome Isolation Results

C. testosteroni KF1 genome was successfully isolated in high amount using Qiagen Genomic DNA extraction kit, confirmed by Agarose Gel Electrophoresis. DNA bands can be seen in the wells 2 confirming presence of genomic DNA.

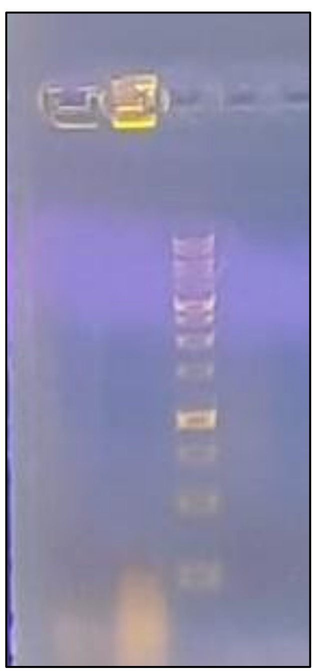


Figure 4.6: Genome Isolation results

4.2.2 Primer Designing

Primer design was done through OligoAnalyzer and restriction sites were obtained from NEBcutter2.0. Further evaluation results for analyzing nature of primers, heterodimer formation, hairpin structure, etc. was also done and following results were obtained.

Restriction sites chosen from zero cutters list on NEBcutter2.0 for our sequence:

XhoI	C TCGA G
NdeI	CA TA TG

<u>Results obtained from OligoAnalyzer</u>

- **Forward Primer**

FP: ACCGAC CAT ATG TCAATTCCGCATCTTCTGGGGGCC

SEQUENCE	5'-ATG TCA ATT CCG CAT CTT CTG GGG GCC-3'
COMPLEMENT	5'-GGC CCC CAG AAG ATG CGG AAT TGA CAT-3'
LENGTH	27
GC CONTENT	55.6%
MELTING TEMP.	64.6°C

- **Reverse Primer**

RP: **AACCGA** CTCGAG CTAGGTGGCAACCGGCAGGGC

SEQUENCE	5'-GCT AGG TGG CAA CCG GCA GGG C-3'
COMPLEMENT	5'-GCC CTG CCG GTT GCC ACC TAG C-3'
LENGTH	22
GC CONTENT	72.7%
MELTING TEMPERATURE	67.6°C

4.2.3 Gene Amplification results

Gene for protein was successfully amplified by PCR using the designed primers. Following results were obtained on the agarose gel showing band of ~3Kbp confirming amplification of the Nitrate Reductase gene.

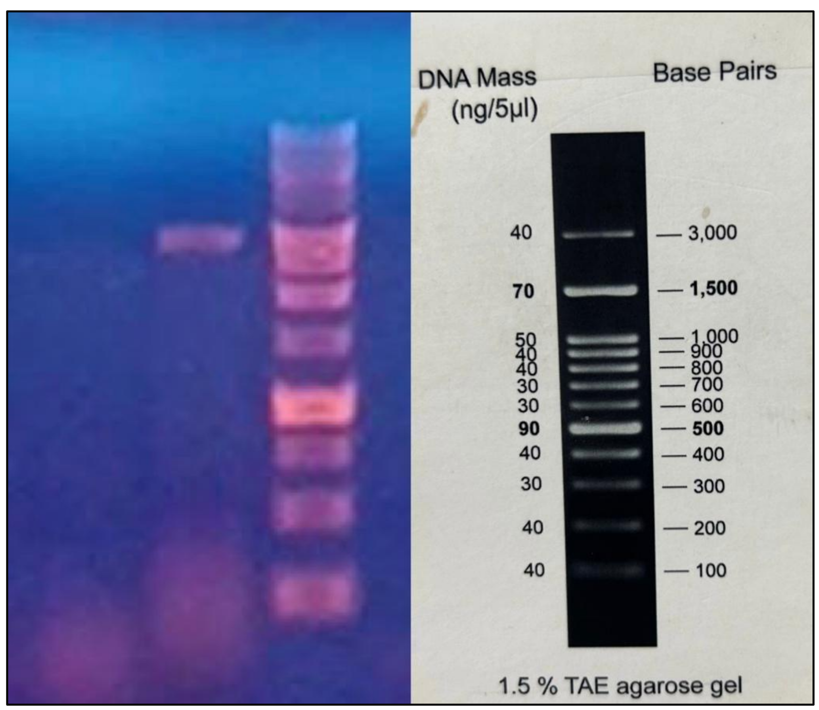


Figure 4.7: Gene Amplification results

4.2.4 Plasmid Isolation results

Three plasmid configurations were seen on gel. Hence, plasmid isolation was successfully done.

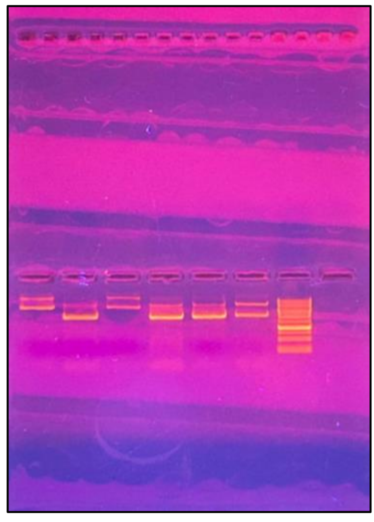


Figure 4.8: Plasmid Isolation results

4.2.5 Transformation of Recombinant vector into *E. coli DH5α*

After completion of the digestion, ligation and competent host cell preparation is done. The plasmid vector with Molybdopterin Oxidoreductase gene were transformed into *E. coli DH5α* cells. Colonies were obtained on the plates that have recombinant plasmid.

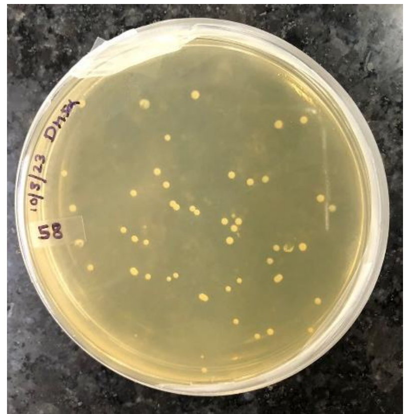


Figure 4.9: Transformed E. coli DH5α colonies

4.2.6 Colony PCR of transformed *E. coli DH5α* cells.

Plasmid with gene for Molybdopterin Oxidoreductase was successfully transformed into E. coli DH5α host cells, and the presence of gene was confirmed by Colony PCR of transformed colonies using the designed primers. Following results were obtained on the Agarose Gel showing a band of ~3Kbp confirming successful cloning of the Molybdopterin Oxidoreductase gene.

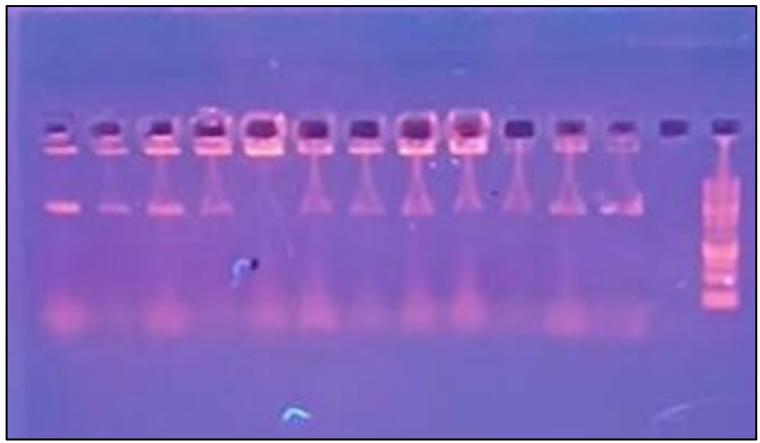


Figure 4.10: Colony PCR results from transformed *E. coli DH5α* colonies

4.2.7 Transformation of recombinant vector into *E. coli BL21*

Recombinant plasmids with Molybdopterin Oxidoreductase gene were transformed into expression host *E. coli BL21* cells for protein expression. Colonies were obtained on the plates (given below) that may have recombinant plasmid.

Figure 4.11: Transformed *E. coli BL21* colonies

4.2.8 Colony PCR of transformed *E. coli BL21*

Recombinant plasmid with gene for Molybdopterin Oxidoreductase was successfully transformed into *E. coli BL21* host cells, and the presence of gene was confirmed by Colony PCR of transformed colonies using the designed primers. Following results were obtained on the Agarose Gel showing a band of ~3Kbp confirming successful cloning of Molybdopterin Oxidoreductase gene.

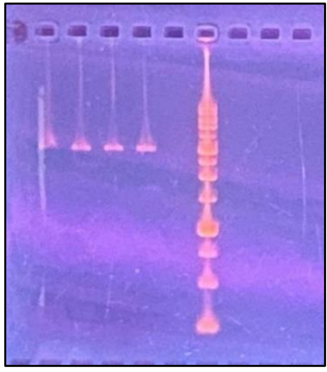


Figure 4.12: *E.coli BL21* Colony PCR

4.3 PROTEIN PURIFICATION RESULTS (SDS PAGE)

After cloning and expression of protein, protein purification was done using Ni NTA Chromatography. Following images (figure 4.13) shows results of SDS PAGE for samples purified through chromatography.

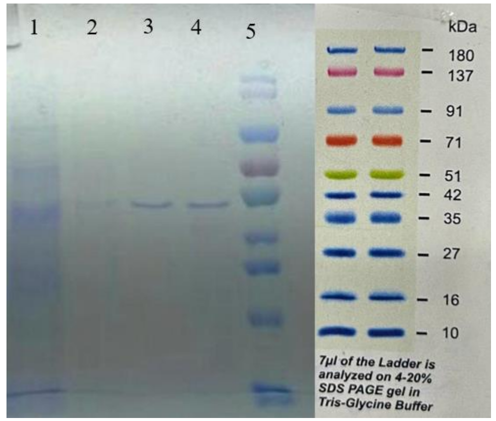


Figure 4.13: Protein Purification Result

The Molybdopterin Oxidoreductase protein's molecular weight is 104kDa, which was observed in high amount on SDS.

1) Flow through
2) 40 mM fraction
3) 80 mM fraction
4) 300 mM fraction
5) ladder

Hence, purification of protein was achieved. The visible bands show that the protein obtained with a molecular weight of approximately 104kDa.

CHAPTER 5
SUMMARY AND CONCLUSIONS

In this project, Molybdopterin Oxidoreductase enzyme gene from *Comamonas testosteroni KF1* which was found to be homologous to Arsenite Oxidase from Alcaligens faecalis was cloned and expressed successfully. Arsenite Oxidase enzyme belongs to Molybdopterin Oxidoreductase family and is able to convert As^{3+} form to As^{5+} form which is hundred times less toxic. Hence, can be utilised in bioremediation methods. Further, through IPTG induction the protein was attempted to express in higher amounts and purified through Ni NTA Affinity Chromatography. The purification was analyzed on SDS PAGE which showed presence of a 104 kDa protein. Hence, protein purification was achieved after many attempts. The structure for Molybdopterin Oxidoreductase from *C. testosteroni KFI* was also predicted through AlphaFold2, structure assessment of Ramachandran Plot was done using Swiss- Model. The protein purification was optimized to obtain a homogeneous solution which was further subjected to crystallization. For future, the protein crystals will be subjected to X-ray diffraction to obtain structural insights into the protein structure. Then the purified protein will further be used for structural biology studies on this enzyme through X-ray crystallography.

APPENDICES

EQUIPMENT	MODEL	MANUFACTURER
sCentrifuge	Minispin Plus Z 326 K	Eppendrof Hermle
Gel Documentation systems	Bioimaging systems	Gene Genius
Circular dichroism spectrophotometer	J-1500	JASCO
Fluorescence spectrophotometer	Spectramaxi i3x	Molecular Devices
Dry Bath	MK-20	Genaxy scientific pvt. Ltd.
Magnetic Stirrer	5MLH	Remi Equipment Ltd.
PCR Themal cycler	Mastercycler	Eppendorf
Nanodrop	Nanodrop200	Thermo Scientific
SDS-PAGE apparatus	-	Biorad
Shaker Incubator	OLS200 Innova – 3100	Grant New Brunswick Laboratories (USA)
Water distillation plant	Milli Q	Millipore
Sonicator	Sonifier-450	Branson
Surface plasmon resonance	Biacore 3000	Biacore

REFERENCES

1. Syed Zaghum AbbasMehwish RiazNaseem RamzanM. Tariq ZahidFarah R. Shakoori, Mohd. Rafatullah (2014). Isolation and characterization of arsenic resistant bacteria from wastewater. Brazilian Journal of Microbiology, 45(4), 1309-1315.
2. Aitio A, Becking G. *Environmental Health Criteria.* Vol. 224. World Health Organization; Geneva: 2001. Arsenic and arsenic compounds. Aitio A, Becking G (2001) Arsenic and arsenic compounds. Environmental Health Criteria 224, World Health Organization, Geneva.
3. Boyd Claude E. (2020) Water Quality: An Introduction, Third Edition Springer International Publishing.
4. Cervantes, C., Ji, G., Ramirez, J. L, & Silver, S. (1994). Resistance to arsenic compounds in microorganisms. FEMS microbiology reviews, 15(4), 355-367.
5. Dopp, E., Kligerman, A. D., & Diaz-Bone, R.A. (2010). Organoarsenicals. Uptake, metabolism, and toxicity. Organometallics in Environment and Toxicology, 7.
6. Mitiku, A. A. (2020). A review on water pollution: causes, effects and treatment methods. Int J Pharm Sci Rev Res, 60(2), 94-101.
7. Kurniawan, Tonni Agustiono, et al. "Remediation technologies for contaminated groundwater due to arsenic (As), mercury (Hg), and/or fluoride (F): A critical review and way forward to contribute to carbon neutrality." Separation and Purification Technology (2023): 123474.
8. Duruibe, J.O. Ogwuegbu, M.O.C., & Egwurugwu, J.N. (2007). Heavy metal pollution and human biotoxic effects. International Journal of physical sciences, 2(5), 112-118.
9. Banerjee, Goutam, et al. "Bioremediation of heavy metals by a novel bacterial strain Enterobacter cloacae and its antioxidant enzyme activity, flocculant production, and protein expression in presence of lead, cadmium, and nickel." Water, Air, & Soil Pollution 226 (2015): 1-9.
10. Zakhar, Ronald, Ján Derco, and František Čacho. "An overview of main arsenic removal technologies." Acta chimica slovaca 11.2 (2018): 107-113.
11. Dilpazeer, Fariha, et al. "A Comprehensive Review of the Latest Advancements in Controlling Arsenic Contaminants in Groundwater." Water 15.3 (2023): 478.
12. Ellis, PJ., Conrads, T, Hille, R., & Kuhn P (2001) Crystal structure of the 100 kDa arsenite oxidase from Alcaligenes faecalis in two crystal forms at 1.64 A and 2.03 A. Structure, 9(2), 125-132.

13. Silver, S., & Phung, L. T. (2005). Genes and enzymes involved in bacterial oxidation and reduction of inorganic arsenic. Applied and environmental microbiology, 71(2), 599- 608.
14. Drewniak, Lukasz, Aleksandra Styczek, Malgorzata Majder-Lopatka, and Aleksandra Sklodowska. "Bacteria, hypertolerant to arsenic in the rocks of an ancient gold mine, and their potential role in dissemination of arsenic pollution." Environmental pollution 156, no. 3 (2008): 1069-1074.
15. Fan, H., C. Su, Y. Wang, J. Yao, K. Zhao, Y. Wang, and G. Wang. "Sedimentary arsenite-oxidizing and arsenate-reducing bacteria associated with high arsenic groundwater from Shanyin, Northwestern China." Journal of Applied Microbiology 105, no. 2 (2008): 529-539.
16. Cai, Lin, Guanghui Liu, Christopher Rensing, and Gejiao Wang. "Genes involved in arsenic transformation and resistance associated with different levels of arsenic-contaminated soils." BMC microbiology 9 (2009): 1-11.
17. Liao, Vivian Hsiu-Chuan, Yu-Ju Chu, Yu-Chen Su, Sung-Yun Hsiao, Chia-Cheng Wei, Chen-Wuing Liu, Chung-Min Liao, Wei-Chiang Shen, and Fi-John Chang. "Arsenite-oxidizing and arsenate-reducing bacteria associated with arsenic-rich groundwater in Taiwan." Journal of contaminant hydrology 123, no. 1-2 (2011): 20-29.
18. Drewniak, Lukasz, and Aleksandra Sklodowska. "Arsenic-transforming microbes and their role in biomining processes." Environmental Science and Pollution Research 20 (2013): 7728-7739.
19. Sanders, Talia, Yiming Liu, Virginia Buchner, and Paul B. Tchounwou. "Neurotoxic effects and biomarkers of lead exposure: a review." Reviews on environmental health 24, no. 1 (2009): 15-46.
20. Rosen, Barry P., and Zijuan Liu. "Transport pathways for arsenic and selenium: a minireview." Environment international 35, no. 3 (2009): 512-515.
21. Macy, Joan M., Joanne M. Santini, Björg V. Pauling, Andrew H. O'Neill, and Lindsay I. Sly. "Two new arsenate/sulfate-reducing bacteria: mechanisms of arsenate reduction." Archives of microbiology 173 (2000): 49-57.
22. Gihring, Thomas M., and Jillian F. Banfield. "Arsenite oxidation and arsenate respiration by a new Thermus isolate." FEMS Microbiology Letters 204, no. 2 (2001): 335-340.
23. Bleicher, Konrad H., Hans-Joachim Böhm, Klaus Müller, and Alexander I. Alanine. "Hit and lead generation: beyond high-throughput screening." Nature reviews Drug discovery 2, no. 5 (2003): 369-378.
24. Goel, P. (2006). Water Pollution: Causes, Effects and Control India: New Age International.

25. Hoekstra, AY., & Mekonnen, M. M. (2012). The water footprint of humanity. Proceedings of the national academy of sciences, 109(9), 3232-3237.
26. Lenntech Water Treatment and Air Purification (2004). Water Treatment, Published by Lenntech, Rotterdamseweg, Netherlands.
27. Meybeck M., Chapman D. and Helmer R(Eds.) (1989) Global Fresh Water Quality: A First Assessment, Basil Blackwell: Oxford.
28. Meybeck, Michel, Peters, N.E., & Chapman, D.V. (2005). 91: Water Quality.
29. Mukhopadhyay, R., Rosen, B.P., Phung, L. T., & Silver, S. (2002) Microbial arsenic: from geocycles to genes and enzymes. FEMS microbiology reviews, 26(3), 311-325.
30. Nakamuro, K., & Sayato, Y. (1981) Comparative studies of chromosomal aberration induced by trivalent and pentavalent arsenic. Mutation Research/Genetic Toxicology, 88(1), 73-80.
31. Owa, F.D. (2013). Water pollution: sources, effects, control and management. Mediterranean journal of social sciences, 4(8) 65-65.
32. Norman E. Peters & Michel Meybeck (2000) Water Quality Degradation Effects on Freshwater Availability: Impacts of Human Activities, Water International, 25:2, 185- 193.
33. Oremland, R.S. & Stolz, J.F. (2005). Arsenic microbes and contaminated aquifers. Trends in microbiology, 13(2)45-49.
34. Rosen B.P. Biochemistry of arsenic detoxification. FEBS Lett. 2002; 529:86-92. doi: 10.1016/S0014-5793(02)03186-1.
35. Richey, C., Chovanec, P., Hoeft, S.E., Oremland, R.S. Basu, P., & Stolz J.F.(2009). Respiratory arsenate reductase as a bidirectional enzyme. Biochemical and biophysical research communications, 382(2), 298-302.
36. Sar, P., Paul, D., Sarkar, A., Bharadwaj, R., & Kazy S.K. (2015). Microbiology of Arsenic-Contaminated Groundwater And The Environment, 477.
37. Sarkar, A., Kazy, S.K. & Sar, P. (2014) Studies on arsenic transforming groundwater bacteria and their role in arsenic release from subsurface sediment. Environ Sci Pollut Res 21, 8645-8662.
38. Vollenweider, R. A. (1971). Scientific fundamentals of the eutrophication of lakes and flowing waters, with particular reference to nitrogen and phosphorus as factors in eutrophication (p. 250). Paris: Organisation for economic co-operation and development.
39. Slyemi, Djamila and Violaine Bonnefoy. "How prokaryotes deal with arsenic(†)." Environmental microbiology reports 4 6 (2011): 571-86.
40. Sanyal, Santonu Kumar. (2014). Diversity of Arsenite Oxidase Gene and Arsenotrophic Bacteria from Arsenic Contaminated Soils of Faridpur, Bangladesh.

10.13140/RG.2.1.4535.0640.

41. https://webstor.srmist.edu.in/web_assets/srm_mainsite/files/files/BT0210%20-%20MOLECULAR%20BIOLOGY%20LABORATORYMANUAL.pdf
42. https://www.labtools.us/nebcutter-v2-0/\
43. Increased rigidity of eglin c at acidic pH: evidence from NMR spin relaxation and MD simulations. Hu H, Clarkson MW, Hermans J, Lee AL. Biochemistry. 2003 Dec 2. 42(47):13856-68. 10.1021/bi035015z PubMed 14636053.
44. Langlais, C., Korn, B. (2005). Recombinant Protein Expression in Bacteria. In: Encyclopedic Reference of Genomics and Proteomics in Molecular Medicine. Springer, Berlin, Heidelberg . https://doi.org/10.1007/3-540-29623-9_4800

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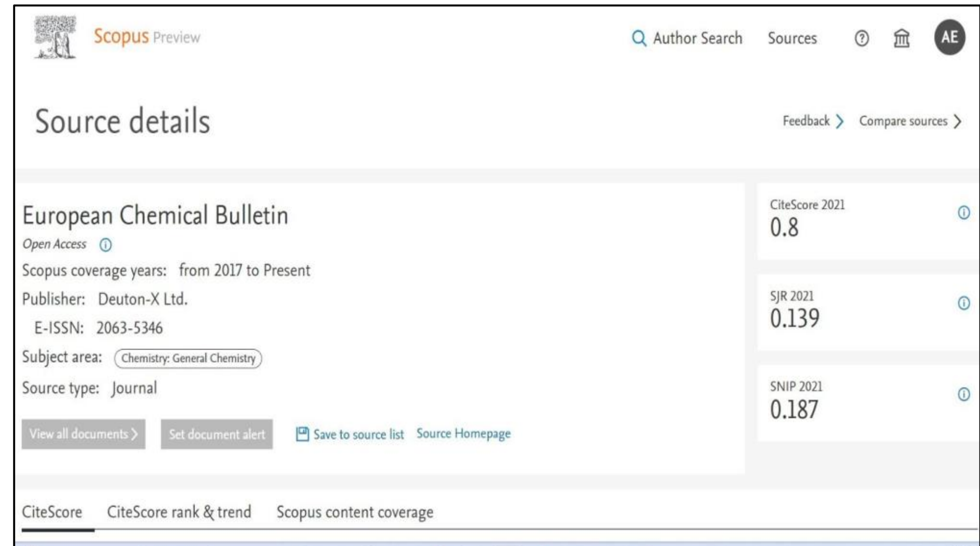

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RECENT FINDINGS ON PLASTIC WASTE POLLUTION AND BIOTRANSFORMATION

Tushar Agarwal and Jai Gopal Sharma*

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Abstract

Plastic waste is generated in large quantities globally, and its disposal poses significant environmental challenges as it is non-biodegradable and can persist in the environment for hundreds of years. It is estimated that over 8 million tons of plastic waste end up in our oceans every year. Biodegradation is an eco-friendly approach to managing plastic waste as it does not result in the formation of toxic by-products. Types of plastic waste, degradation methods, and different microorganisms impacting biodegradation are discussed in this review. Overall, microbial bioremediation has the potential to provide an eco-friendly solution to the problem of plastic waste, and recent advances in this field have shown promising results. However, further research is needed to optimize bioremediation processes and to develop new microorganisms that can efficiently degrade and transform plastic waste.

Keywords: Bioremediation; Environment awareness; Biotransformation; Biodegradation; Plastic waste

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Introduction

Plastic waste is generated in large quantities globally, and its disposal poses significant environmental challenges as it is non-biodegradable and can persist in the environment for hundreds of years [1,2]. It is estimated that over 8 million tons of plastic waste end up in our oceans every year [3]. Plastic waste can also release toxic chemicals and greenhouse gases when burned, contributing to air pollution and climate change [4,5]. As plastic waste continues to be a major environmental problem, innovative solutions are required to address the challenges posed by plastic waste. This includes the development of new technologies and methods for the efficient and sustainable management of plastic waste, as well as awareness-raising and education efforts to encourage individuals and businesses to reduce their plastic consumption and waste. Efforts to manage plastic waste include recycling, reuse, reduction, and disposal [6]. Recycling involves the conversion of plastic waste into new plastic products [7]. Reuse involves finding new uses for plastic products, such as using plastic bags for shopping multiple times. Reduction involves reducing the amount of plastic used in products, such as by using reusable water bottles instead of disposable plastic ones. Finally, disposal involves the safe and environmentally friendly disposal of plastic waste, such as through landfilling or incineration.

Plastics can also be degraded into simpler compounds that can be absorbed or assimilated by the environment [8,9]. This process can occur naturally through physical, chemical, or biological means. Physical degradation occurs due to the action of external factors such as sunlight, heat, and mechanical stress [10]. Exposure to UV light and heat can cause the degradation of plastic, leading to the breaking of chemical bonds, which results in the formation of smaller molecules. Mechanical stress, such as grinding or shredding, can also break down plastic waste into smaller fragments. Chemical degradation involves the use of chemicals to break down the polymer chains of plastic into simpler compounds [9]. Chemicals such as acids, bases, and oxidizing agents can be used to degrade plastic waste. For example, hydrolysis is a common chemical method used to break down the ester linkages in PET plastics. However, not all plastics are easily biodegradable, and some may require special conditions or pre-treatments to enhance biodegradation. For example, polyethylene (PE) and polypropylene (PP) are difficult to biodegrade, and it can take hundreds of years for them to degrade in the environment [8]. This has led to the development of new technologies, such as the use of engineered microorganisms or enzymes to degrade specific types of plastics.

Bioremediation is an eco-friendly approach to tackle this problem, and it involves using microorganisms such as bacteria, fungi, and algae to break down plastic waste into simpler, non-toxic compounds (Figure 1) [11]. These microorganisms secrete enzymes that can break down the polymer chains of plastic into smaller molecules. Microbial bioremediation of plastic waste can be classified into two categories: biodegradation and biotransformation [12]. Biodegradation involves the complete breakdown of plastic waste into carbon dioxide and water [13]. This process is carried out by microorganisms such as bacteria and fungi, which secrete enzymes that break down the polymer chains of plastic into smaller molecules that can be used as an energy source for the microorganisms. The biodegradation of plastic waste has been extensively studied, and several microorganisms have been identified that can efficiently degrade plastic waste [14,15]. Biotransformation, on the other hand, involves the conversion of plastic waste into non-toxic compounds by microorganisms. This process is particularly useful for plastics that are difficult to biodegrade, such as polyvinyl chloride (PVC) and polystyrene (PS). Biotransformation can be carried out by microorganisms such as white rot fungi, which secrete enzymes that can break down the chemical bonds of plastic waste, resulting in the formation of non-toxic compounds.

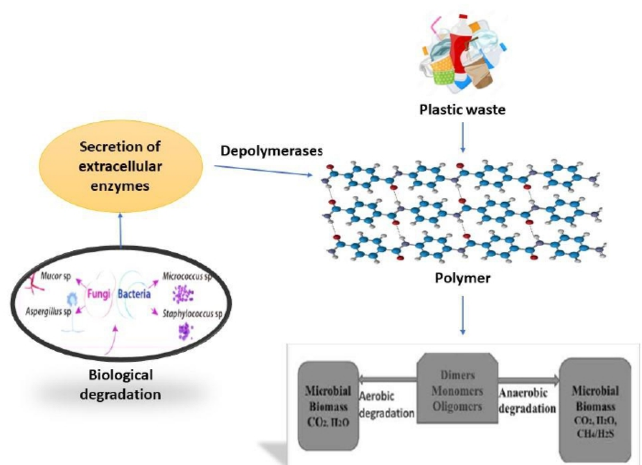

Figure 1: Process of biodegradation of plastic waste

Recent advances in microbial bioremediation for the disposal of plastic waste include the development of new microorganisms that can efficiently degrade and transform plastic waste, as well as the optimization of bioremediation processes to increase their efficiency. For example, genetic engineering has been used to develop microorganisms that can degrade specific types of plastics, such as polyethylene terephthalate (PET), which is commonly used in plastic bottles. In addition, bioremediation processes have been optimized by using pre-treatments such as mechanical shredding or chemical treatments to increase the surface area of plastic waste and make it more accessible to microorganisms. Overall, microbial bioremediation has the potential to provide an eco-friendly solution to the problem of plastic waste, and recent advances in this field have shown promising results. However, further research is needed to optimize bioremediation processes and to develop new microorganisms that can efficiently degrade and transform plastic waste.

Types of plastic waste

Plastic waste refers to any discarded or abandoned plastic material that has served its intended purpose and is no longer useful. It can accumulate in landfills, oceans, rivers, and other water bodies, causing harm to wildlife and ecosystems [16]. Plastics vary in their properties and degradation rates, and some are more environmentally friendly than others. Some plastics are easily recyclable, while others are difficult to recycle or not recyclable at all. It is essential to properly sort and dispose of plastic waste according to its type and to make efforts to reduce plastic waste in general. Plastics can be classified into various types based on their chemical structure and properties. The following are the most common types of plastic:

1. **Polyethylene (PE):** PE is a type of polymer made from the monomer ethylene. It is one of the most widely used plastics in the world and can be found in a variety of applications, including packaging, toys, pipes, and medical devices. PE is popular due to its low cost, versatility, and ease of manufacturing. It is also lightweight, moisture-resistant, and can be recycled, making it a popular choice for sustainable products. It can be produced in various forms, including high-density polyethylene (HDPE), low-density polyethylene (LDPE), and linear low-density polyethylene (LLDPE). HDPE is known for its strength, stiffness, and resistance to impact, while LDPE is known for its flexibility and transparency. LLDPE is a blend of the two and is used for

applications that require a combination of the properties of HDPE and LDPE [17,18].

2. **Polypropylene (PP):** PP is a thermoplastic polymer made from the monomer propylene. It is a versatile material with a wide range of applications due to its excellent chemical and heat resistance, high stiffness, and low density. PP is commonly used in packaging, textiles, automotive parts, yogurt cups, bottle caps, straws and consumer goods. It is also used in the medical industry for applications such as surgical instruments and medical devices. Its resistance to chemicals and sterilization makes it a popular choice for these applications. PP is often produced in a variety of forms, including homopolymer, copolymer, and random copolymer. Homopolymer is the most common form and is used in applications that require high strength and stiffness. Copolymer and random copolymer have improved impact resistance and are often used in applications that require more flexibility. PP is a highly recyclable material and is often used in products that promote sustainability. It is also lightweight, which makes it an attractive option for manufacturers looking to reduce the weight of their products [19].
3. **Polyvinyl Chloride (PVC):** PVC is a synthetic thermoplastic polymer made from the monomer vinyl chloride. It is one of the most widely used plastics in the world due to its versatility, durability, and low cost. PVC is commonly used in a variety of applications, including construction, electronics, automotive, pipes, vinyl flooring, window frames, and healthcare. In the construction industry, PVC is used for pipes, flooring, roofing, and siding due to its durability, weather resistance, and ease of installation. In the healthcare industry, PVC is used for medical tubing, blood bags, and other medical devices due to its biocompatibility and sterilization resistance [20].

   PVC can be produced in different forms, including rigid and flexible PVC. Rigid PVC is used in applications that require strength, durability, and rigidity, while flexible PVC is used in applications that require flexibility, such as electrical cables and hoses. However, PVC has also been associated with environmental concerns due to the release of dioxins during its manufacturing process and when it is burned. Efforts are being made to improve the sustainability of PVC production and reduce its impact on the environment. Additionally, PVC can be recycled, although it requires specialized processing to remove additives and impurities.

4. **Polystyrene (PS):** PS is a synthetic thermoplastic polymer made from monomer styrene. It is a widely used plastic due to its versatility, low cost, and good insulation properties. PS is commonly used in a variety of applications, including packaging, disposable insulation, foam cups, disposable cutlery, and consumer electronics. Its excellent insulation properties make it a popular choice for insulation boards and packaging materials. In the food industry, PS is used for disposable food containers and packaging due to its low cost and durability. PS can be produced in different forms, including crystal and expandable. Crystal PS is a clear and rigid plastic used for products such as disposable cutlery and CD cases. Expandable PS is a foam plastic used for packaging materials and insulation. However, PS is also associated with environmental concerns due to its slow rate of decomposition and the difficulty of recycling. When disposed of improperly, PS can contribute to litter and pollution. Efforts are being made to reduce the environmental impact of PS through recycling programs and the development of biodegradable alternatives [21].

5. **Polyethylene Terephthalate (PET):** PET is a type of thermoplastic polymer that is commonly used in the manufacture of plastic bottles, food containers, and other products. It is a clear, strong, and lightweight material that is resistant to moisture and many chemicals. PET is made by polymerizing ethylene glycol and terephthalic acid or by using dimethyl terephthalate and ethylene glycol as starting materials. PET has several advantages as a packaging material, including its low cost, versatility, and recyclability. It is widely used in the food and beverage industry because it is safe for food contact and does not react with the contents of the container. PET can also be easily recycled, which makes it an environmentally friendly choice. However, PET also has some disadvantages. It is not heat-resistant and can deform at high temperatures, and it is also susceptible to UV degradation. PET can also take a long time to decompose in the environment, which can lead to pollution if not properly disposed of or recycled [22,23].
6. **Polycarbonate (PC):** PC is a type of thermoplastic polymer that is known for its strength, durability, and clarity. It is commonly used in the manufacture of a wide range of products, including electronic components, automotive parts, medical equipment, and sports equipment. PC is made by reacting bisphenol A (BPA) and phosgene, which produces a polymer that is tough, lightweight, and resistant to impact. It has a high melting point and is able to withstand high temperatures, making it suitable for use in applications where high heat resistance is required [24].

   One of the most notable properties of PC is its clarity. It is often used in place of glass in applications where impact resistance is needed, such as in safety glasses, windows, and skylights. PC is also used in the manufacture of electronic components because of its excellent electrical insulation properties. However, there are concerns about the potential health risks associated with PC, particularly regarding the use of BPA in its production. BPA is a chemical that has been linked to a range of health problems, including reproductive disorders, diabetes, and cancer. As a result, some manufacturers have begun to shift away from using PC in their products and are instead exploring alternatives such as PET or PP.
7. **Acrylonitrile Butadiene Styrene (ABS):** ABS is a thermoplastic polymer that is commonly used in the manufacture of a wide range of products, including automotive parts, toys, appliances, and piping systems. It is a copolymer made by polymerizing styrene and acrylonitrile in the presence of polybutadiene. ABS has several desirable properties, including its strength, impact resistance, and toughness. It is also resistant to heat and chemicals, making it suitable for use in harsh environments. ABS has a relatively low melting point, which means that it can be easily molded into complex shapes using injection molding or extrusion techniques. ABS is also known for its aesthetic qualities. It can be easily colored and has a shiny, glossy appearance that makes it a popular choice for consumer products such as toys and electronic devices.

   One of the disadvantages of ABS is that it can release toxic fumes when burned, which can be harmful to health. This makes it important to use proper ventilation and safety precautions when working with or disposing of ABS products. Additionally, ABS is not biodegradable, which can lead to environmental concerns if it is not properly disposed of or recycled [25].

Degradation methods of plastic waste

There are several methods available for the degradation of plastic waste, including

physical, chemical, and biological methods [26,27].

1. **Physical methods**

Physical methods of plastic degradation involve breaking down plastic waste into smaller pieces through mechanical or physical means. These methods do not involve any chemical reactions and are typically less energy-intensive than chemical methods. Physical methods of plastic degradation have some advantages over chemical methods, such as being less energy-intensive and producing fewer emissions. However, they may not be suitable for all types of plastic waste, and some methods may produce by-products that are difficult to dispose of. Additionally, physical methods may not completely break down plastic waste and may only produce smaller particles that can still pose environmental risks. Here are a few examples of physical methods of plastic degradation:

Shredding or Grinding: This involves shredding or grinding plastic waste into smaller pieces, which can then be used as feedstock for other processes, such as recycling or fuel production.

Pyrolysis: This involves heating plastic waste in the absence of oxygen to break down the long chains of polymers into smaller hydrocarbons, which can be used as feedstock for fuel production.

Hydrogenation: This involves adding hydrogen to plastic waste under high pressure and temperature, which breaks down the long chains of polymers into smaller molecules that can be used as feedstock for fuel production.

Mechanical recycling: This involves sorting and cleaning plastic waste and then mechanically processing it into pellets or flakes, which can be used as feedstock for the production of new plastic products.

2. **Chemical methods**

Chemical methods of plastic degradation involve breaking down plastic waste through chemical reactions. These methods typically require more energy and resources than physical methods but can be more effective in breaking down plastic waste into its constituent molecules. Chemical methods of plastic degradation can be effective in breaking down plastic waste into its constituent molecules, which can then be used as feedstock for the production of new plastic products. However, these methods typically require more energy and resources than physical methods and can produce hazardous by-products if not properly managed. Additionally, some chemical methods may not be suitable for all types of plastic waste. Here are a few examples of chemical methods of plastic degradation:

Depolymerization: This involves breaking down the long chains of polymers that make up plastic waste into their constituent monomers using chemical reactions. The monomers can then be purified and used as feedstock for the production of new plastic products.

Solvolysis: This involves breaking down plastic waste using a solvent, such as an acid or a base, to dissolve the polymers into their constituent molecules. The resulting solution can then be purified and used as feedstock for the production of new plastic products.

Gasification: This involves heating plastic waste in the presence of oxygen or steam to break down the polymers into their constituent gases, such as methane and carbon dioxide. These gases can then be used as fuel or feedstock for the production of new plastic products.

Hydrolysis: This involves breaking down plastic waste using water and an acid or a base to break the long chains of polymers into their constituent monomers. The resulting monomers

can then be purified and used as feedstock for the production of new plastic products.

3. **Biological methods**

Biological methods of plastic degradation involve using microorganisms, such as bacteria or fungi, to break down plastic waste. Biological methods of plastic degradation are considered more environmentally friendly than physical and chemical methods, as they use natural processes to break down plastic waste. However, these methods may require specific environmental conditions to be effective and may not be suitable for all types of plastic waste. Additionally, some methods may produce hazardous by-products if not properly managed. Here are a few examples of biological methods of plastic degradation:

Biodegradation: This involves using microorganisms to break down plastic waste into simpler compounds, such as carbon dioxide and water. Some microorganisms, such as certain bacteria and fungi, are capable of breaking down certain types of plastic, such as polyhydroxyalkanoates (PHAs) or polylactic acid (PLA).

Composting: This involves using microorganisms, such as bacteria and fungi, to break down organic waste, including certain types of plastic, into nutrient-rich compost. Composting requires specific environmental conditions, such as moisture, temperature, and oxygen levels, to be effective.

Anaerobic digestion: This involves breaking down organic waste, including certain types of plastic, using microorganisms in the absence of oxygen. This process produces biogas, which can be used as fuel, and a nutrient-rich digestate that can be used as fertilizer.

Enzymatic degradation: This involves using enzymes, such as lipases and proteases, to break down certain types of plastic, such as PET and polyurethane (PU), into smaller molecules.

Plastic-degrading microorganisms

Biodegradation of plastic waste by microorganisms involves the breakdown of the plastic polymer into smaller compounds by the action of enzymes produced by microorganisms. There are various microorganisms capable of degrading plastic waste, including bacteria, fungi, and algae. The following are some examples of microorganisms that can degrade plastic:

***Pseudomonas putida*:** This bacterium has been found to have the ability to degrade certain types of plastic. Specifically, it has been shown to be capable of degrading PET, which is commonly used in the production of plastic bottles and packaging. The degradation process involves the production of certain enzymes, such as PET hydrolase, by *Pseudomonas putida*. PET hydrolase is able to break down the long chains of polymers that make up PET into smaller, more manageable molecules. These smaller molecules can then be used as feedstock for the production of new plastic products or for other purposes, such as fuel production. Overall, *Pseudomonas putida*'s application in plastic breakdown has the potential to be a successful and sustainable way to cut down on plastic waste [28,29].

***Bacillus subtilis*:** *Bacillus subtilis* is a type of bacteria that has been found to have the ability to degrade certain types of plastic. Specifically, it has been shown to be capable of degrading polyethylene, which is commonly used in the production of plastic bags and packaging. The degradation process involves the production of certain enzymes, such as lipases and esterases. These enzymes are able to break down the long chains of polymers that make up polyethylene into smaller, more manageable molecules. These smaller molecules can then be used as feedstock for the production of new plastic products or for other purposes, such as fuel production. Overall, the use of Bacillus subtilis in plastic degradation has the potential to be an effective and environmentally-friendly method for reducing plastic waste [30].

***Aspergillus niger*:** *Aspergillus niger* is a type of fungus that has been found to have the ability to degrade LDPE types of plastic. Specifically, it has been shown to be capable

of degrading polyurethane, which is commonly used in the production of foams, coatings, and adhesives. The degradation process involves the production of certain enzymes, such as polyurethanease and esterase. These enzymes are able to break down the long chains of polymers that make up polyurethane into smaller, more manageable molecules. These smaller molecules can then be used as feedstock for the production of new plastic products or for other purposes, such as fuel production [31,32].

Rhizopus arrhizus: *Rhizopus arrhizus* has been found to be effective in degrading PU plastics. Specifically, it has been shown to be capable of degrading PS, which is commonly used in the production of disposable cups, containers, and packaging materials. The degradation process involves the production of certain enzymes, such as styrene monooxygenase and laccase, by *Rhizopus arrhizus*. These enzymes are able to break down the long chains of polymers that make up PS into smaller, more manageable molecules. These smaller molecules can then be used as feedstock for the production of new plastic products or for other purposes, such as fuel production [33].

Penicillium simplicissimum: *Penicillium simplicissimum* has been found to be effective in degrading PVC plastics. Specifically, it has been shown to be capable of degrading PET, which is commonly used in the production of plastic bottles and packaging materials. The degradation process involves the production of certain enzymes, such as PETase and MHETase. These enzymes are able to break down the long chains of polymers that make up PET into smaller, more manageable molecules. These smaller molecules can then be used as feedstock for the production of new plastic products or for other purposes, such as fuel production. Overall, the use of *Penicillium simplicissimum* in plastic degradation has the potential to be an effective and environmentally-friendly method for reducing plastic waste, particularly in the case of PET plastics [34,35].

Saccharomyces cerevisiae: This yeast has been shown to degrade polycaprolactone (PCL) plastics. While *S. cerevisiae* is not known to have any direct involvement in plastic degradation, it is possible that it could be used indirectly in the production of bio-based plastics. Bio-based plastics are made from renewable sources such as plant materials or microorganisms like algae, and *S. cerevisiae* has been used in the fermentation of various types of plant materials for bio-based plastic production. Although there is no proof that *Saccharomyces cerevisiae* can break down plastics directly, a recent study indicates that it might be viable to use this yeast as a component of a biohybrid system for breaking down plastic [36].

Chlorella vulgaris: *Chlorella vulgaris* is a type of freshwater microalgae that has been studied for its potential in bioremediation, including the degradation of two common types of plastic, i.e., PE and PVC. The degradation process involves the production of certain enzymes by the algae, which are able to break down the long chains of polymers that make up the plastic into smaller, more manageable molecules. In addition to degrading plastic, *Chlorella vulgaris* has also been shown to have potential in other areas of bioremediation, such as wastewater treatment and carbon capture. However, more research is needed to fully understand the potential of this microalga in plastic degradation and other applications [37].

Conclusion

Due to biodegradation, thermo-oxidative degradation, photodegradation, thermal, and hydrolysis processes in the ecosystem, the concentration of in the aquatic environment has grown dramatically, which is very detrimental for both marine and freshwater organisms as well as humans owing to their impact on the food chain. To completely remove these polymers from the environment, effective biodegradable strategies are required. Polymers are generally difficult to eliminate or break down due to their hydrophobic and inert properties. Microbes have also shown promise as a means of degrading these polymers, complementing physical and chemical approaches. The use of microorganisms in plastic degradation has shown promising results in laboratory settings. However, there are still challenges that need to be addressed before this method can be scaled up for large-scale industrial applications. These challenges

include optimizing the conditions for the growth and activity of microorganisms, developing cost-effective methods for producing and using the necessary enzymes, and ensuring that the by-products of the degradation process are safe and environmentally friendly. However, it is important to note that the efficiency of plastic degradation by microorganisms can be affected by various factors such as temperature, pH, nutrients, and the structure and properties of plastic waste. Therefore, further research is required to optimize the conditions for the efficient and sustainable biodegradation of plastic waste by microorganisms (Figure 2).

The Future of Biodegradable Plastics

Advancements

Scientists are constantly developing new materials and technologies to improve the biodegradability of plastic.

Environmental Benefits

Biodegradable plastics offer a promising solution to reducing plastic waste and its negative impact on the environment.

Challenges

The production cost of biodegradable plastics is higher than traditional plastics, and there is limited infrastructure to support their proper disposal.

Figure 2: Future of biodegradable plastics

More evaluation utilizing original polymers polluted wastewater is required to assess the possible usage of microorganisms for polymer removal. Further work is needed on issues related to plastic cleanup, plastic toxicity, and microbe use. It is important to effectively advocate for the transfer of plastic polymers from wastewater to a suitable area for deposition/incineration to prevent them from entering the aquatic environment, which includes rivers and seas. Evaluating the cumulative impact on ecosystems requires coordinated long-term cleaning efforts.

References

[1] H. Sahni, N. Chopra, P. Gadhavi, The Social Rendition of Plastic Waste Management Initiatives in India, in: 2023: pp. 31–58. https://doi.org/10.1108/S2043-052320230000019003.

[2] T.D. Moshood, G. Nawanir, F. Mahmud, F. Mohamad, M.H. Ahmad, A. AbdulGhani, Sustainability of biodegradable plastics: New problem or solution to solve the global plastic pollution?, Curr. Res. Green Sustain. Chem. 5 (2022) 100273. https://doi.org/10.1016/j.crgsc.2022.100273.

[3] A.T. Williams, N. Rangel-Buitrago, The past, present, and future of plastic pollution, Mar. Pollut. Bull. 176 (2022) 113429. https://doi.org/10.1016/j.marpolbul.2022.113429.

[4] M. Bardales Cruz, E. Saikawa, M. Hengstermann, A. Ramirez, J.P. McCracken, L.M. Thompson, Plastic waste generation and emissions from the domestic open burning of plastic waste in Guatemala, Environ. Sci. Atmos. 3 (2023) 156–167. https://doi.org/10.1039/D2EA00082B.

[5] A. Gómez-Sanabria, G. Kiesewetter, Z. Klimont, W. Schoepp, H. Haberl, Potential for future reductions of global GHG and air pollutants from circular waste management systems, Nat. Commun. 13 (2022) 106. https://doi.org/10.1038/s41467-021-27624-7.

[6] T.-D. Bui, J.-W. Tseng, M.-L. Tseng, M.K. Lim, Opportunities and challenges for solid waste reuse and recycling in emerging economies: A hybrid analysis, Resour. Conserv. Recycl. 177 (2022) 105968.

https://doi.org/10.1016/j.resconrec.2021.105968.

[7] O. Dogu, M. Pelucchi, R. Van de Vijver, P.H.M. Van Steenberge, D.R. D'hooge, A. Cuoci, M. Mehl, A. Frassoldati, T. Faravelli, K.M. Van Geem, The chemistry of chemical recycling of solid plastic waste via pyrolysis and gasification: State-of-the-art, challenges, and future directions, Prog. Energy Combust. Sci. 84 (2021) 100901. https://doi.org/10.1016/j.pecs.2020.100901.

[8] A.P. Asiandu, A. Wahyudi, S.W. Sari, A Review: Plastics Waste Biodegradation Using Plastics-Degrading Bacteria, J. Environ. Treat. Tech. 9 (2020) 148–157. https://doi.org/10.47277/JETT/9(1)157.

[9] H. Sharma, D.K. Neelam, Understanding challenges associated with plastic and bacterial approach toward plastic degradation, J. Basic Microbiol. 63 (2023) 292–307. https://doi.org/10.1002/jobm.202200428.

[10] M. Brebu, Environmental Degradation of Plastic Composites with Natural Fillers—A Review, Polymers (Basel). 12 (2020) 166. https://doi.org/10.3390/polym12010166.

[11] R. Husain, N. Vikram, S. Pandey, G. Yadav, S.K. Bose, A. Ahamad, M. Shamim, K.G. Kumar, N.A. Khan, M. Zuhaib, T. Hussain, Microorganisms: An Eco-Friendly Tools for the Waste Management and Environmental Safety, in: Biotechnol. Innov. Environ. Bioremediation, Springer Nature Singapore, Singapore, 2022: pp. 949–981. https://doi.org/10.1007/978-981-16-9001-3_36.

[12] N. Tiwari, B. Garua, J.G. Sharma, Microbial diversity, interactions, and biodegradation/biotransformation of organic and inorganic contaminants, in: Wastewater Treat. React., Elsevier, 2021: pp. 341–372. https://doi.org/10.1016/B978-0-12-823991-9.00001-0.

[13] A. Priya, K. Dutta, A. Daverey, A comprehensive biotechnological and molecular insight into plastic degradation by microbial community, J. Chem. Technol. Biotechnol. 97 (2022) 381–390. https://doi.org/10.1002/jctb.6675.

[14] T. Matjašič, T. Simčič, N. Medvešček, O. Bajt, T. Dreo, N. Mori, Critical evaluation of biodegradation studies on synthetic plastics through a systematic literature review, Sci. Total Environ. 752 (2021) 141959. https://doi.org/10.1016/j.scitotenv.2020.141959.

[15] Z. Qin, J. Mou, C.Y.H. Chao, S.S. Chopra, W. Daoud, S. Leu, Z. Ning, C.Y. Tso, C.K. Chan, S. Tang, Z.J. Hathi, M.A. Haque, X. Wang, C.S.K. Lin, Biotechnology of Plastic Waste Degradation, Recycling, and Valorization: Current Advances and Future Perspectives, ChemSusChem. 14 (2021) 4103–4114. https://doi.org/10.1002/cssc.202100752.

[16] L.K. Ncube, A.U. Ude, E.N. Ogunmuyiwa, R. Zulkifli, I.N. Beas, An Overview of Plastic Waste Generation and Management in Food Packaging Industries, Recycling. 6 (2021) 12. https://doi.org/10.3390/recycling6010012.

[17] R.M. Patel, Polyethylene, in: Multilayer Flex. Packag., Elsevier, 2016: pp. 17–34. https://doi.org/10.1016/B978-0-323-37100-1.00002-8.

[18] S. Dai, C. Chen, Direct Synthesis of Functionalized High-Molecular-Weight Polyethylene by Copolymerization of Ethylene with Polar Monomers, Angew. Chemie. 128 (2016) 13475–13479. https://doi.org/10.1002/ange.201607152.

[19] Hisham A. Maddah, Polypropylene as a Promising Plastic: A Review, Am. J. Polym. Sci. 6 (2016) 1–11. https://doi.org/10.5923/j.ajps.20160601.01.

[20] S. Oberoi, M. Malik, Polyvinyl Chloride (PVC), Chlorinated Polyethylene (CPE), Chlorinated Polyvinyl Chloride (CPVC), Chlorosulfonated Polyethylene (CSPE), Polychloroprene Rubber (CR)—Chemistry, Applications and Ecological Impacts—I, in: Ecol. Heal. Eff. Build. Mater., Springer International Publishing, Cham, 2022: pp. 33–52. https://doi.org/10.1007/978-3-030-76073-1_3.

[21] K. Kik, B. Bukowska, P. Sicińska, Polystyrene nanoparticles: Sources, occurrence in the environment, distribution in tissues, accumulation and toxicity to various organisms, Environ. Pollut. 262 (2020) 114297. https://doi.org/10.1016/j.envpol.2020.114297.

[22] R. Nisticò, Polyethylene terephthalate (PET) in the packaging industry, Polym. Test. 90 (2020) 106707. https://doi.org/10.1016/j.polymertesting.2020.106707.

[23] T.N. Tsironi, S.M. Chatzidakis, N.G. Stoforos, The future of polyethylene terephthalate bottles: Challenges and sustainability, Packag. Technol. Sci. 35 (2022) 317–325. https://doi.org/10.1002/pts.2632.

[24] T. Artham, M. Doble, Biodegradation of Aliphatic and Aromatic Polycarbonates, Macromol. Biosci. 8 (2008) 14–24. https://doi.org/10.1002/mabi.200700106.

[25] P. Singh, P. Katiyar, H. Singh, Impact of compatibilization on polypropylene (PP) and acrylonitrile butadiene styrene (ABS) blend: A review, Mater. Today Proc. 78 (2023) 189–197. https://doi.org/10.1016/j.matpr.2023.01.350.

[26] A. Lee, M.S. Liew, Tertiary recycling of plastics waste: an analysis of feedstock, chemical and biological degradation methods, J. Mater. Cycles Waste Manag. 23 (2021) 32–43. https://doi.org/10.1007/s10163-020-01106-2.

[27] F. Zhang, Y. Zhao, D. Wang, M. Yan, J. Zhang, P. Zhang, T. Ding, L. Chen, C. Chen, Current technologies for plastic waste treatment: A review, J. Clean. Prod. 282 (2021) 124523. https://doi.org/10.1016/j.jclepro.2020.124523.

[28] O.F. Brandenberg, O.T. Schubert, L. Kruglyak, Towards synthetic PETtrophy: Engineering Pseudomonas putida for concurrent polyethylene terephthalate (PET) monomer metabolism and PET hydrolase expression, Microb. Cell Fact. 21 (2022) 119. https://doi.org/10.1186/s12934-022-01849-7.

[29] S. Fontanazza, A. Restuccia, G. Mauromicale, A. Scavo, C. Abbate, Pseudomonas putida Isolation and Quantification by Real-Time PCR in Agricultural Soil Biodegradable Mulching, Agriculture. 11 (2021) 782. https://doi.org/10.3390/agriculture11080782.

[30] Y. Xu, X. Liu, J. Zhao, H. Huang, M. Wu, X. Li, W. Li, X. Sun, B. Sun, An efficient phthalate ester-degrading Bacillus subtilis: Degradation kinetics, metabolic pathway, and catalytic mechanism of the key enzyme, Environ. Pollut. 273 (2021) 116461. https://doi.org/10.1016/j.envpol.2021.116461.

[31] R. Sangeetha Devi, V. Rajesh Kannan, D. Nivas, K. Kannan, S. Chandru, A. Robert Antony, Biodegradation of HDPE by Aspergillus spp. from marine ecosystem of Gulf of Mannar, India, Mar. Pollut. Bull. 96 (2015) 32–40. https://doi.org/10.1016/j.marpolbul.2015.05.050.

[32] B. Mousavi, M.T. Hedayati, N. Hedayati, M. Ilkit, S. Syedmousavi, Aspergillus species in indoor environments and their possible occupational and public health hazards, Curr. Med. Mycol. 2 (2016) 36. https://doi.org/10.18869/ACADPUB.CMM.2.1.36.

[33] B. Nowak, J. PająK, S. Labuzek, G. Rymarz, E. Talik, Biodegradation of poly(ethylene terephthalate) modified

with polyester “Bionolle®” by Penicillium funiculosum, Polimery/Polymers. 56 (2011) 35–44. https://doi.org/10.14314/POLIMERY.2011.035.

[34] H. V. Sowmya, Ramalingappa, M. Krishnappa, B. Thippeswamy, Degradation of polyethylene by Penicillium simplicissimum isolated from local dumpsite of Shivamogga district, Environ. Dev. Sustain. 17 (2015) 731–745. https://doi.org/10.1007/S10668-014-9571-4.

[35] H. Sowmya, M. Krishnappa, B.T.-Environment, undefined development, undefined 2015, Degradation of polyethylene by Penicillium simplicissimum isolated from local dumpsite of Shivamogga district, Springer. (n.d.). https://link.springer.com/article/10.1007/s10668-014-9571-4 (accessed November 25, 2022).

[36] S. Jaiswal, B. Sharma, P. Shukla, Integrated approaches in microbial degradation of plastics, Environ. Technol. Innov. 17 (2020) 100567. https://doi.org/10.1016/j.eti.2019.100567.

[37] P.K. Akao, H. Mamane, A. Kaplan, I. Gozlan, Y. Yehoshua, Y. Kinel-Tahan, D. Avisar, Iohexol removal and degradation-product formation via biodegradation by the microalga Chlorella vulgaris, Algal Res. 51 (2020) 102050. https://doi.org/10.1016/j.algal.2020.102050.

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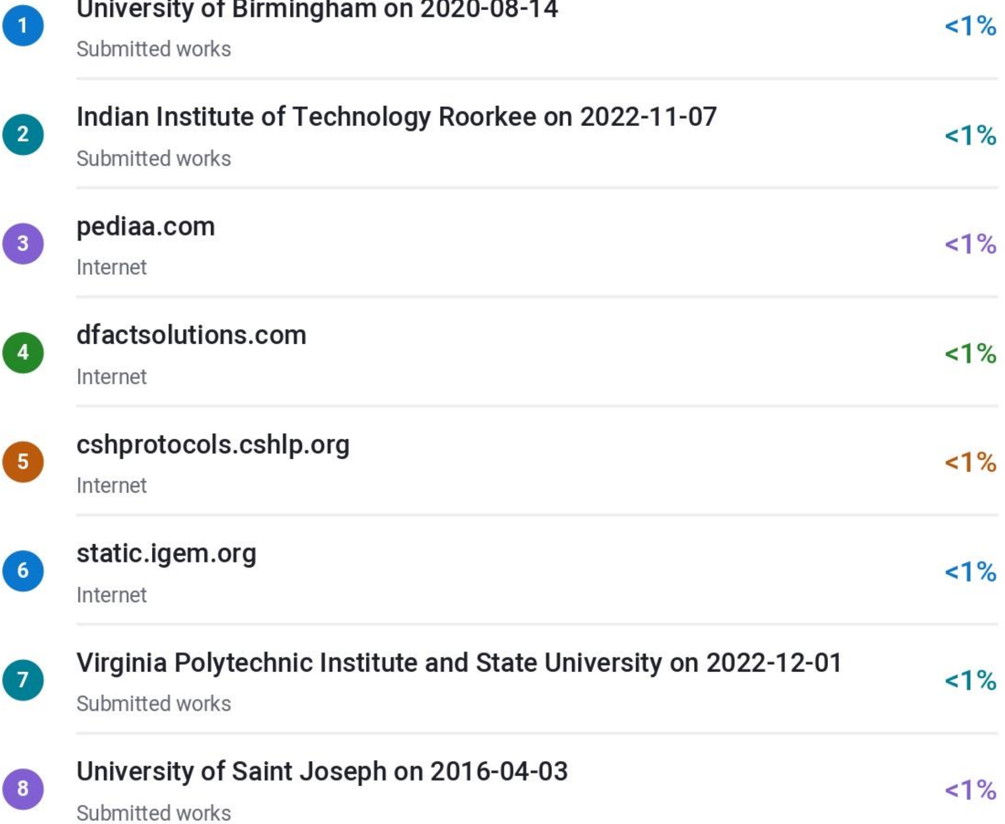

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