B.Tech. Project Report

on

Isolation, Amplification and Cloning of Arginase Producing Gene from *Bacillus megaterium*

at

Jaypee University of Information Technology

Under the guidance of -

Dr. Saurabh Bansal
Associate Professor
Faculty In-Charge, JUIT Alumni Cell
Member, IQAC, Board of Studies
Department of Biotechnology & Bioinformatics

Submitted by –

Tanya Bansal
191820
Undergraduate student (IV year)
Department of Biotechnology

Preface

This project on cancer represents an earnest endeavour to shed light on one of the most pressing challenges in the field of healthcare and medicine. Cancer, a multifaceted and complex disease, continues to impact the lives of millions of individuals worldwide, necessitating dedicated efforts to understand its underlying mechanisms, develop effective treatments, and improve patient's outcomes.

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Isolation, Amplification and Cloning of Arginase Producing Gene from *Bacillus megaterium*

by

Tanya Bansal

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Abstract

An important cycle that operates partly in the cytosol and partly in the mitochondria of a cell is the urea cycle. The ureohydrolase enzyme arginase (Arg) catalyzes the conversion of arginine to ornithine, releasing urea as a by-product. Increased metabolic activity of cancer cells amounts for increased demand of amino acids for their growth. Cancer types like hepatocellular carcinoma, prostate cancer are auxotrophic for arginine. Cancer cells fail to match the production of amino acids with their depletion. Hence, growth of afore mentioned cancer types cease upon non-availability of arginine, and consequently dependency on surrounding cells increases. Artificial administration of Arg to completely devoid such cells of arginine is a budding therapeutic approach for cancer treatment. Arg from human source is already under clinical trials. But what distinguishes this project is the production of Arg from microbial source. The objective is to discover a novel microbial source for Arg and simultaneously reduce the cost of Arg, thereby impacting overall cancer treatment with respect to availability of drug and cost.

The focus is on isolation of Arg producing gene from a Gram-positive thermophilic bacterium *Bacillus megaterium*, its amplification and cloning, followed by monitoring of expression in the host and purification. Comparison of clinical implications of Arg derived from human source versus Arg derived from microbial source is subject to the availability of microbial Arg in its purest form.

Project supervisor : Dr. Saurabh Bansal
Title : Associate Professor

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List of abbreviations

ADI : Arginine deiminase
ALL : Acute lymphoblastic leukemia
ASL : Argininosuccinate lyase
ASS : Argininosuccinate synthase
Arg : L-Arginase
b0,+AT : b0, + amino acid transporter
CATs : Cationic amino acid transporters
ChT : Chemotherapy
GoI : Gene of interest
hArg : Arginase from human source
HCC : Hepatocellular carcinoma
HNSCCs : Head and neck squamous cell carcinomas
mArg : Arginase from microbial source
mAsp : Asparaginase from microbial source
MDSCs : Myeloid-derived suppressor cells
NFW : Nuclease free water

MHC	: Major histocompatibility complex
MTCC	: Microbial Type Culture Collection and Gene Bank
NCBI	: National Center of Biotechnology Information
NK cells	: Natural killer cells
NO	: Nitric oxide
NOS	: Nitric oxide synthase
OTC	: Ornithine transcarbamylase
PCR	: Polymerase chain reaction
RT	: Radiation therapy
TAMs	: Tumor-associated macrophages
TILs	: Tumor-infiltrating lymphocytes
y+LAT1	: y+ L-amino acid transporter 1

Chapter 1
Introduction

Cancer, a word capable of turning individual's life upside down, is a word all of us are aware of, because either it has been a part of our own life or has been a part of life of someone we know. Hardly there exists an individual unaware of this word, for it has gripped each and every section of the society. According to the National Cancer Registry Programme Report 2022, the estimated number of incident cases of cancer in India for the year 2022 was found to be 14,61,427 (Sathishkumar et al., 2023). The current measures used for the treatment of cancer primarily make use of only chemicals (chemotherapy, ChT) and radiations (radiation therapy, RT). But there is a set of complications that accompanies these approaches, like ChT induced neurotoxicity, RT induced neurotoxicity, RT induced mucosal necrosis, among many others (Albano et al., 2021). In this view, there is an absolute need for discovering a novel therapeutic approach that not only provides treatment for cancer, but also can drastically decrease the amount of complications that are inevitable with existing traditional approaches.

Targeted approach for cancer treatment has received attention of researchers from all over the world. Metabolic rate of cancer cells is higher as compared to the normal cells. Certain amino acids which are essential for their growth like asparagine, arginine, glutamine, proline, are not synthesized at the rate they are degraded. Consequently, cancer cells become dependent on surrounding cells for nutrition. Targeted approach for cancer treatment aims to devoid such cells of the amino acid they are auxotrophic for (Kumari and Bansal, 2021).

Much of the advancement till date is only seen for the cancer types auxotrophic for asparagine, like acute lymphoblastic leukemia (ALL), Hodgkin's lymphoma (Muneer et al., 2020). Asparagine degrading enzyme asparaginase has passed clinical trials, and is currently used for cancer treatment (Müller, 1998). Asparaginase from microbial source (mAsp) is available in the market, and is significantly impacting the overall cancer treatment, with respect to decreased complications and long-term disease free survival (Zhen et al., 2021). But not all the cancer types are auxotrophic for asparagine. Hepatocellular carcinoma (HCC), prostate cancer are some of the cancer types which are auxotrophic for arginine (Patil et al., 2016). The ureohydrolase enzyme arginase (Arg) catalyzes the conversion of arginine to ornithine, releasing urea as a by-product (Caldwell et al., 2018). Pegylated human recombinant Arg (PEG-BCT-100) is under clinical trials to determine its safety and effectiveness in the treatment of advanced HCC (ClinicalTrials.gov Identifier : NCT01092091).

But as compared to the rise in incident cases of HCC each year (Llovet et al., 2021), having only single source of Arg will lead to shortage in the availability of the drug in the future, leading to rise in the price and increase in the cost for cancer treatment. The objective is to discover a novel source of Arg, having safety and effectiveness comparable to the human Arg (hArg), and significantly reduce the cost of treatment by meeting the demand for the drug. The focus is on isolation of Arg producing gene from a Gram-positive thermophilic bacterium *Bacillus megaterium*, its amplification and cloning, followed by monitoring of expression in the host and purification. Comparison of clinical implications of Arg derived

from human source versus Arg derived from microbial source (mArg) is subject to the availability of mArg in its purest form.

Cancer is a broad term that refers to a group of diseases characterized by the uncontrolled growth and spread of abnormal cells in the body. These abnormal cells form tumors, which can invade nearby tissues and, in some cases, metastasize (spread) to other parts of the body through the bloodstream or lymphatic system. There are many different types of cancer, and they can occur in various organs and tissues. Some common types of cancer include breast cancer, lung cancer, colorectal cancer, prostate cancer, and leukemia, among others. Each type of cancer has its own characteristics, behavior, and treatment options. Cancer can develop due to a combination of genetic factors and environmental exposures. Certain risk factors, such as tobacco use, exposure to carcinogens (substances that can cause cancer), unhealthy diet, lack of physical activity, chronic infections, and family history of cancer, can increase the likelihood of developing the disease. However, it's important to note that not all individuals with risk factors will develop cancer, and some people without known risk factors can still develop the disease.

The development of cancer typically involves multiple stages, including initiation, promotion, and progression. In the initiation stage, genetic mutations or alterations occur within a cell's DNA, which can be caused by external factors or errors during cell division. These mutations disrupt normal cell growth and division control mechanisms. In the promotion stage, the mutated cells undergo further changes that enable them to multiply and form a pre-cancerous lesion or tumor. During progression, the tumor cells acquire additional genetic changes that allow them to invade nearby tissues or spread to other parts of the body. The diagnosis and treatment of cancer involve various approaches, including imaging tests, biopsies, laboratory analysis, surgery, radiation therapy, chemotherapy, targeted therapy, immunotherapy, and hormone therapy. The choice of treatment depends on factors such as the type and stage of cancer, the overall health of the patient, and their individual preferences.

Advances in cancer research and treatment have significantly improved outcomes for many cancer patients. Early detection, lifestyle modifications, and prevention efforts, such as smoking cessation and vaccinations against cancer-causing viruses, also play important roles in reducing cancer incidence and mortality rates. It's essential for individuals to be aware of potential signs and symptoms of cancer and to undergo regular screenings as recommended by healthcare professionals. If you have specific concerns about cancer, it's important to consult with a healthcare provider who can provide personalized information and guidance based on your individual situation.

Chapter 2

Literature Review

2.1. Normal versus cancer cells

Each cell, either normal or cancerous, undergoes cell cycle. Cell cycle is defined as the sequence of events that take place when a cell undergoes division, could be mitotic or meiotic. Cell cycle is under tight regulation in case of normal cells, and the disruption of this tight regulation gives rise to cancer cells. Cell cycle (referring here to mitosis) comprises of four phases, G_1, S, G_2 and M. Approximate time taken by cells to go through these phases is 5 hours, 7 hours, 3 hours and 1 hour respectively. Hence, a normal cell undergoes cell division in approximately 16 hours. Diagrammatic representation of mitotic cell cycle is shown below (Figure 2-1).

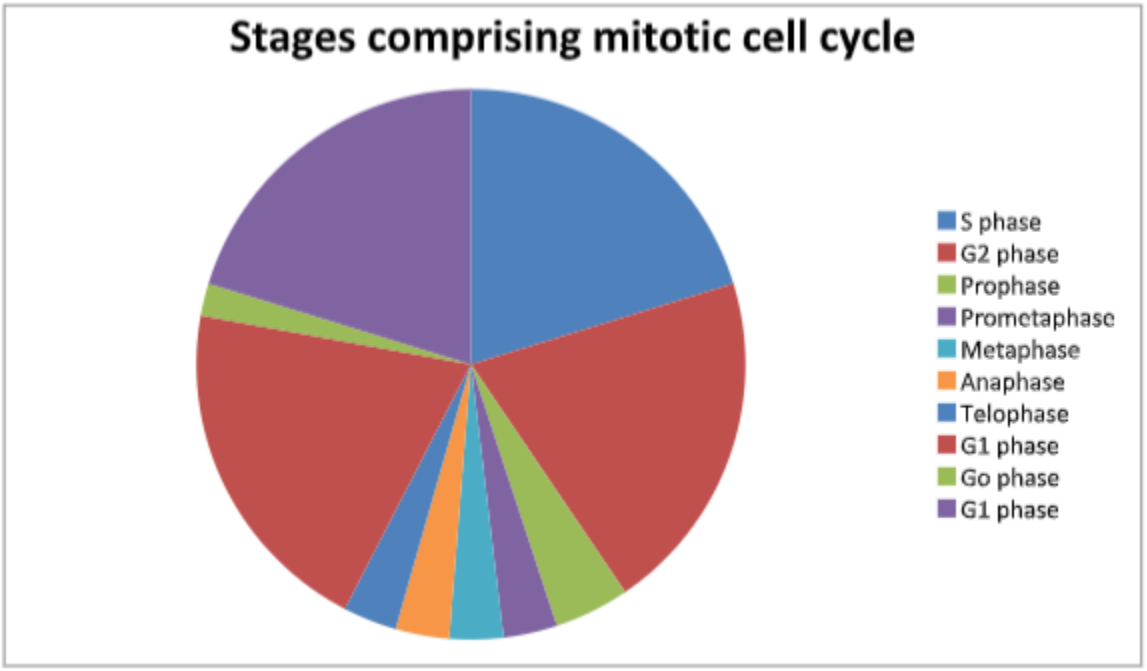

Figure 2-1 Stages comprising mitotic cell cycle

The present knowledge of how many genes are involved in the control of the cell cycle came after the rigorous research efforts of about 15 years, of Lee Hartwell, Paul Nurse and Tim Hunt. Their work was recognized by the awarding of Nobel Prize in Medicine or Physiology to them in the year 2001. A great feat! From their findings, it became evident that three checkpoints keep the cell cycle under control. These checkpoints have been named according to their occurrence in the cell cycle. The first checkpoint G_1/S occurs at the end of G_1 phase and before the beginning of S phase. This checkpoint checks whether the cell has achieved its normal size post previous mitosis or not and also evaluates the condition of the DNA. If cell size and condition of the DNA is found to be satisfactory, the cell is allowed to proceed from G_1 phase to S phase. Discrepancy in even any one of the conditions of the two, results in

arresting of the cell cycle at this checkpoint and cell cycle does not proceed further. Similarly, the second checkpoint G_2/M occurs at the end of G_2 phase and before the beginning of M phase which monitors the DNA. Incomplete DNA replication or detection of DNA damage results in the arresting of cell cycle at this checkpoint. Conversely, if neither of the above mentioned two conditions is encountered, the cell is allowed to proceed from G_2 phase to M phase. The last checkpoint occurs during M phase, known as M checkpoint. This checkpoint is also known as Spindle Assembly checkpoint because it monitors the formation of spindle fiber system.

Sometimes, these checkpoints fail to arrest the cell cycle even after encountering undesirable conditions such as damaged DNA, incomplete DNA replication etc. In those circumstances, cells formed after division are not as healthy as normal cells. These cells will keep on dividing uncontrollably owing to the defects in their genome. Mass of such cells form tumor (Klug, 2012).

Table 2-1 Normal versus cancer cells

Criterion	Normal cells	Cancerous cells	References
Condition of genetic material	Complete and not damaged	Incomplete and damaged	(Klug et al., 2019)
Duration of cell cycle	Approximately 16 hours	Lesser than 16 hours	(Klug et al., 2019)
Nutrient demand	Comparatively lesser	Comparatively higher	(Nenkov et al., 2021)

The cell cycle duration of cancer cells is shorter in comparison to the cell cycle duration of normal cells. Consequently, cancer cells demand more nutrients (Nenkov et al., 2021). This is the approach behind considering various enzymes such as asparaginase, Arg, arginine deiminase (ADI) etc. as potential therapeutic enzymes for cancer treatment.

2.2. Strategies for cancer treatment

2.2.1. Traditional methods

These treatments are widely accepted and used by the majority of healthcare providers. These strategies are being used for long period of time. It differs from alternative or complementary therapies, which are less common. The most common traditional methods are explained below (Stub et al., 2018; Tannock FRCPC, 1998).

2.2.1.1. Chemotherapy

A chemotherapy drug is used to kill cancer cells. It is possible to administer the drugs orally or intravenously. Various types of drugs may be administered simultaneously or sequential. Based on Cecil's Textbook of Medicine (1988), chemotherapy can achieve long-lasting and complete remission in part of the cancer cases. Chemotherapy drugs can be administered in a variety of ways depending on the type of cancer and where it is found, including injections into muscles, injections under the skin, injections into arteries, injections into veins (intravenous treatment), pills taken by mouth and injections into the spinal cord or brain.

During chemotherapy, a thin catheter is inserted into a large vein near the heart. Such a line is known as a central line. Minor surgery is needed to place the catheter. Most chemotherapy treatments are given in cycles. These cycles may last a few days, a few weeks, or even several months. Each cycle is usually be followed by a rest period without chemotherapy. During this time, the body and blood counts recover.

2.2.1.2. Surgery

Tumors that are cancerous can be removed by surgery. Using lasers and laparoscopic techniques, microsurgical procedures are becoming one of the safest forms of treatment. A physician can use these techniques to operate on a patient without having to open the patient. In addition to being a common form of treatment, surgery is also indispensable for diagnosing a patient. Surgery involves the removal of cancer cells (tumor) and some of the nearby tissue. Some people undergo surgery to relieve tumor-related side-effects (Debela et al., 2021) (Gelband et al., 2015).

2.2.1.3. Radiation therapy

Radiation therapy is reserved for a tumor by irradiating only part of the body. Radiation involves a high local radioactive beam for tumor. Radiation treatment is considered on the basis of the position of the tumor. At high doses, radiation therapy kills cancer cells or slows their growth by damaging their DNA. Cancer cells that have damaged DNA beyond their ability to repair will stop dividing or die. When damaged cells die, they are broken down and eliminated by the body. Radiation therapy does not kill cancer cells immediately. It takes days or weeks of treatment before the DNA is damaged enough for the cancer cells to die. Then, the cancer cells continue to die for several weeks or months after the radiation treatment is over. There are two main forms of radiation therapy, external rays and internal rays. Radiation therapy ranks the most effective treatment after surgery (Arruebo et al., 2011; Baskar et al., 2012a) (Gelband et al., 2015).

Table 2-2 Types of cancer treated and limitations of the traditional methods

S no.	Type of cancer treatment	Types of cancer successfully treated	Limitations
1.	Chemotherapy	Many cancer types are treated with chemotherapy. Some people may receive only chemotherapy treatment. Usually, chemotherapy and other cancer treatments are combined. Treatment depends on the type of cancer you have, whether it has spread, and if you have other	A chemotherapy treatment kills or slows not only fast-growing cancer cells, but also healthy cells that grow and divide rapidly. Side-effects from damage to healthy cells may include mouth sores, nausea, and hair loss. These effects usually subside after chemotherapy is completed. Common side-effects include anaemia, appetite loss, bleeding and bruising (thrombocytopenia), constipation, delirium, diarrhea, edema (swelling), fatigue, fertility issues in boys and

		health problems (Amjad et al., 2022).	men, fertility issues in girls and women, flu-like symptoms, hair loss (alopecia), infection and neutropenia, lymphedema, memory or concentration problems, mouth and throat problems, nausea and vomiting, nerve problems (peripheral neuropathy), immunotherapy and organ-related inflammation, pain, sexual health issues in men, sexual health issues in women, skin and nail changes, sleep problems , urinary and bladder problems (Nurgali et al., 2018).
2.	Surgery	Surgery is most effective for solid tumors that are contained in one area. The cancer is treated locally, which means that only the cancerous part of your body is treated. Tumors that have spread or leukaemia (a type of blood cancer) should not be treated with this method (Gelband et al., 2015).	Usually, people will experience pain in the area of their body that was operated on after surgery. Surgery can also cause infection. Surgery also involves risks such as bleeding, tissue damage, and anesthesia reactions (Tohme et al., 2017).
3.	Radiation therapy	Stomach and abdomen, rectum, pelvis, head and neck, chest, breast, brain, cervix (Baskar et al., 2012b; Gelband et al., 2015; Mohan et al., 2019).	The side-effects of radiation therapy depend on the part of the body that is being treated. Some side-effects include fatigue, hair loss, memory or concentration problems, nausea and vomiting, skin changes, headache, blurry vision, tenderness, less active thyroid gland, cough, shortness of breath, fertility problems (men), sexual problems (women), fertility problems (women) (Majeed & Gupta, 2022).

2.2.1. Modern methods

Traditional methods of cancer treatment have undoubtedly saved many lives. But the limitations they possess drove the community towards finding better approaches, which not only provide the cure, but also remove the compromise on life that is made by traditional methods. With the advancement in the field of science, following modern methods of cancer treatment have emerged (Pucci et al., 2019).

2.2.1.1. Hormone therapy

Hormone therapy is that cancer treatment that slows or stops the growth of cancer that uses hormones to grow. This therapy is also known as hormonal therapy or hormone treatment or endocrine therapy. Hormone therapy targets the hormones that circulate in the body; hence this therapy is also known as systemic treatment. The drugs used travel throughout the body to find and target the hormones. Most types of surgery and radiation therapy affect only a certain part of body, whereas hormone therapy targets the entire body and this is how it differs from above mentioned traditional methods (Nordenström et al., 2018).

2.2.1.2. Hyperthermia

Also known as thermal therapy or thermal ablation or thermotherapy, tissue is heated to temperature as high as $113^{o}F$ in this type of cancer treatment with an aim to kill cancer cells with minimal harm to normal tissue (Otte, 1988).

2.2.1.3. Immunotherapy

Immunotherapy engages immune system to fight against cancer. Since this type of cancer treatment uses substances made from living organisms, it is also classified as a type of biological therapy. In this therapy, immune system plays the vital role. It first detects and then destroys abnormal cells. Immune cells are sometimes found in and around tumors. These cells are called tumor-infiltrating lymphocytes or TILs. Presence of TILs is a sign that the immune system is responding to the tumor. People whose tumors contain TILs often do better than people whose tumors don't contain them. Immunotherapy helps the immune system to act better against cancer (Esfahani et al., 2020).

2.2.1.4. Stem cell transplant

Stem cells are destroyed in people who are exposed to high doses of chemotherapy or radiation therapy or both. To restore these blood-forming stem cells is the aim of stem cell transplant, a modern method of cancer treatment. This also implies that stem cell transplant does not work directly against cancer, but helps in recovering body's ability to produce stem cells (Chu et al., 2020).

2.2.1.5. Targeted therapy

Targeted therapy is known so ('targeted') because it targets proteins that help in progression of cancer cells. There are many types of targeted therapy and most types treat cancer by interfering with specialised proteins that help tumors grow and spread throughout the body. They treat cancer in many ways as follows -

(a) Help the immune system destroy cancer cells:

Prominent reason behind striving of cancer cells in patient's body is because they can hide from immune system. Targeted therapy can mark cancer cells, which makes it easier for

immune system to find and destroy them. Alternatively, targeted therapy can boost the immune system to work better against cancer.

(b) Stop cancer cells from growing:

Healthy cells usually divide to make new cells only when they receive strong signals to do so. These signals bind to proteins on the cell surface, telling the cells to divide. New cells are formed only as the body needs them. But some cancer cells have changes in the proteins on their surface that tell them to divide whether or not signals are present. Few targeted therapies interfere with these proteins, thereby preventing them from telling the cells to divide. This helps in slowing cancer uncontrolled growth.

(c) Stop signals that help form blood vessels:

Cancer cells require formation of new blood vessels to grow beyond a certain size. In a process called angiogenesis, new blood vessels form in response to signals from the tumor. Some targeted therapies are designed to interfere with these signals to prevent blood supply. In absence of blood supply, tumor remains small, or in presence of blood supply, these treatments can cause blood vessels to die, which causes the tumor to shrink.

(d) Deliver cell-killing substances to cancer cells:

This includes combination of monoclonal antibodies with toxins, chemotherapy drugs, and radiation. Cells take up the cell-killing substances upon attachment of monoclonal antibodies with targets on the surface of cancer cells, thereby causing cancer cells to die.

(e) Enzymatic approach:

In the enzymatic approach of targeted therapy, enzymes are used to specifically deplete the amino acids on which cancer cells show dependency. Depletion of amino acids means depleting the nutrient reserve of cancer cells, without which they cannot grow. Consequently, cancer cells die. This approach will be considered in detail (Padma, 2015).

Table 2-3 Modern methods of cancer treatment, types of cancer successfully treated and limitations

S no.	Type of cancer treatment	Types of cancer successfully treated	Limitations
1.	Hormone therapy	Prostate cancer (Mazzucchelli et al., 2014).	Common side-effects seen in men include hot flashes, loss of interest in or ability to have sex, weakened bones, diarrhea, nausea, enlarged and tender breasts and fatigue. Common side-effects seen in women include hot flashes, vaginal dryness, changes in periods if have not yet reached menopause, loss of interest in sex,

			nausea, mood changes and fatigue (Nordenström et al., 2018).
2.	Hyperthermia	Advanced cancers like appendix cancer, bladder cancer, brain cancer, breast cancer, cervical cancer, oesophageal cancer, head and neck cancer, liver cancer, lung cancer, melanoma, mesothelioma, sarcoma and rectal cancer (Crezee et al., 2021).	Perfusion techniques can cause swelling, blood clots, bleeding and other damage to the normal tissues in the treated area. Diarrhea, nausea and vomiting are common after whole-body hyperthermia. It can also cause more serious side-effects that are not common, including heart and blood vessel problems (Sharma et al., 1990).
3.	Immunotherapy	Head and neck squamous cell carcinomas (HNSCCs) (Sahu & Suryawanshi, 2021).	Immunotherapy can cause side-effects like pain, swelling, soreness, redness, itchiness and rash, many of which happen when the immune system that has been revved-up to act against the cancer also acts against healthy cells and tissues in your body (Okwundu et al., 2021).
4.	Stem cell transplant	Lymphoma (Benekou & Montoto, 2017), neuroblastoma (Jain et al., 2020) and multiple myeloma (Fiala et al., 2021).	If you have an allogeneic transplant, you might develop a serious problem called graft-versus-host disease. Graft-versus-host disease can occur when white blood cells from your donor (the graft) recognize cells in your body (the host) as foreign and attack them. This problem can cause damage to your skin, liver, intestine and many other organs. It can occur a few weeks after the transplant or much later (Aladağ et al., 2020).
5.	Targeted therapy	Prostate cancer (Crawford et al., 2019), breast cancer (Lev, 2020), ALL (Salvaris & Fedele, 2021).	Side-effects include high blood pressure, proteinuria, thrombotic microangiopathy, kidney failure etc. (Izzedine et al., 2011).

2.3. Understanding synthesis, circulation and metabolism of arginine inside a human body

L-arginine is a cationic (Figure 2-2), semi-essential amino acid, which is derived primarily from dietary sources and synthesis in the kidney (Lind, 2004). In most mammalian cells, including tumor cells, L-arginine circulating in the body is transported across the plasma membrane via the Na^+ independent cationic amino acid transport system y^+ (Cendan et al., 1995). Once transported across the plasma membrane, this versatile amino acid serves as a

precursor for the synthesis of urea, nitric oxide (NO), polyamines, proline, glutamate, creatine and agmatine (Morris, 2006).

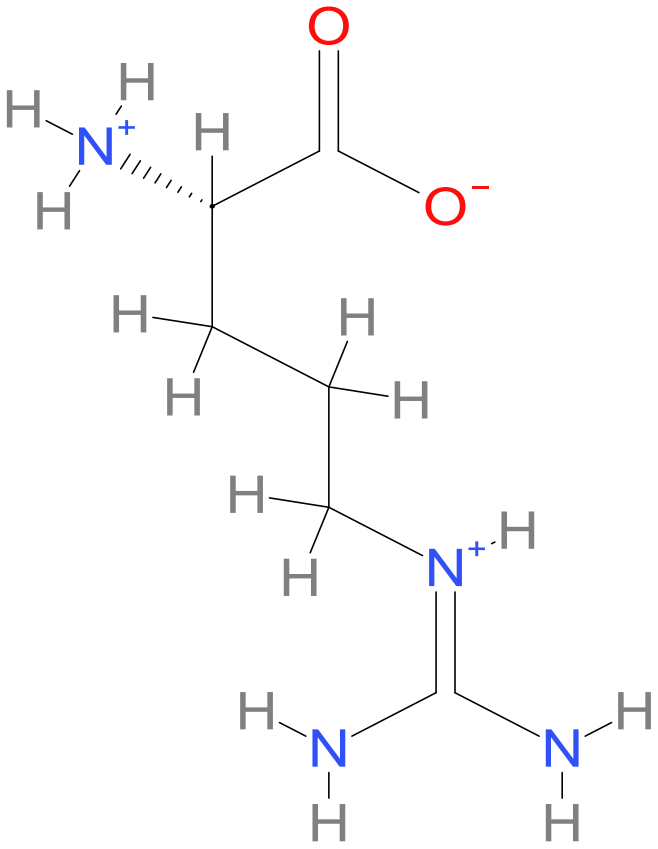

Figure 2-2 Chemical structure of L-arginine at physiological pH

2.3.1. Sources of L-arginine

(a) Dietary sources - L-arginine is an amino acid that can be found in a number of dietary sources, such as:

1. Meat: Turkey, beef, hog, and chicken are all excellent sources of L-arginine. Particularly, lean meat cuts typically contain more L-arginine than fatty meat portions.
2. Fish: There are numerous varieties of fish, such as salmon, tuna, and halibut. Omega-3 fatty acids, which are found in fish, are a good source of health and can aid to lower inflammation in the body.
3. Dairy items including milk, cheese, and yoghurt. These foods also include good amounts of calcium and other essential elements.
4. Almonds, peanuts, sunflower seeds, and pumpkin seeds are among the nuts and seeds. These meals are also rich in fibre, other minerals, and good fats.
5. Beans, lentils, and chickpeas are examples of legumes. These foods also contain a lot of fibre and other vital elements.

6. Grains: Wheat germ, quinoa, and oats. Additionally, these meals contain a lot of fibre, vitamins, and minerals.

It is important to note that while L-arginine is found in many different dietary sources, it is not always present in high quantities. If an individual is looking to increase intake of L-arginine, then he/she must consider taking a dietary supplement or consulting with a healthcare professional to develop a dietary plan that meets their specific needs.

(b) Endogenous production – The body's ability to produce L-arginine is greatly influenced by the kidneys. In particular, the kidneys are home to enzymes necessary for the synthesis of L-arginine from other amino acids including citrulline and ornithine. The 'arginine-citrulline route' is the mechanism through which the kidneys create L-arginine. Citrulline and aspartate are transformed into argininosuccinate in this pathway by the enzyme argininosuccinate synthase (ASS), which is then further turned into L-arginine by the enzyme argininosuccinate lyase (ASL). Numerous physiological activities, including the control of blood pressure, the creation of NO, and the elimination of waste from the body, depend on the kidney's generation of L-arginine. In addition, L-arginine is also important for the production of proteins and for the maintenance of a healthy immune system.

2.3.2. Transport of L-arginine across the plasma membrane

Numerous transporters, including cationic amino acid transporters (CATs), y+ L-amino acid transporter 1 (y+LAT1), and b0, + amino acid transporter (b0,+AT), move L-arginine across the plasma membrane of cells. These transporters are in charge of bringing L-arginine from the bloodstream and extracellular fluid into the cells. L-arginine is one of the cationic amino acids that are transported by CATs, a family of transporters. CATs come in three main subtypes, each with a unique tissue distribution and transport profile. The kidneys, liver, and intestines are just a few of the tissues that have significant levels of CAT expression. L-arginine is one of the cationic amino acids that are taken up by the transporter y+LAT1 into cells. In tissues like skeletal muscle and the liver, which need a lot of L-arginine for protein synthesis and the creation of NO, this transporter is abundantly expressed. L-arginine is one of the neutral and cationic amino acids that are transported into cells by the b0,+AT transporter. The small intestine, where this transporter is extensively expressed, is in charge of absorbing amino acids from food. Several physiological activities, such as the creation of NO, the synthesis of proteins, and the preservation of a cell's membrane potential, depend on the transport of L-arginine across the plasma membrane.

2.3.3. Metabolic fates of L-arginine inside a cell

L-arginine is a crucial amino acid with numerous functions in cellular metabolism. Numerous biologically active compounds, such as NO, urea, polyamines, and creatine, can be made from it as a precursor. L-arginine also contributes to the production of proteins and the maintenance of a strong immune system. L-arginine's metabolic fate within a cell is influenced by a number of variables, including the type of cell, the metabolic state of the cell, and the accessibility of additional nutrients and cofactors.

2.3.3.1. Protein synthesis

The incorporation of L-arginine into proteins is one of the amino acid's main functions in cellular metabolism. One of the 22 common amino acids that cells need to create proteins is L-arginine. L-arginine can be incorporated into proteins during translation after being delivered into the cell. Cellular structure, function, and signalling all depend on proteins.

2.3.3.2. Nitric oxide synthesis

NO, a signalling molecule that is essential for controlling blood pressure, blood flow, and immunological responses, can only be produced from L-arginine. Nitric oxide synthase (NOS) is an enzyme found inside of cells that changes L-arginine into NO. Numerous cellular activities, including vasodilation, neurotransmission, and inflammation, are influenced by NO. Additionally, it has been demonstrated that NO has a role in the regulation of apoptosis (programmed cell death) and has anti-tumor capabilities.

2.3.3.3. Urea cycle

L-arginine participates in the urea cycle, a metabolic process that eliminates ammonia from the body. The metabolism of amino acids produces ammonia, a poisonous by-product that can build up in the blood and harm the brain and other organs. L-arginine is transformed into ornithine in the liver, which is then used to make urea. Urea is a crucial part of the body's nitrogen balance and is eliminated from the body in urine.

2.3.3.4. Polyamine synthesis

Polyamines are small, positively charged molecules that are involved in a variety of cellular processes, including cell growth, differentiation, and apoptosis. L-arginine is involved in the synthesis of polyamines through its conversion to ornithine. Ornithine is used to produce putrescine, which is then converted to spermidine and spermine. Polyamines are essential for cell growth and proliferation, and they have been shown to be involved in the regulation of gene expression and protein synthesis.

2.3.3.5. Creatine synthesis

Additionally, L-arginine contributes to the creation of creatine, a chemical necessary for cellular energy production. L-arginine is transformed into guanidinoacetate in the kidneys, and from there, creatine is made. The creation of ATP, the main source of energy for cells, requires the high-energy molecule creatine. Creatine has been demonstrated to have neuroprotective effects and is crucial for muscle function.

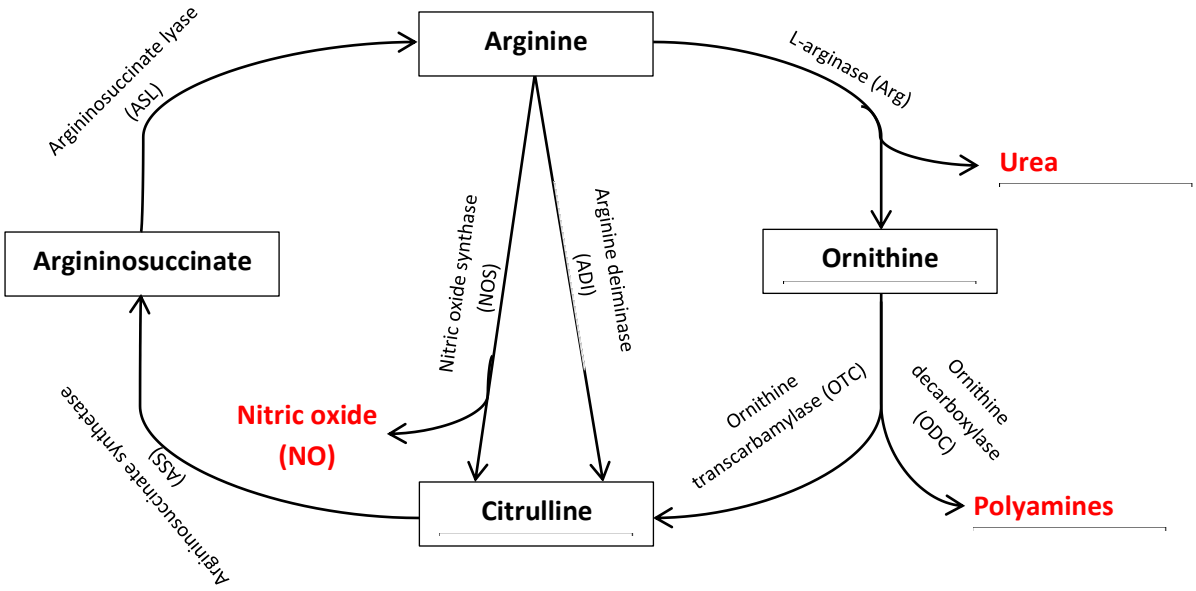

Figure 2-3 Metabolic fates of L-arginine inside a cell

2.4. Versatility of L-arginine in health and diseases

L-arginine is an important amino acid that is used by the body in a number of physiological functions. L-arginine has been thoroughly researched for its possible therapeutic uses in treating a range of medical disorders.

2.4.1. Cardiovascular diseases

For its possible significance in the prevention and treatment of cardiovascular disorders, L-arginine has been the subject of substantial research. In patients with hypertension and other cardiovascular disorders, it has been demonstrated to increase endothelial function, lower blood pressure, and improve lipid profiles. L-arginine is a sole precursor of NO, which is essential for controlling blood flow and blood pressure. Vasodilation, which improves blood flow and lowers blood pressure, is facilitated by NO. Supplementing with L-arginine has been demonstrated to promote NO synthesis and enhance endothelial function, which can aid in the treatment and prevention of cardiovascular disorders. L-arginine has also been demonstrated to improve exercise tolerance and lessen angina and heart failure symptoms in patients. After angioplasty and stenting, L-arginine supplementation has also been found to lower the risk of restenosis. L-arginine use in acute myocardial infarction patients is debatable, nevertheless, as some individuals may experience more negative side-effects as a result.

2.4.2. Immune function

L-arginine has been investigated for its possible use in the treatment of a number of inflammatory and infectious illnesses. It also plays a role in immunological function. The growth and activation of T-cells, which are essential for the immune system's reaction to infections and tumors, depend on L-arginine. Additionally, it helps macrophages produce

NO, which is vital for the defence against pathogens. Patients with numerous infectious disorders, such as HIV, leprosy, and tuberculosis, have showed improved immune function when given L-arginine supplements. Additionally, it has been demonstrated to lessen the intensity of inflammation in people with sepsis and other inflammatory diseases.

2.4.3. Cancer

Cancer prevention and treatment have both been investigated as potential applications for L-arginine. It has been demonstrated to possess anti-tumor capabilities and to stop the growth of many cancer cell types, including breast, colon, and prostate cancer. By making cancer cells more susceptible to chemotherapy and radiotherapy, L-arginine may also improve their effectiveness. The effects of L-arginine on cancer, however, may vary depending on the type and stage of the disease as well as other variables like the accessibility of other amino acids and nutrients. According to certain research, high amounts of L-arginine may encourage the growth of specific cancer cells, especially those that lack the enzyme ASS, which is involved in L-arginine metabolism.

2.4.4. Diabetes

L-arginine has been demonstrated to enhance type 2 diabetic patients' insulin sensitivity and glucose metabolism. In addition, diabetic nephropathy, a common consequence of diabetes that can result in kidney failure, may be protected by L-arginine. Additionally, it has been demonstrated that diabetic patients' wound healing is enhanced by L-arginine supplementation. L-arginine may aid in promoting the formation of new blood vessels and enhancing the transport of nutrients and oxygen to the location of the lesion. Diabetes is linked to impaired wound healing.

2.5. Role of L-arginine in tumor biology

Numerous researches have recently examined the function that L-arginine may have in the initiation and spread of cancer. L-arginine may have anti-tumor properties, according to some studies, but it may also encourage the growth of tumors, according to other studies. The role of L-arginine in cancer will be discussed in this subtopic along with its potential as a therapeutic target for cancer treatment.

L-arginine controls the growth, survival, and immune evasion of cancer cells, which is essential for tumor biology. Particularly, numerous aspects of tumor growth and metastasis are influenced by L-arginine metabolism by the Arg and NOS enzymes. For instance, it has been demonstrated that the production of Arg by tumor cells aids in the growth of the tumor by inhibiting the immune system and encouraging angiogenesis. On the other hand, by increasing the synthesis of NO, which activates immune cells and slows tumor growth, NOS activity can support anti-tumor immunity.

The exact metabolic pathways and enzymes involved in L-arginine's metabolism may also have an impact on how it affects tumor growth and metastasis. For instance, cancer cells can synthesise L-arginine from citrulline and enhance tumor growth by boosting cell proliferation and inhibiting immune cells if they display high levels of ASS and ornithine

transcarbamylase (OTC). Contrarily, cancer cells lacking these enzymes rely on the microenvironment for the uptake of L-arginine and may be more vulnerable to L-arginine deprivation or Arg inhibition.

In numerous pre-clinical cancer models, L-arginine has been demonstrated to have anti-tumor properties. In animal models of melanoma, HCC, and lung cancer, for instance, L-arginine deprivation by Arg production or inhibition has been demonstrated to reduce tumor growth and spread. It is believed that a number of processes, including the suppression of angiogenesis, activation of apoptosis, and inhibition of cell proliferation, are responsible for the anti-tumor effects of L-arginine deprivation.

Lack of L-arginine can also improve the immune system's ability to fight tumors by boosting the number of effector T-cells and natural killer (NK) cells that infiltrate the tumor microenvironment. The suppression of regulatory T-cells and myeloid-derived suppressor cells (MDSCs), which can decrease anti-tumor immunity, is likely to be the cause of this. Depriving the body of L-arginine can also boost the expression of MHC class I molecules, which are crucial for cytotoxic T-lymphocytes to recognise tumor cells.

Lack of L-arginine can make cancer cells more sensitive to chemotherapeutic agents and radiation treatment. In animal models of breast cancer and ovarian cancer, for instance, it has been demonstrated that L-arginine deprivation increases the effectiveness of cisplatin and paclitaxel. The exact mechanism causing this effect is unknown, although it could be due to apoptosis being induced or DNA repair pathways being inhibited.

In addition to L-arginine deprivation, it has been demonstrated in numerous pre-clinical cancer models that inhibiting Arg activity or expression has anti-tumor effects. By raising L-arginine levels in the tumor microenvironment and boosting the immune response to tumors, Arg inhibition can lessen tumor growth and spread. By decreasing the expression of anti-apoptotic proteins and boosting the expression of pro-apoptotic proteins, arginine inhibition can also make cancer cells more susceptible to chemotherapy and radiation treatment.

2.6. Arginase and cancer

Recently, attention has been drawn to the possible therapeutic approach of arginine depletion employing Arg for the treatment of cancer. L-arginine, a crucial amino acid for tumor growth and survival, is being depleted using Arg in this method. Arg can prevent tumor cell proliferation and cell death while also increasing anti-tumor immunity by preventing the availability of L-arginine. L-arginine is hydrolyzed by the enzyme Arg into L-ornithine and urea. MDSCs, regulatory T-cells, and tumor-associated macrophages (TAMs) are a few of the cells in the tumor microenvironment that express it. By inhibiting the immune response and encouraging angiogenesis, these cells aid in the growth and spread of tumors. Arg is hypothesised to have immunosuppressive effects by depleting L-arginine, an amino acid that is crucial for the growth and operation of immune cells. Using Arg to reduce L-arginine levels is a promising therapeutic approach for the treatment of cancer. Arg can prevent tumor cell proliferation and cell death while also increasing anti-tumor immunity by preventing the availability of L-arginine.

2.6.1. Pre-clinical studies

The effectiveness of arginine depletion utilising Arg in several cancer models has been shown in pre-clinical investigations. Arg-based arginine depletion was demonstrated in a study by Rodriguez et al. to limit the growth of melanoma tumors in mice. This result was linked to the stimulation of anti-tumor immunity and the induction of apoptosis in tumor cells.

Similar to this, it has been demonstrated that arginine depletion using Arg can stop the growth of breast cancer tumors in mice by causing cell cycle arrest, death, and anti-tumor immunity in the tumor cells.

The anti-tumor activity of T-lymphocytes in a mouse model of pancreatic cancer was demonstrated to be enhanced by arginine depletion using Arg in a different study by Munoz-Espin et al. This effect was attributed to the restoration of T-cell function and the inhibition of MDSCs in the tumor microenvironment.

2.6.2. Clinical trials

The safety and effectiveness of arginine depletion utilising Arg for the treatment of cancer have been examined in a number of clinical trials. Different types of Ag, including Arg-expressing MDSCs and human recombinant Arg I (PEG-Arg), have been employed in these studies.

Patients with advanced HCC received PEG-Arg as part of a phase I/II clinical trial. The outcomes demonstrated that PEG-Arg had a good safety profile and was well tolerated. Additionally, PEG-Arg was demonstrated to cause arginine depletion and foster anti-tumor immunity in HCC patients.

Patients with metastatic melanoma received Arg-expressing MDSCs in a different phase I/II clinical trial. The outcomes demonstrated the safety and tolerability of MDSCs that express Arg. Arg-expressing MDSCs have also been demonstrated to boost anti-tumor immunity in patients and to cause arginine depletion.

Chapter 3

Materials & Methods

3.1. Screening for L-arginine depletion activity

Bacillus megaterium was grown on a medium with composition given in Table 3-1 (Nadaf et al., n.d.). *Lactobacillus helveticus* doesn't carry a gene that translates to Arg, so it was also grown as a positive control, along with many other bacterial isolates.

S no.	Ingredient	Composition
1	KCl	0.5%
2	$MgSO_4$	0.5%
3	KH_2PO_4	1.0%
4	$FeSO_4$	0.1%
5	$ZnSO_4$	0.1%
6	L-arginine	1.0%
7	Phenol red	2.0%
8	Agar	2.8%
9	Nutrient agar	1.0%

Table 3-1 Media composition for screening for L-arginine depletion activity

3.2. Purity check by Gram's staining technique

Bacillus megaterium is a Gram-positive bacterium (Bunk et al., 2010). Gram's staining procedure also confirmed the same (Figure 4-2).

3.2.1. Protocol for purity check by Gram's staining –

3.2.1.1. Requirements –

1. Crystal violet solution
2. Gram's iodine staining solution
3. Ethanol
4. Safranin

3.2.1.2. Procedure –

1. Created a smear by taking the sample and spread it on slide. Let it dry completely.
2. Stained the sample with crystal violet solution for 1 minute.
3. Washed the slide with water to remove excess stain.
4. Stained the sample with Gram's iodine staining solution for 1 minute.
5. Again washed the slide with water.
6. Washed the slide with few drops of ethanol.
7. Stained the sample with few drops of safranin for 30 seconds.
8. Washed again with water and observed the slide under microscope.

3.3. Genomic DNA isolation from *Bacillus megaterium*

It's more difficult to lyse Gram-positive cells than Gram-negative cells. The genomic DNA was isolated as per the following modified protocol.

3.3.1. Protocol for genomic DNA isolation –

3.3.1.1. Requirements –

1. Extraction buffer composition (SDS lysis buffer) for 1 l –
   (a) 1 M tris-Cl (pH 7.5) : 200 ml
   (b) 0.5 M EDTA (pH 8.0) : 50 ml
   (c) 10% SDS : 50 ml
   (d) 5 M NaCl : 50 ml
   (e) dH_2O : 650 ml
2. 5 mM potassium acetate solution
3. Phenol:chloroform:isoamylalcohol (25:24:1)
4. Chloroform:isoamylalcohol (24:1)
5. Chilled isopropanol
6. 70% ethanol
   Recipe for 70% ethanol –
   (a) Ethyl alcohol : 70 ml
   (b) dH_2O : 30 ml
7. TE (pH 8.0)
   10X TE (pH 8.0) recipe –
   (a) 100 mM tris-Cl (pH 8.0)
   (b) 10 mM EDTA (pH 8.0)
   Sterilize the solution by autoclaving for 20 minutes at 15 psi. Store at room temperature.

3.3.1.2. Procedure –

1. Bacterial isolates were grown in LB broth and inoculated at 37^{o}C for overnight with vigorous shaking.
2. Took 20 ml of culture in a centrifuge tube.
3. Centrifuged at 12000 RPM for 5 minutes at 4^{o}C. Repeated 3 times.
4. Discarded the supernatant and collected the pellet.
5. To the pellet, added 650 µl of SDS lysis buffer, followed by vortexing.
6. Kept the tube in water bath at 80^{o}C for 2 hours.
7. Added 100 µl of 5 mM potassium acetate solution.
8. Kept at ice for 30 minutes.
9. Centrifuged at 12000 RPM for 5 minutes at 4^{o}C.
10. Collected the supernatant and added equal volume of PCIA and mixed well.
11. Centrifuged at 12000 RPM for 5 minutes at 4^{o}C.
12. Collected the upper aqueous layer and added equal volume of CIA.
13. Mixed well and centrifuged at 12000 RPM for 5 minutes at 4^{o}C.
14. Collected the supernatant and added equal volume of chilled isopropanol through the walls of the tube and kept at -20^{o}C overnight.

15. Next day, centrifuged at 12000 RPM for 5 minutes at 4^{o}C.
    Pellet of the DNA that remained attached to the wall of the tube was found.
16. Washed the tube with 100 µl of 70% ethanol.
17. Centrifuged at 12000 RPM for 2 minutes at 4^{o}C.
18. Discarded the supernatant and pellet was dissolved in 40 µl of TE buffer and stored at 4^{o}C.

3.4. Isolation of plasmid DNA from DH5-α competent cells containing pET-28(a)+

3.4.1. Protocol for plasmid DNA isolation –

3.4.1.1. Requirements –

1. Alkaline lysis solution I –
   (a) 50 mM glucose
   (b) 25 mM tris-Cl (pH 8.0)
   (c) 10 mM EDTA (pH 8.0)

   Prepare solution I from standard stocks in batches of ~100 ml, autoclave for 20 minutes at 15 psi and store at 4^oC.

2. Alkaline lysis solution II –
   (a) 0.2 N NaOH (freshly diluted from 10 N stock solution)
   (b) 1% (w/v) SDS

   Prepare solution II fresh and use at room temperature.

3. Alkaline lysis solution III –
   (a) 5 M potassium acetate : 60 ml
   (b) Glacial acetic acid : 11.5 ml
   (c) dH_2O : 28.5 ml

   Store solution at 4^oC and transfer it to an ice-bucket just before use.

4. Kanamycin (working $conc^n$ 50 µg/ml)
5. 70% ethanol
6. Phenol:chloroform (1:1)
7. STE –
   (a) 10 mM tris-Cl (pH 8.0)
   (b) 0.1 M NaCl
   (c) 1 mM EDTA (pH 8.0)

   Sterilize the solution by autoclaving for 20 minutes at 15 psi. Store at 4^oC.

8. TE (pH 8.0) containing 20 µg/ml RNase A
9. Isopropanol

3.4.1.2. Procedure –

1. Inoculated 10 ml of LB broth containing kanamycin with a single colony of bacteria. Incubated the culture overnight at 37^oC with vigorous shaking.
2. Poured culture into a 15 ml centrifuge tube. Centrifuged at 4000 RPM for 10 minutes at 4^oC.
3. Removed the supernatant, leaving the bacterial pellet as dry as possible.
4. Resuspended the bacterial pellet in 2.5 ml of ice-cold STE and centrifuged again at 4000 RPM for 10 minutes at 4^oC. Removed the supernatant, leaving the bacterial pellet as dry as possible.

*Volume of STE added = 0.25*Starting culture volume. In this case, starting culture volume was 10 ml, so amount of STE added was 0.25*10 ml, which is 2.5 ml.*

5. Resuspended the bacterial pellet in 200 µl of ice-cold alkaline lysis solution I by vigorous vortexing, and transferred to micro-centrifuge tube (MCT).
6. Added 400 µl of freshly prepared alkaline lysis solution II to bacterial suspension. Closed the tube tightly, and mixed the contents by inverting the tube rapidly five times. *Do not vortex!* Stored the tube on ice.
7. Added 300 µl of ice-cold alkaline lysis solution III. Closed the tube and dispersed alkaline lysis solution III through viscous bacterial lysate by inverting the tube several times. Stored the tube on ice for 3-5 minutes.
8. Centrifuged at maximum speed for 5 minutes at 4^{o}C. Transferred 600 µl of the supernatant to a fresh tube.
9. Added equal volume of phenol:chloroform. Mixed organic and aqueous phases by vortexing and then centrifuged at maximum speed for 2 minutes at 4^{o}C. Transferred upper aqueous layer to a fresh tube.
10. Precipitated nucleic acids from supernatant by adding 600 µl of isopropanol at room temperature. Mixed solution by vortexing and then allowed the mixture to stand for 2 minutes at room temperature.
11. Collected precipitated nucleic acids by centrifugation at maximum speed for 5 minutes at room temperature.
12. Removed the supernatant and allowed all the fluid to drain away.
13. Added 1 ml of 70% ethanol to pellet. Recovered the DNA by centrifugation at maximum speed for 2 minutes at room temperature.
14. Again removed all of the supernatant.
15. Stored open tube at room temperature until the ethanol was evaporated and no fluid was visible.
16. Dissolved nucleic acids in 100 µl of TE (pH 8.0). Stored at -20^{o}C.

3.5. Manual primer designing

Two genes present in *Bacillus megaterium* translate to L-arginase. Of them, gene of following characteristics was chosen for further study.

Gene : rocF
Locus tag : BG04_2464

Gene sequence was found using National Center of Biotechnology Information (NCBI) database. Gene comprised of 897 nucleotides.

ATGAAAAAAGACATTTCAATTATCGGTGTACCCATGGATTACGGACAAACAAGA
AGGGGTGTAGACATGGGGCCGAGCGCGATCCGCTATGCAGGCATGAATACACGC
TTAGAAGCTCTTGGCTATACCGTACACGATGAAGGCGATATTAAAGTAGAAATTA
AAGAGCGGGCAGACGTAGATAAAAATACGAATTTGAAAAACTTAGCGGCTGTAG
CCAGCGGAAATGAACAGCTTGCTGCACGAGTTGAAGAAGTGAGAGAGCAAGAC
CGCTTTCCGTTAATATTAGGCGGAGACCACAGCATTGCGATTGGAACACTTGCGG
GAGTCGCAAAAGGTTCAGAAAATTTAGGCGTTATTTGGTATGATGCTCATGGTGA
TTTAAACACAGCTGAAACATCTCCATCAGGCAACATTCACGGCATGCCATTAGCG
GTGAGCCTTGGTATTGGACATCCTGTACTTTTAAATATTGGAGGGTATACCCCAA
AAATTAAACCTGAAAATCTCGTTATTATCGGTGCACGCTCATTGGATGATGGTGA
AAAAGAGTTAATTAAAGAAAAAGGAATTAAAGTGTATACCATGCATGAAATTGA
TCGTTTAGGTATGACGCAAGTGATGAAAGAAACGATTGATTATTTAAGCGGCAC
AGACGGCGTGCACCTGTCGCTGGACTTAGACGGCTTAGATCCGGATGATGCACC
AGGCGTAGGTACACCTGTAAAAGGCGGCATCAGCTACCGAGAAAGTCATTTAGC
AATGGAAATGCTAGCGGAAGCAGACATAGTTACATCTGCTGAATTTGTAGAGGT
GAACCCCATTTTAGATCAGCATAACAAAACTGCAGAAGTAGCGGTTGCGTTAAT
GAGTTCATTGTTTGGTGATAAGCTTTTA

Using NEBcutter V2.0, following enzymes were shown to not be cutting gene of interest (GoI) at any point.

#	Enzyme	Specificity
1	AatII	G▴ACGT▾C
2	Acc65I	G▾GTAC▴C
3	AclI	AA▾CG▴TT
4	AcuI	CTGAAG(N)$_{14}$▴NN▾
5	AfeI	AGC▾GCT
6	AflII	C▾TTAA▴G
7	AflIII	A▾CRYG▴T
8	AgeI	A▾CCGG▴T
9	AhdI	GACNN▴N▾NNGTC
10	AleI	CACNN▾NNGTG
11	AlwNI	CAG▴NNN▾CTG
12	ApaI	G▴GGCC▾C
13	AscI	GG▾CGCG▴CC
14	AseI	AT▾TA▴AT
15	AsiSI	GCG▴AT▾CGC
16	AvaI	C▾YCGR▴G
17	AvaII	G▾GWC▴C
18	AvrII	C▾CTAG▴G
19	BamHI	G▾GATC▴C
20	BanI	G▾GYRC▴C
21	BanII	G▴RGCY▾C
22	BbsI	GAAGACNN▾NNNN▴
23	BbvCI	CC▾TCA▴GC
24	BcgI	▴NN▾(N)$_{10}$CGA(N)$_6$TGC(N)$_{10}$▴NN▾
25	BciVI	GTATCC(N)$_5$▴N▾
26	BclI	T▾GATC▴A
27	BfuAI	ACCTGCNNNN▾NNNN▴
28	BglI	GCCN▴NNN▾NGGC
29	BglII	A▾GATC▴T
30	BlpI	GC▾TNA▴GC
31	BmgBI	CAC▾GTC
32	BmrI	ACTGGGNNNN▴N▾
33	BpmI	CTGGAG(N)$_{14}$▴NN▾
34	Bpu10I	CC▾TNA▴GC
35	BpuEI	CTTGAG(N)$_{14}$▴NN▾
36	BsaAI	YAC▾GTR
37	BsaBI	GATNN▾NNATC
38	BsaHI	GR▾CG▴YC
39	BsaXI	▴NNN▾(N)$_9$AC(N)$_5$CTCC(N)$_7$▴NNN▾
40	BseRI	GAGGAG(N)$_8$▴NN▾
41	BseYI	C▾CAG▴C
42	BsiEI	CG▴RY▾CG
43	BsiWI	C▾GTAC▴G
44	BsmBI	CGTCTCN▾NNNN▴
45	BsmFI	GGGAC(N)$_{10}$▾NNNN▴
46	BsmI	GAATG▴CN▾
47	BsoBI	C▾YCGR▴G
48	BspCNI	CTCAG(N)$_7$▴NN▾
49	BspDI	AT▾CG▴AT
50	BspHI	T▾CATG▴A
51	BspMI	ACCTGCNNNN▾NNNN▴
52	BspQI	GCTCTTCN▾NNN▴
53	BsrFI	R▾CCGG▴Y
54	BsrGI	T▾GTAC▴A
55	BsrI	ACTG▴GN▾
56	BssHII	G▾CGCG▴C
57	BstAPI	GCAN▴NNN▾NTGC

58	BstBI	TT^CG_AA
59	BstEII	G^GTNAC_C
60	BstXI	CCAN_NNNN^NTGG
61	Bsu36I	CC^TNA_GG
62	BtgZI	GCGATG$(N)_{10}$^NNNN_
63	BtsI	GCAGTG_NN^
64	BtsIMutI	CAGTG_NN^
65	ClaI	AT^CG_AT
66	CspCI	_NN^$(N)_{11}$CAA$(N)_5$GTGG$(N)_{10}$_NN^
67	DraIII	CAC_NNN^GTG
68	DrdI	GACNN_NN^NNGTC
69	EaeI	Y^GGCC_R
70	EagI	C^GGCC_G
71	EarI	CTCTTCN^NNN_
72	Eco53kI	GAG^CTC
73	EcoO109I	RG^GNC_CY
74	EcoRI	G^AATT_C
75	EcoRV	GAT^ATC
76	Esp3I	CGTCTCN^NNNN_
77	FseI	GG_CCGG^CC
78	FspI	TGC^GCA
79	HaeII	R_GCGC^Y
80	HincII	GTY^RAC
81	HpaI	GTT^AAC
82	Hpy99I	_CGWCG^
83	KasI	G^GCGC_C
84	KpnI	G_GTAC^C
85	MfeI	C^AATT_G
86	MluI	A^CGCG_T

87	MmeI	TCCRAC$(N)_{18}$_NN^
88	MscI	TGG^CCA
89	MslI	CAYNN^NNRTG
90	NaeI	GCC^GGC
91	NarI	GG^CG_CC
92	NciI	CC^S_GG
93	NdeI	CA^TA_TG
94	NgoMIV	G^CCGG_C
95	NotI	GC^GGCC_GC
96	NruI	TCG^CGA
97	PaeR7I	C^TCGA_G
98	PaqCI	CACCTGCNNNN^NNNN_
99	PciI	A^CATG_T
100	PflFI	GACN^N_NGTC
101	PflMI	CCAN_NNN^NTGG
102	PluTI	G_GCGC^C
103	PmeI	GTTT^AAAC
104	PmlI	CAC^GTG
105	PpuMI	RG^GWC_CY
106	PshAI	GACNN^NNGTC
107	PsiI	TTA^TAA
108	PspOMI	G^GGCC_C
109	PspXI	VC^TCGA_GB
110	PvuI	CG_AT^CG
111	RsrII	CG^GWC_CG
112	SacI	G_AGCT^C
113	SacII	CC_GC^GG
114	SalI	G^TCGA_C
115	SapI	GCTCTTCN^NNN_

116	SbfI	CC_TGCA^GG
117	ScaI	AGT^ACT
118	SexAI	A^CCWGG_T
119	SfiI	GGCCN_NNN^NGGCC
120	SfoI	GGC^GCC
121	SgrAI	CR^CCGG_YG
122	SmaI	CCC^GGG
123	SmlI	C^TYRA_G
124	SnaBI	TAC^GTA
125	SpeI	A^CTAG_T
126	SrfI	GCCC^GGGC
127	StuI	AGG^CCT
128	SwaI	ATTT^AAAT
129	TaqI	T^CG_A
130	TfiI	G^AWT_C
131	Tsp45I	^GTSAC_
132	TspMI	C^CCGG_G
133	TspRI	_NNCASTGNN^
134	Tth111I	GACN^N_NGTC
135	XbaI	T^CTAG_A
136	XcmI	CCANNNN_N^NNNNTGG
137	XhoI	C^TCGA_G
138	XmaI	C^CCGG_G
139	XmnI	GAANN^NNTTC
140	ZraI	GAC^GTC

Table 3-2 Tables showing list of enzymes that don't cut GoI at any point (0 cutters)

Plasmid pET-28a(+) was chosen as suitable vector for carrying out transformation. Of the restriction enzymes that cut pET-28(a) plasmid but not GoI, NdeI and BamHI were chosen for forward and backward primer respectively.

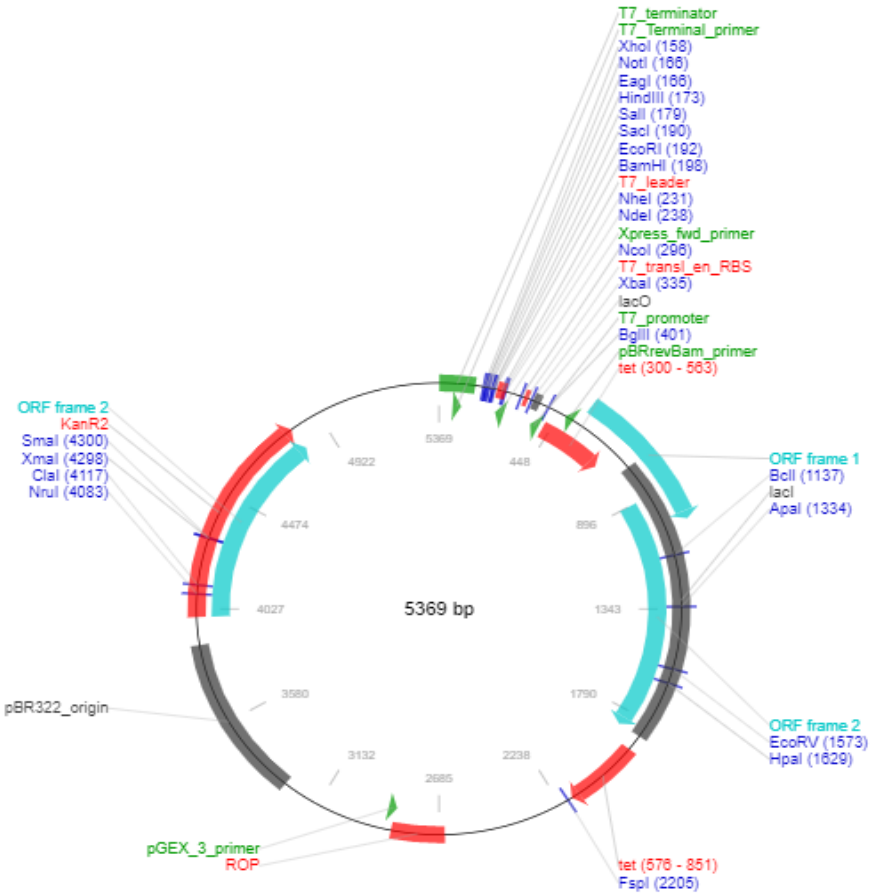

Figure 3-1 pET-28a(+) plasmid map

3.6. Polymerase chain reaction

To amplify the GoI, polymerase chain reaction (PCR) was carried out for two reaction volume of 20 µl, whose composition is given in the table 3-3.

Table 3-3 Composition for 20 µl reaction volume for PCR

S no.	Reagent	For 20 µl*6
1.	Nuclease free water (NFW)	40 µl
2.	Forward primer	1 µl
3.	Reverse primer	1 µl
4.	Buffer	12 µl
5.	Master mix	60 µl
6.	Template DNA	5 µl
7.	DNA polymerase	1 µl

Ran temperature gradient PCR for six temperatures 52^{o}C, 55^{o}C, 57^{o}C, 59^{o}C, 61^{o}C and 64^{o}C, under the following conditions.

Table 3-4 Reaction conditions for PCR

S no.	Phase of PCR	Temperature	Duration	Cycles
1.	Initial denaturation	94^{o}C	1:30 min	
2.	Denaturation	94 ^{o}C	45 s	X40
3.	Annealing (subject to temperature gradient)	52 ^{o}C-64 ^{o}C	45 s	
4.	Extension	72^{o}C	50 s	
5.	Final extension	72^{o}C	7 min	
		4^{o}C	∞	

3.7. Re-ordered culture of *Bacillus megaterium* from MTCC, revived the culture and isolated genomic DNA

Despite repeated attempts of obtaining PCR products, after following various troubleshooting guides, we were unable to amplify the GoI. Once searching through the MTCC website, we got to know that the culture that we were using was ordered from MTCC before the year 2020, and MTCC renamed the same culture as *Bacillus wiedmannii* in the year 2020 after whole genome sequencing results. Upon discussion with the project supervisor, Dr. Saurabh Bansal sir, we put forward an application to the Head of the Department of Biotechnology & Bioinformatics Prof. Dr. Sudhir Syal sir to please kindly provide us with the new culture. Our request was accepted, and the order was placed. We received our culture in the month of May 2023. Owing to the shortage of time, we were only able to successfully revive the culture and isolate genomic DNA.

Chapter 4

Results

4.1. Screening for L-arginine depletion activity

Results were visible after 24 hours of incubation at 37^{o}C (Figure 4-1). Medium turned black for *Bacillus megaterium* and *Lactobacillus rhamnosus* as they both contain gene/genes that translate to Arg, so Arg converted L-arginine present in the medium to urea, in accordance with urea cycle discussed above. Remaining bacterial isolates, i.e. *Lactobacillus helveticus*, *Chromobacterium violaceum*, *Bacillus subtilis* and *Lactobacillus plantarum* couldn't turn the medium black as they all lack a gene that can translate to Arg. *Bacillus megaterium* was chosen for further study.

Figure 4-1 Screening for L-arginine depletion activity. Medium turned black for *Bacillus megaterium* and *Lactobacillus rhamnosus* as they both contain gene/genes that translate to Arg, so Arg converted L-arginine present in the medium to urea, in accordance with urea cycle discussed above. Remaining bacterial isolates, i.e. *Lactobacillus helveticus*, *Chromobacterium violaceum*, *Bacillus subtilis* and *Lactobacillus plantarum* couldn't turn the medium black as they all lack a gene that can translate to Arg

4.2. Purity check by Gram's staining technique

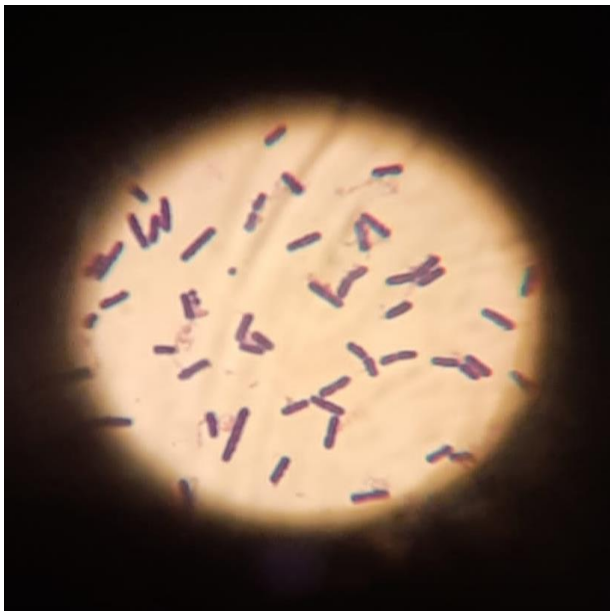

Figure 4-2 Gram stain of *Bacillus megaterium*

4.2.1. Inferences –

1. *Bacillus megaterium* is a Gram-positive bacterium.
2. It is a rod-shaped bacterium.

4.3. Genomic DNA isolation from *Bacillus megaterium*

4.3.1. DNA quantification –

Absorbance of DNA was measured at 260 nm and 280 nm using NanoDrop spectrophotometer (Table 4-1) (Desjardins and Conklin, 2010).

Table 4-1 Absorbance of genomic DNA at 260 nm and 280 nm

Parameter	Sample 1	Sample 2
Absorbance at 260 nm	0.188	0.177
Absorbance at 280 nm	0.140	0.126
A_{260}/A_{280}	1.34	1.40
DNA concentration (A_{260}*50 µg/ml)	9.4	8.85

4.3.2. Gel electrophoresis –

DNA was visualized in 0.8% agarose gel (Figure 4-3) (Lee et al., 2012)

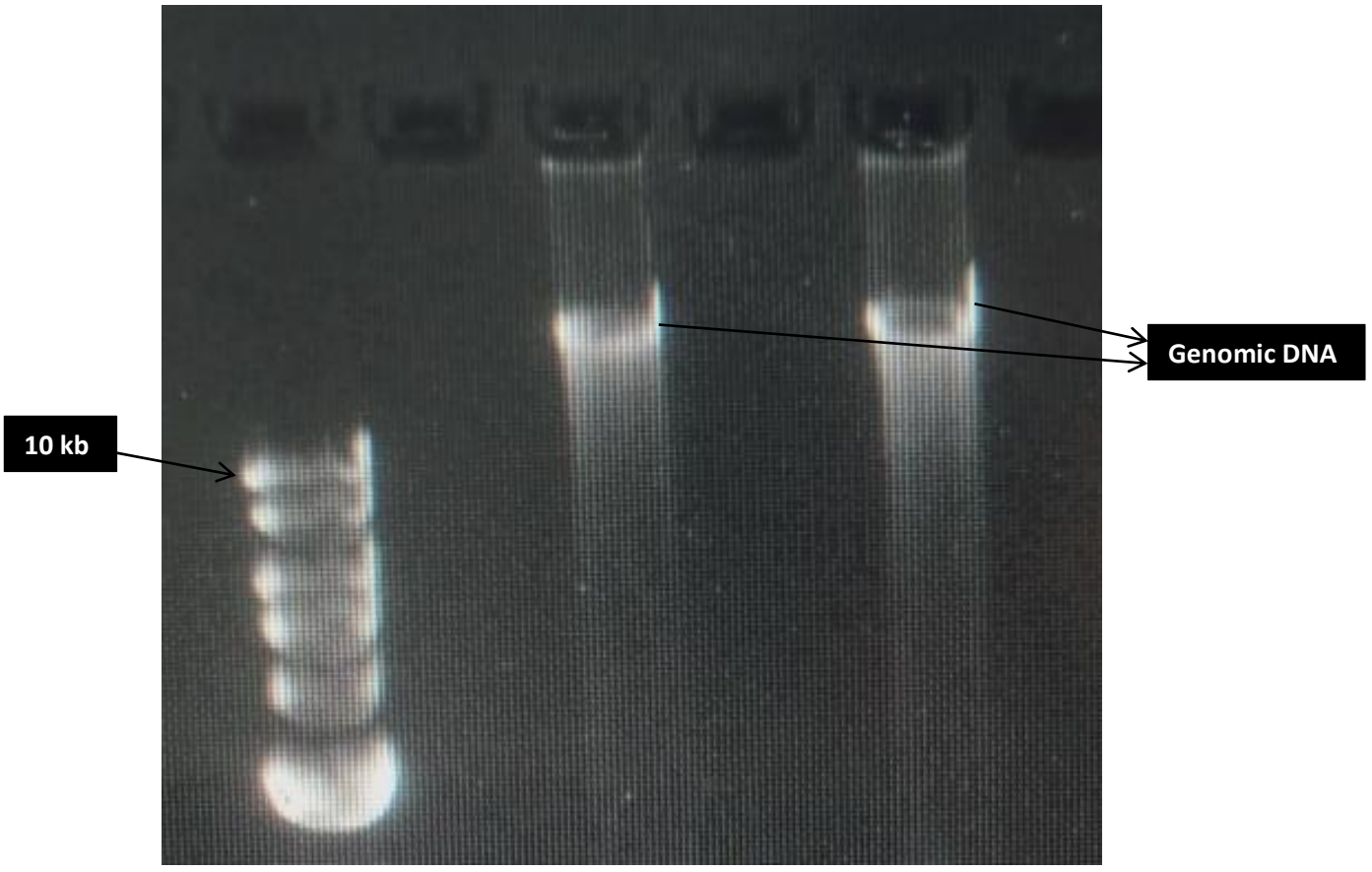

Figure 4-3 Visualization of genomic DNA in 0.8% agarose gel

4.3.3. Inferences –

1. DNA is contaminated with protein, as indicated by value of A_{260}/A_{280} (Sik Kim et al., 2005).
2. Size of genomic DNA of *Bacillus megaterium* is much greater than 10 kb.

4.4. Isolation of plasmid DNA from DH5-α competent cells containing pET-28(a)+

4.4.1. DNA quantification –

Absorbance of DNA was measured at 260 nm and 280 nm using NanoDrop spectrophotometer (Table 4-2).

Table 4-2 Absorbance of plasmid DNA at 260 nm and 280 nm

Parameter	Sample 1	Sample 2
Absorbance at 260 nm	1.872	1.665
Absorbance at 280 nm	0.913	0.762
A_{260}/A_{280}	2.050	2.185
DNA concentration (A_{260}*50 µg/ml)	93.60	83.25

4.4.2. Gel electrophoresis –

DNA was visualized in 1% agarose gel (Figure 4-4) (Lee et al., 2012)

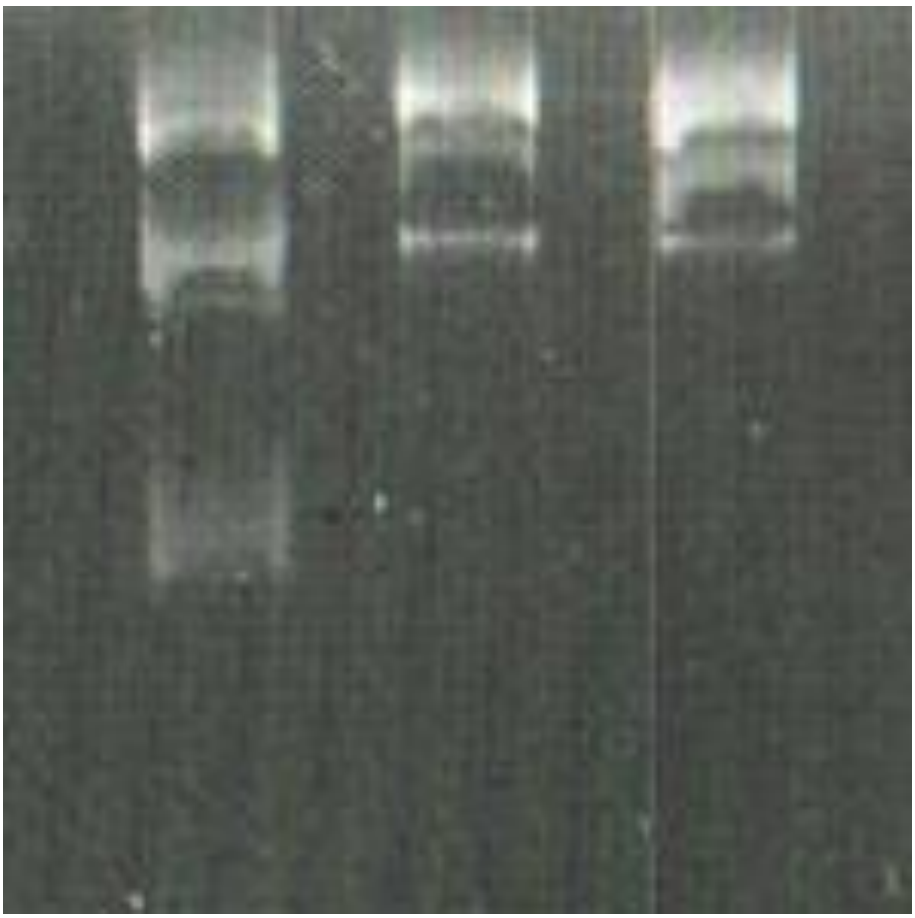

Figure 4-4 Visualization of plasmid DNA in 1.2% agarose gel

4.5. Manual primer designing

Forward primer : 5’ ACACATATGAAAAAAGACATTTCAATTATC 3’
Reverse primer : 5’ ACAGGATCCTAAAAGCTTATCACCAAACAATG 3’

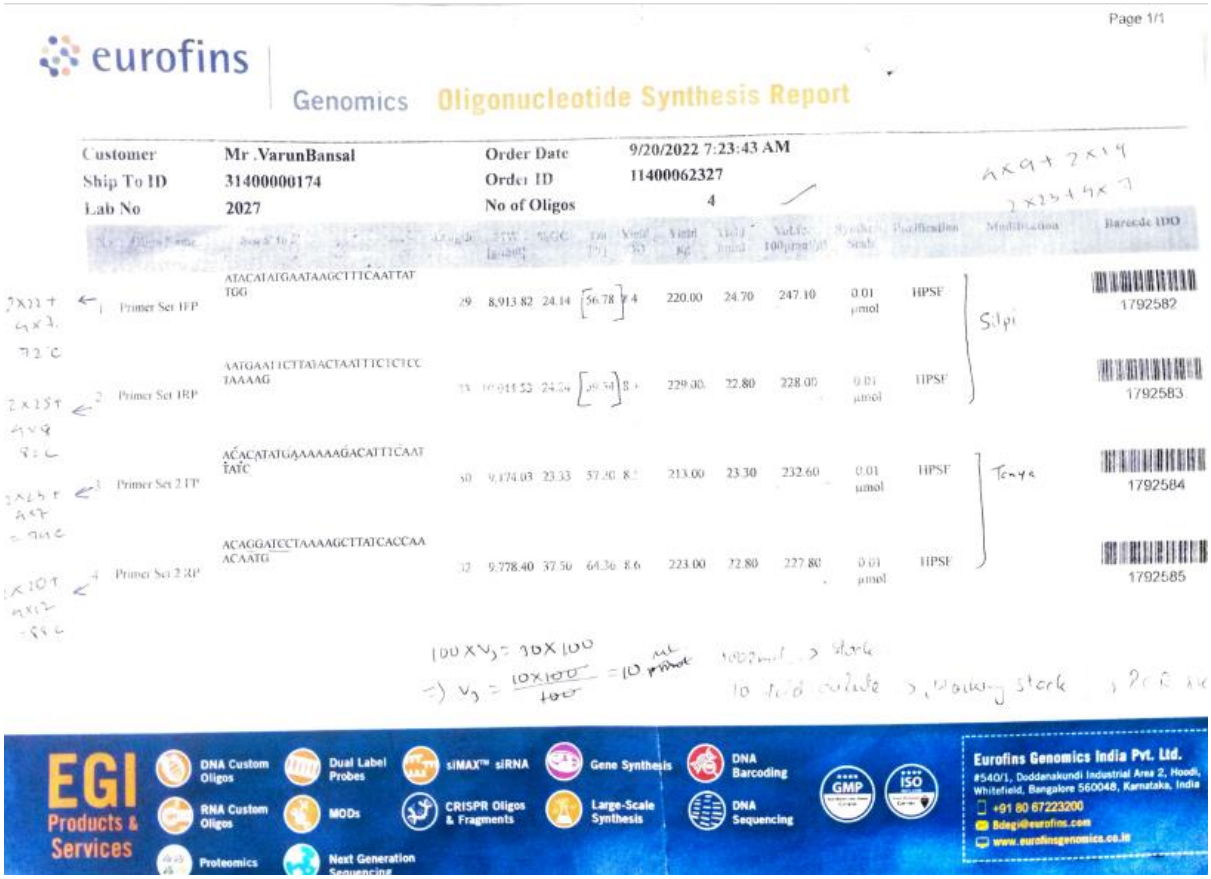

Figure 4-5 Report of primers ordered. Primer set 2 FP and primer set 2 RP are forward and reverse primers respectively for amplification of GoI

4.6. Polymerase chain reaction

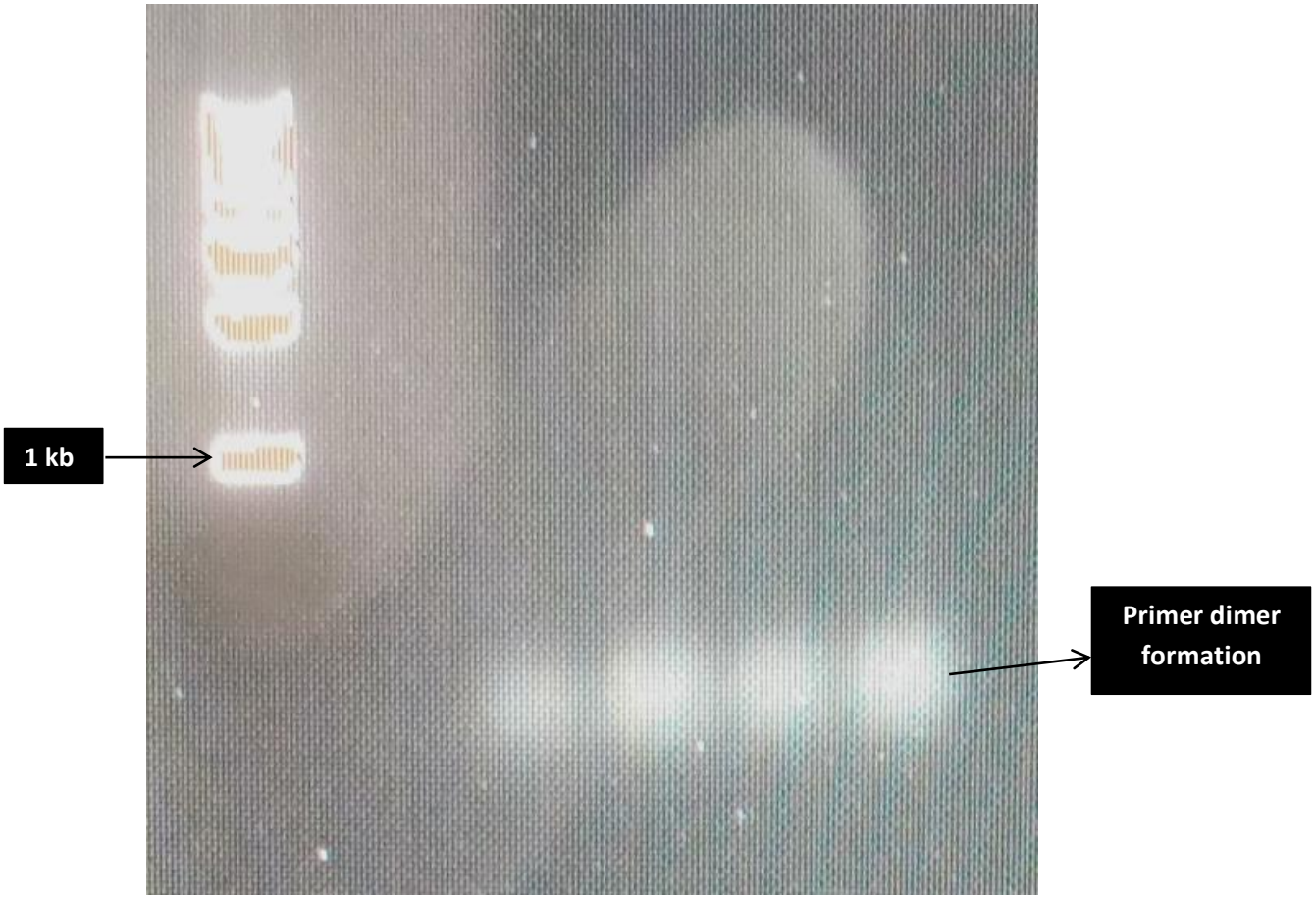

Figure 4-6 Visualization of PCR products in 1.2% agarose gel

4.7. Re-ordered culture of *Bacillus megaterium* from MTCC, revived the culture and isolated genomic DNA

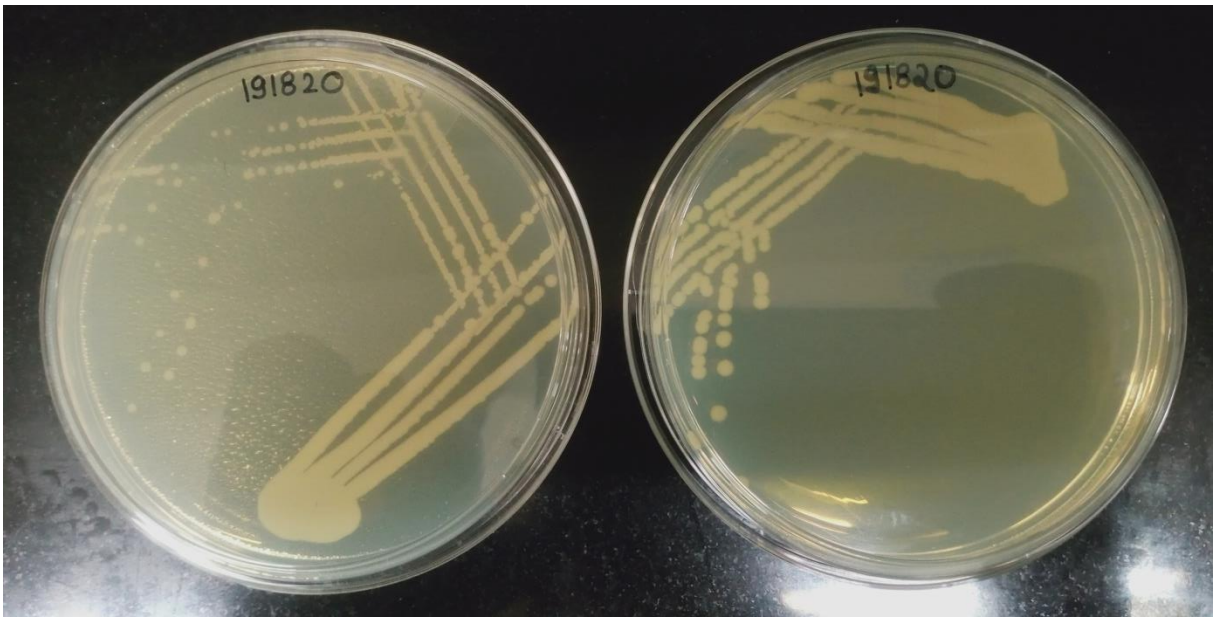

Figure 4-7 Revival of new culture of *Bacillus megaterium*

We were also able to successfully isolate the genomic DNA from this new culture, but don't have its photo to be presented here.

Chapter 5

Conclusions and future work

Being an undergraduate student, having less than a year to work on this project, I wasn't able to amplify Arg producing gene from *Bacillus megaterium*, and so was unable to proceed towards cloning. Also, with more than half of my Bachelors programme spent online due to COVID-19 pandemic, before the beginning of the project, I didn't know most of the skills that were utmost necessary to carry out this project work. Hence, during the duration of the project, most of my time was also spent in learning those techniques, and project work didn't proceed at the pace it should have. Also, to perform well in national-level examinations to have a secure future, my determination got diverted at times. But this project proved to be life-changing for me, in terms of my vision and the way that I now look towards the field of research.

We all know that the field of biotechnology is blooming, but when we compare the situation of biotechnology in India with the developed countries like United States, we stumble thinking about our future if we cannot move to foreign country for higher education. There's family to look after, who always looked after us. When we pursue any research-oriented programme from a private institution, our exposure to lab facilities is limited, and that's a fact. But if you are reading this, and you are really interested in the field of biotechnology and research, but sometimes feel discouraged listening to people or after reading on Quora about this field, its situation in India, then mark my words, "Every field in every country is challenging. I often hear people saying that computer science graduates are the luckiest ones because they get good placements right after their graduation, or even earlier. Well, that's simply a scenario of opportunities. If there are more opportunities in a particular field, then there will also be more number of people pursuing that field, hence more the competition. Similarly, if there are fewer opportunities in a particular field, then there will also be lesser number of people pursuing that field, but the competition will be equivalent because of lesser opportunities. So, either way, you have to work hard and smart. Running away because of competition will put you in an infinite loop that will never stop. Take a deep breath, and think what you really want to do, or where do you want to see yourself in coming decade, and then just don't stop. Don't let this race stop you, let yourself finish that race first".

Though the resources that I had for my major project were limited, to give a simple example, all the centrally-funded research oriented institutions order pre-formulated kits for DNA isolation, but due to less resources here, I had to make all the reagents first, then optimize them for pH, and then isolate DNA. But is that a big reason to disown this field. Hell no. Though the time it took here is considerably greater than time it would have taken there, but I know all the reagents and their usage in DNA isolation. So I can optimize my protocol whenever I feel so, because I have basic practical knowledge. Through this, I simply want to convey that don't let resources stop you. Sir A.P.J. Abdul Kalam went on to became what he became by studying newspapers that he used to sell. Had he stopped himself saying I don't have books, we would have never got to know such a great personality, whom we idolize.

Make most out of the resources that you have at present, as someone in the world is surely praying to have them.

Having said that, whosoever is going to work on this project, I whole-heartedly wish you the very best and that you take this project to completion. All the equipment that I have collected during the course is available in the lab, and you are absolutely free to use them. They are all yours. I hope that you see the potential that this project has. In case you need me, I am just a call away.

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