CLONING AND EXPRESSION OF ARGINASE IN *E. coli*

Thesis submitted in the partial fulfilment of the requirements for the degree of

Masters of Science In Biotechnology

By

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Under the supervision of

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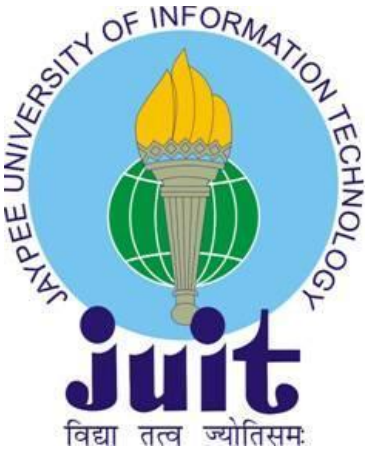
to



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SELF- DECLARATION

I hereby declare that the work reported in the M. Sc. Dissertation thesis entitled **"Cloning and Expression of Arginase in *E.coli*"** submitted to Jaypee University of Information Technology, Waknaghat, is an authentic record of my work carried out under the supervision of Dr. Saurabh Bansal (Associate professor) At *Dept of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat.*

I have not submitted this work elsewhere for any other degree or diploma.

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JUIT, Waknaghat, H.P, INDIA

CERTIFICATE

This is to certify that the work reported in the M. Sc. Dissertation thesis entitled **"Cloning and Expression of arginase in *E.coli*"** submitted by Silpi Sikha Borah (217812) at Jaypee University of Information Technology, Waknaghat is a bonafide record of her original work and has not been submitted elsewhere for any other degree or diploma programme.

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ACKNOWLEDGEMENT

I take this opportunity to express my gratitude to numerous people who has supported me and encouraged me to carry on my work efficiently.

First and foremost, I would like to express my heartfelt gratitude and appreciation to my project supervisor, **Dr. Saurabh Bansal** for his guidance, assistance and supervision throughout this journey. His expertise and knowledge contributed a major part in making this project a success. I thank him for giving insightful advices and suggestions and also for continuous source of inspiration.

I would also like to take this opportunity to thank **Dr. Sudhir Kumar Syal**, Head of the department of Biotechnology and Bioinformatics, Jaypee University of Information Technology for his knowledge and support in making this project and also for allowing me to work on such a diverse project.

I am deeply indebted to Lab Technicians **Sh. Baleshwar Prasad** and **Ms. Mamta Mishra** for their love and support, which has been a source of motivation throughout the process. Also, I am grateful and would like to take this opportunity to thank my classmates and PhD Scholars of the department who have supported and encouraged me during this journey.

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LIST OF ABBREVIATIONS

ASS	Arginosuccinate Synthase
ASL	Arginosuccinate Lyase
OCT	Ornithine Transcarbomyl Transferase
ADC	Arginine decarboxylase
ADI	Arginine deiminase
OAT	Ornithine aminotransferase
ODC	Ornithine decarboxylase

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ABSTRACT

As per the statistics published in 2022 by the American Cancer Society, cancer is the second leading cause of death worldwide and although there are a number of treatment methods developed, they have several side effects like heart problems, dysphagia, chronic bladder spams associated with it. Also, these techniques to treat cancers are very expensive, so with the increasing research in the field of cancer a new treatment method was identified that is depletion of semi essential amino acids that are needed by cancerous cells. There were several cancer cells that were identified to have an important feature of arginine auxotrophy, for which those cancer cell lines were dependent on the extracellular nutritional pool to fulfil their nutrient demand and with the increasing research it was found that enzymatic degradation of arginine is one of the most efficient approaches to deprive arginine. Among the enzymes involved in arginine degradation arginase was one of the enzymes which is a metalloenzyme belonging to the ureohydrolase class was found to have several advantages over others. This, study was carried out to isolate bacterial arginase from *Bacillus megaterium* (strain ATCC 14581) and clone in pET28a vector. The enzyme activity and protein estimation were also studied using enzyme assay and protein estimation protocols to characterise the arginase. The arginase of *Bacillus megaterium* is an extracellular enzyme and shows maximum activity in the presence of metal ion manganese, 3.84U/mL. The estimated protein and specific activity of the enzyme was calculated to be 1.089 mg/mL and 3.52 U/mg.

Chapter 1–

INTRODUCTION

Chapter 1 – INTRODUCTION

1.1 Introduction - According to statistics of 2022 that is published by the American Cancer Society, cancer which is caused because of the defect in cellular processes which ultimately leads to uncontrolled cell growth and defect in normal cellular function is the second leading cause of death worldwide [1,2]. It is seen in the reports of 2018 that there is a large incidence of cancer cases to around 18.1 million globally which is expected to be around 25million till 2040 [3]. There are a number of researches that took place from late 1700s which led to the increase understanding of cancer, this ultimately led to the development of several treatment methods which includes chemotherapy, radiotherapy, immunotherapy, hormone therapy, etc. But although these treatments have proved effective in treating many cancer patients but there were several side-effects that have been seen to be associated with it. It is reported that approximately 90% of cancer patients who have been treated by surgeries experience severe pain and patients who have received radiotherapy and chemotherapy faces adverse side-effects like heart problems, chronic bladder spams, diarrhoea, flu-like symptoms, dysphagia, inflammation of the spinal cord and neurological damage [1]. Also, there are several cases that have been reported which showed evidence of cancer cells developing resistance towards these conventional treatments [1]. So, these limitations led to the need for development of new cancer treatments which are more-effective and have less side-effects.

One of the promising alternatives that was identified in the field of cancer treatment was enzymatic treatment and the type of enzymatic treatment that have been famous is the depletion of semi essential amino acids that are needed by the cancerous cells to survive like asparagine, arginine, methionine and glutamine [7]. This idea of depletion of semi essential amino acids dates back to 1930s, when Gilroy in his pioneering work showed that mice when receives a diet that is enriched in arginine develops tumours faster and larger than the mice which are supplied with standard diet [8]. These method of depletion of semi essential amino acids were considered because there were many differences observed between normal and malignant cells one of the significant differences was their differences in the metabolism [9]. Like, in case of normal cells catabolic metabolism fulfils the bio-energetic requirements for homeostasis but in tumor cells these metabolic pathways are altered for which to maintain the balance between elevated biosynthesis of macromolecules and ATP levels they need to consume additional nutrients [9]. As their nutrition demand increases because of their rapid growth the endogenous supply of nutrient becomes inadequate to fulfil their demand and they ultimately depend on the

exogenous nutrient that are present in the microenvironment [9]. This particular characteristic of tumor cells which was identified was then used as a key point in developing this new treatment for cancer.

The amino acid which has been selected to work upon is L-arginine because it has been reported by studying several human malignancies like pancreatic, breast, prostate, lung etc. that cancer cells have an important feature of arginine auxotrophy, i.e., these cancer cells were auxotrophic to enzymes involved in Arginine synthesis (ASS, ASL, and OCT), it has opened a new gateway in the field of research [11,2]. Also arginine auxotrophic cancer cells were found to have defect in cellular mechanism for which they were dependent on the extracellular nutritional pool to fulfil their nutrient demand [2]. Arginine is an essential amino acid that is primarily synthesised via urea cycle in the proximal renal tubule and from citrulline [10,11]. It is a basic amino acid because of the complex guanidinium group in the end of its side chain which remains positively charged in basic, neutral and most acidic environments [11]. It has many important functions like stimulating release of growth hormones, expression of T cell receptors, improving wound healing, killer T cells activation, dispose of ammonia and is also important in synthesis of NO [11, 12]. There are three different approaches that have been identified for arginine deprivation therapy: Arginine restricted diet, enzymatic degradation of arginine and inhibition of arginine sensing and transport [11]. Among all the three, the enzymatic degradation is currently considered as the most effective method [11]. Enzymatic degradation of Arginine involves three major enzymes, they are Arginine deiminase (ADI), Arginine decarboxylase (ADC), and Arginase [9]. The study of arginase began after arginase was purified from ox liver by precipitation and it was seen that the arginase that was purified was able to decrease 77% tumor growth [2].

Arginase also known as Arg amidohydrolase (EC 3.5.3.1) which belongs to the ureohydrolase class of enzymes is a metalloenzyme as it uses manganese to catalyse the conversion of ornithine and urea [17,2]. Arginase has been studied to have two functions which involves detoxification of ammonia and production of ornithine which is used in the polyamines and proline synthesis that play an essential role in growth, neuronal development, inflammation, tissue repair, wound healing and cell proliferation [17]. rhArg has been developed and widely studied on different cancer cell lines like prostrate, HCC, breast, etc and after proving effective it is in phase I clinical trial but the bacterial arginase from *B. megaterium* is still unexplored, so this project focusses on the cloning and expression of arginase from *B. megaterium* in *E. coli*.

1.2 Methodology - As the objective of this project is cloning and expression of bacterial arginase to exploit its beneficial uses in the cancer treatment and produce a cost effective and minimal side effect treatment method, here we have chosen *Bacillus megaterium* as our model organism. *Bacillus megaterium* which is one of the widely used and well-studied microorganisms in the field of microbiology is a aerobic, gram-positive and spore forming bacterium [7,8]. Some of its important characteristics are that it has large size, can grow in a wide temperature range from 3-45 degree Celsius and has stable plasmid replication system with the ability of surviving in diverse conditions [7,8]. There are many studies that have been reported over the past decades on the genetics of *B. megaterium* and also a number of strains have already been developed so far [8].

Firstly, the protein sequence of the rocF gene of *B. megaterium* (strain ATCC 14581) with ORF BG04_2464 will be procured from UniProt database and the gene sequence of the same will also be procured from the databases. Then based on the gene sequence forward and reverse primers need to be designed with restriction enzyme sites NdeI and EcoRI. Using the DNA isolation protocol, the DNA from the bacteria needs to be isolated and then PCR amplification will be done using the designed primers. The required portion of the DNA will get amplified which will be ligated in pet28a vector. Then using this pet28a vector the strain of *E. coli* will be transformed. The gene of *Bacillus megaterium* that codes for arginase will be then induced for expression using IPTG (Isopropyl ß-D-1-thiogalactopyranoside).

1.3 Objectives – The objectives of the work were:

I. Screening of arginase producing bacteria
II. Cloning and expression of arginase gene of selected bacteria

Chapter 2 –

LITERATURE SURVEY

Chapter 2 – LITERATURE SURVEY

According to statistics of 2022 that is published by the American Cancer Society, cancer that is caused due to defect in cellular processes which ultimately leads to uncontrolled cell growth and defect in normal cellular function is the second leading cause of death worldwide [1,2]. It is seen in the reports of 2018 that there is a large incidence of cancer cases to around 18.1 million globally which is expected to be around 25million till 2040 [3]. Cancers which are considered as one of the most devastating causes of death, after a various method of investigation is reported to be caused due to genetic mutations in the normal cells [4]. Although there are many trials that have been going on to understand, investigate and define the cause and nature of cells affected by cancer through various approaches like immunological, genomic, genetic, proteomic, biochemical, molecular etc. but there has not been much success that is obtained till now in developing a less harmful and more efficient method [4]. Hence, there is a vast scope of availability of research in this field.

As it was known in the development of cancer that first the normal cells begin to undergo uncontrolled cell division which results in the formation of a mass of cells, called as tumors it ultimately led to the understanding of two different types of cancer conditions, i.e., in situ cancer and invasive cancer [5]. When a tumor remains in the same tissue where it was originated than that condition is called as in situ cancer and when the tumor begin to spread and invade the cells nearby than it is called as invasive cancer [5]. Cancer which is a Latin word derived by an observation of Hippocrates more than 2300 years ago was seen to have different level of incidence in different populations. It was observed in 1775 that men who were working in chimney sweeps were more susceptible to scrotal cancer and in 1800s people who work in the pitch blende mines of Germany were seen to have incidence towards lung cancer [5]. By the end of 19th century, it was also observed that people who use cigars suffered from mouth and throat cancers [5]. All these observations led to the conclusions that the agents that cause cancer may lie outside the body. Finally, in 1971, a scientists named Alfred Knudson provided explanations to the fact that cancer is caused by the external agents by proposing a simple genetic model [5]. Model provided by A. Knudson stated that children's those who suffered from retinoblastoma without their parents having any history of the disease (sporadic retinoblastoma) were normal during conception but later on experienced two somatic mutations and these somatic mutations led to the development of this eye cancer [5]. Also, those children's suffering from retinoblastoma whose parents have the history of the disease contains

mutation during conception and at the later stage of age experience one another mutation that led to the cancer [5]. Furthermore, the studies in cell biology also contributed to the understanding of cancer. One of the most important understandings was that cancer cells arise from normal body tissues. This understanding was achieved by observing the attempts of transplantation of tissues from one person to another [5]. It was seen the transplantation of tissues work well when both the people involved in the process are less distantly related as the recipient's immune system has the capability of distinguishing self and non-self-cells [5]. So, when this similar type of experiment was done using cancer cells it was seen that the tumor cells were of same transplantation type which proved that tumors were originated from body's own tissue and not from cells that are introduced by infection [5].

2.1 Difference between normal cells and cancerous cells –

Cancer after proved to be developed from one's own tissue because of mutational events occurring in those cells due to some agents lying outside the body it was also proved that they are monoclonal in origin, i.e., one cell first experiences the transformation because of mutation and the other cells of tumors are the descendants of that cell.

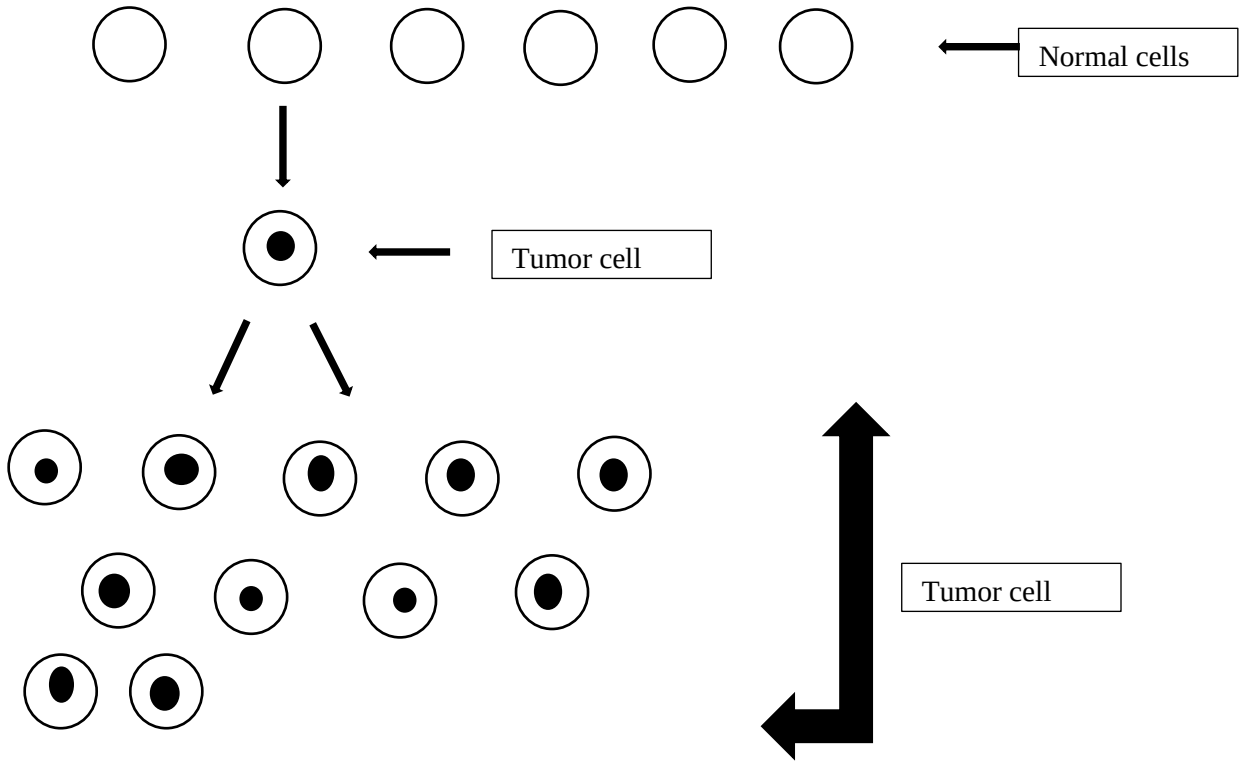


Fig. 1: Monoclonal Origin of Cancer

Cancer cells are also seen to be genetically unstable than normal cells and are also more prone to occurrence of mutational events [5]. So, these cancerous cells may have different characteristics in nature. The size of the nucleus in cancerous cells were also seen to be larger than the normal case and are seen to occupy most of the volume of the cells [5]. However, the most important characteristic that differentiates normal cells from cancerous cells is that in tumor large number of cells are involved in mitosis which is a rare event in normal case [5].

So, from all the research that have been carried out it was confirmed that agent that lie outside the body that can be termed as carcinogens causes the induction of mutation in genes that are critical which ultimately led to the increase rate of mitosis in the cells and the cells than grow abnormally. These abnormal growth results in the formation of tumors in the later period of life. It was also proved until that time that only small number of genes were involved in cancer and this led to a new question that which are the genes that are involved in cancer development [5].

2.2 Genes that play role in Cancer development –

There are different genes that are involve in the development of cancer and these genes that that play major roles are the gens that regulate the cell cycle [5]. These genes are classified into two categories proto-oncogenes and tumor suppressor genes that co-ordinate together to regulate the division and growth of normal cells [5]. Proto-oncogenes are those genes that are involved in cell division and tumor suppressor genes are those that inhibits the cell division [5].

Some of the tumor suppressor genes and proto-oncogenes that are involved in cancer cell development are [5]:

Oncogenes – 1. PDGF (this gene is responsible for a protein called platelet derived growth factor and is involved in the formation of brain cancer)

2. MDM2 (this gene codes for a protein that is an antagonist of the p53 tumor suppressor protein which is involved in certain connective tissue cancers)
3. Ki-ras (this gene codes for a protein that is involved in the stimulatory signalling pathway which is involved in colon, pancreatic and ovarian cancers)

Tumor Suppressor genes – 1. BRCA1 (this gene is involved in ovarian and breast cancers).

2. RB (this gene codes for pRB protein which is the key protein that inhibits the cell cycle. It is involved in bone, breast, bladder cancer and retinoblastoma.

3. NF-1 (this gene codes for a protein that inhibits the stimulatory protein that is involved in myeloid leukaemia).

These genes if mutated results in excessive cell division and also cannot inhibit the cell cycle which ultimately led to uncontrolled cell division and growth of tumors. With all the research and the identification of the genes that are involved in cancer development led to a more detailed understanding of cancer which ultimately helped in developing many treatment methods.

2.3 Several Cancer treatments that have been developed –

As cancer has been proved to be the most dangerous and one of the prevalent causes of maximum deaths worldwide there are a number of researches that took place from late 1700s which led to the increase understanding of cancer, this ultimately led to the development of several treatment methods which includes chemotherapy, radiotherapy, immunotherapy, hormone therapy, etc.

1. Chemotherapy – It is referred to the drugs that are being used to kill cancer cells. In this type of treatment only a single drug or combinations of drug is given either through mouth or directly into blood vessels [6].
2. Surgery – Surgery is one of the most common types of treatment in many cases of cancer. Here, the tumor cells and some of the nearby cells are surgically removed. This process is done to minimise the side effects caused by cancer [6].
3. Radiotherapy - In this treatment method x-rays or radio waves are used to kill the cancer cells. This method is based on the fact that radiation is more harmful to rapidly dividing cells so they are killed more rapidly than normal cells. Radiation therapy is of mainly two types: External beam and internal beam [6].
4. Targeted therapies – In targeted therapies drugs are used either as IV or pill but unlike the targeted therapy it does not harm the cancer cells. This method helps in targeting and damaging specific tumor cells either by turning off the processes that help them to grow and spread or by directly killing them [6].
5. Hormonal therapy – This mode of treatment is used to treat cancers like prostrate, breast and ovarian cancers. Here the body's natural fluid is blocked by either surgery or using

drugs. In case of surgery the organ that is responsible for the production of that hormone is removed and in case of drugs the drugs are either given as pills or injection [6].

6. Laser therapy – Here a narrow and focussed beam of light is passed through a tube into the body. The thin fibers that are present inside the tube direct the light towards the cancer cells and then destroy them [6].

Although these treatments have proved effective in treating many cancer patients but there were several side-effects that have been seen to be associated with it. It is reported that approximately 90% of cancer patients who have been treated by surgeries experience severe pain and patients who have received radiotherapy and chemotherapy faces adverse side-effects like heart problems, chronic bladder spams, diarrhoea, flu-like symptoms, dysphagia, inflammation of the spinal cord and neurological damage [1]. Also, there are several cases that have been reported which showed evidence of cancer cells developing resistance towards these conventional treatments [1]. So, these limitations led to the need for development of new cancer treatments which are more-effective and have less side-effects.

2.4 Enzymatic Therapy: Promising alternative in Cancer treatment –

As it was observed that the traditional methods of cancer treatments were allied with several drawbacks like adverse side effects and lack of precision and non-specificity, enzymatic treatments were seen as a promising alternative in the field of cancer treatments. One of the reasons was that, the enzymes are biomolecules which are very specific towards cancerous cells and also plays role in different proliferative and metabolic pathways of biological systems [2]. Also, these enzymes will act on the well-known checkpoints that regulates the cell division conditions of normal healthy cells and hence will be very specific in their contribution to the cancer therapy [2]. Till date, there are several enzymes that has already been studied like methionase, arginase, arginine deiminase and asparaginase for their anticancer properties and some of them are even in the phase of clinical trials.

One type of enzymatic treatment that has been developed is, the depletion of semi essential amino acids that are needed by the cancerous cells to survive like asparagine, arginine, methionine and glutamine [7]. This idea of depletion of semi essential amino acids dates back to 1930s, when Gilroy in his pioneering work showed that mice when receives a diet that is enriched in arginine develops tumours faster and larger than the mice which are supplied with standard diet [8]. These method of depletion of semi essential amino acids were considered because they were found to play a major role in regulation of important cellular events like

peptide synthesis and hormone synthesis in both normal and tumor cells [9]. Also, amino acids were seen to be playing modulatory roles in various gene expressions by regulating the synthesis of RNA [9]. As there were many differences observed between normal and malignant cells one of the significant differences was their differences in the metabolism [9]. Like, in case of normal cells catabolic metabolism fulfils the bio-energetic requirements for homeostasis but in tumor cells these metabolic pathways are altered for which to maintain the balance between elevated biosynthesis of macromolecules and ATP levels they need to consume additional nutrients [9]. As their nutrition demand increases because of their rapid growth the endogenous supply of nutrient becomes inadequate to fulfil their demand and they ultimately depend on the exogenous nutrient that are present in the microenvironment [9]. This particular characteristic of tumor cells which was identified was then used as a key point in developing this new treatment for cancer.

2.5 Arginine –

Arginine which is active in its L-form is an essential amino acid that is primarily synthesised via urea cycle in the proximal renal tubule and from citrulline [10,11]. It can be classified on the basis of the health and developmental status of the mammals into conditionally essential or semi essential amino acid [11]. Arginine is a basic amino acid because of the complex guanidinium group in the end of its side chain which remains positively charged in basic, neutral and most acidic environments [11]. The positive charge of arginine is delocalized because of the conjugation between the lone pairs of nitrogen and the double bond which makes it able to form multiple H-bonds [11]. It is absolutely necessary for the synthesis of amino acids (like proline and glutamate), proteins, agmatine, urea and polyamines [12]. Arginine has many important functions like stimulating release of growth hormones, expression of T cell receptors, improving wound healing, killer T cells activation, dispose of ammonia and is also important in synthesis of NO [11, 12]. Arginine, when necessary, gets converted into glycogen and glucose and also is known to be the inducer of mTOR pathway [11]. This essential amino acid is found in the proteins of animals and plants like meat, fish dairy products nut and poultry [11].

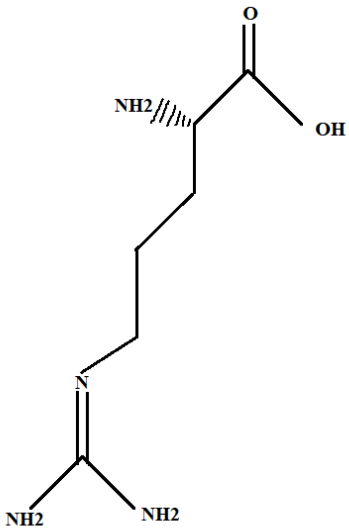


Fig. 2: 2D structure of arginine [11]

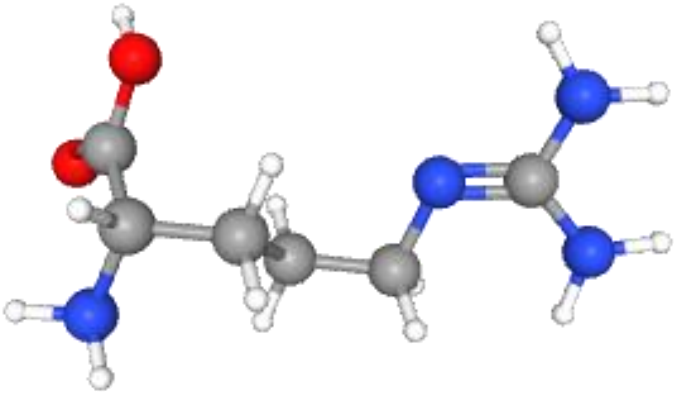


Fig. 3: 3D structure of arginine [11]

Synthesis of Arginine:

Arginine synthesis from citrulline involves two steps [7]:

i) First the L-citrulline and aspartic acid is converted into arginosuccinate and this reaction is catalysed by the enzyme arginosuccinate synthetase (ASS1). The arginosuccinate is then further converted into arginine and fumaric acid by the enzyme arginosuccinate lyase (ASL).

ii) In the second step, the arginine produced is then degraded by the enzyme arginase to L-ornithine and urea of which the L-ornithine is converted back to L-citrulline by the enzyme ornithine transcarbamylase (OCT).

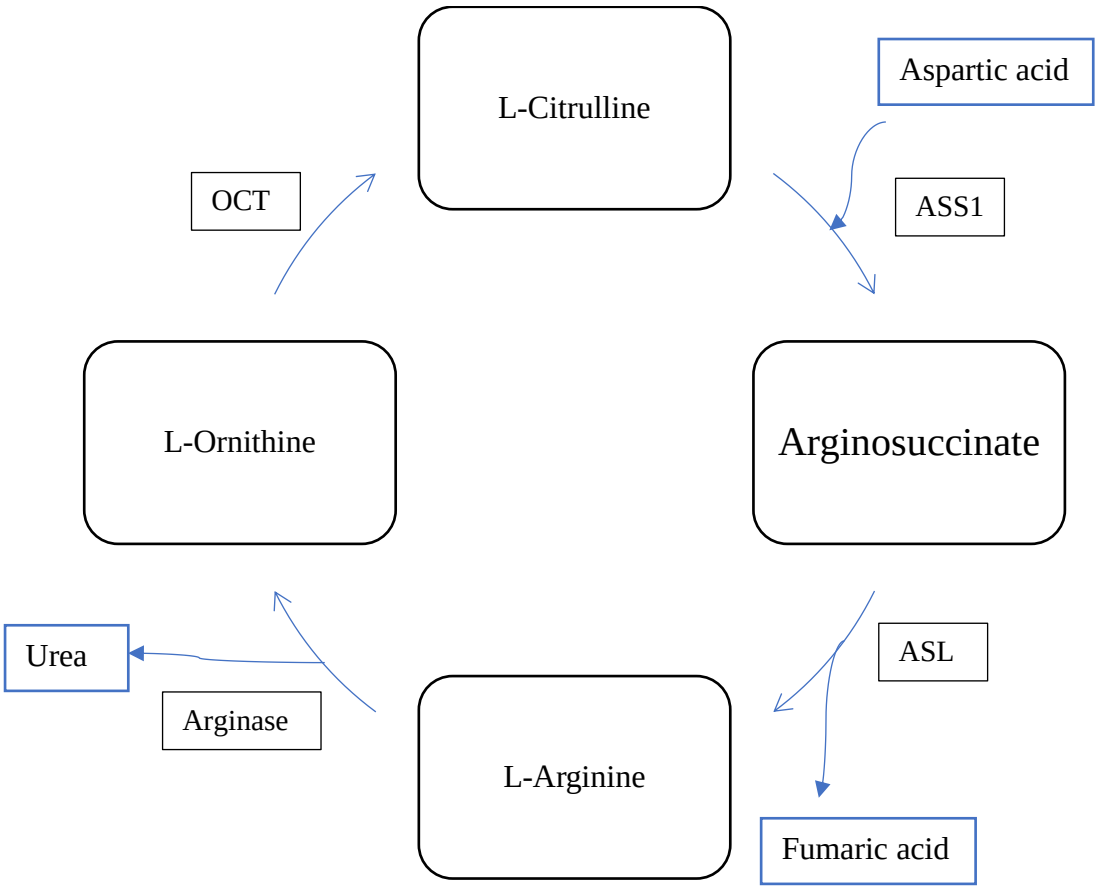


Fig. 4: Synthesis of Arginine

2.6 Arginine Depriving enzyme: a potential window for cancer therapy -

As it has been reported by studying several human malignancies like pancreatic, breast, prostate, lung etc. that cancer cells have an important feature of arginine auxotrophy, i.e., these cancer cells were auxotrophic to enzymes involved in Arginine synthesis (ASS, ASL, and OCT), it has opened a new gateway in the field of research [11,2]. And researchers growing interest in this topic has led to understanding that these arginine auxotrophic cancer cells were found to have defect in cellular mechanism for which they were dependent on the extracellular nutritional pool to fulfil their nutrient demand [2]. Thus, identification of Arginine auxotrophy and its importance to cancer cells led to the identification of a potential window for cancer treatment i.e., arginine deprivation therapy [11]. There are three different approaches that have been identified for arginine deprivation therapy [11]:

i) Arginine restricted diet
ii) Inhibition of arginine sensing and transport
iii) Enzymatic degradation of arginine

- ✓ Arginine restricted diet: This approach of arginine deprivation has been tested in many cancers cell lines but it showed positive results only when tested in the mouse models of skin cancer and colorectal cancer but was not proved effective when tested in human cell lines [11].
- ✓ Inhibition of arginine sensing and transport: With the previous studies it was known that in normal cells CAT transporters along with L-type amino acids are involved in transportation of arginine but there is no inhibitor that have been described till now which blocks all the transporters involved in the arginine transportation [11].
- ✓ Enzymatic degradation of arginine: This approach is currently considered as the most effective method to arginine deprivation therapy [11].

Enzymatic degradation of Arginine involves three major enzymes, they are Arginine deiminase (ADI), Arginine decarboxylase (ADC) and Arginase [9].

- Arginine Decarboxylase (ADC): ADC (EC 4.1.1.19) is an arginine degrading enzyme that catalyses the conversion of arginine to agmatine and carbon dioxide [2]. Some researchers have hypothesised that ADC can be applied to tumor cells as it is responsible in the depletion of arginine, so there were several experiments that were performed using the recombinant ADC [13]. rADC was first tested by two scientist

Leung and Wei on colorectal cancer cell lines for around 72hours [13]. The results obtained was positive as it showed some anti-proliferative effect in-vitro [13]. They also found out by observation that the cell-cycle of tumor cells were arrested during the S-phase when treated with rADC [13]. ADC was also tested in human diploid fibroblast, murine leukaemia and human uterus Hela after isolating it from *E. coli* and a positive response was seen [13]. Although it is proven that treating tumor cells with ADC depletes arginine and inhibits the growth of tumor cells but there are disadvantages associated with it [3]. Agmatine that is formed during this reaction is seen to be retained in healthy cells and it has been observed to have toxic effects on their growth [2]. Also, agmatine when are stored in cells, it results in inhibition of polyamine biosynthesis and arrest of cells at different checkpoints without being selective [2]. It was also seen that agmatine was involved in the inhibition of Nitric oxide synthesis [13]. Because of these reasons ADC is no more considered as a potential candidate to be used in the treatment of cancer cells.

- Arginine Deiminase: ADI also known as Arginine iminohydrolase (EC 3.5.3.6) is also involved in the degradation of arginine and degrades arginine into L-citrulline and ammonia [2]. ADI is found in prokaryotes and plays a critical role in the survival of the bacteria under stress conditions and it interferes with the signalling pathway [2,10]. In normal cells where ASS is present, the citrulline that is formed after degradation of arginine can be recycled back to arginine but this case is not seen in case of tumor cells where ASS is absent, i.e., ASS (-) tumor cells [13]. Some of the tumor cells where this condition is commonly seen are hepatocellular carcinoma, renal, pancreatic, prostrate and melanoma [13]. Although there were certain advantages associated with ADI, ADI was highly instable, had short half-time and as it is absent in mammalian cells it is highly antigenic [10,14]. So, to overcome these limitations Polyethylene glycol of 20kDa was conjugated with ADI (ADI-PEG20). This has not only increased the half-time and reduced the antigenicity but also showed effective anti-cancerous result in various tumors like lung cancer, hepatocellular carcinoma, prostate cancer melanoma, etc [14]. When there is a reduction of arginine in the microenvironment of cancer cells than there is a range of change that occurs within the cell like activation of mTOR pathway [15]. These changes in the cell is then followed by a series of activation and deactivation of which ultimately leads to cell death [15]. After getting positive responses in laboratory trials of ADI, pre-clinical trials were done and to determine the efficacy of ADI in treatment several methods were followed like live/dead analysis,

simple biomass/viability quantification and establishment of stable ASS1-overexpressing/ASS1 knockdown cell lines [10]. Then in March 1999 the US Food and Drug Administration approved ADI-PEG20 for the treatment of HCC and malignant melanomas as an orphan drug and in July 2005, European Agency for the Evaluation of Medicinal Products also granted ADI-PEG20 as an orphan drug [11]. Although ADI showed anti-proliferative effects there were mild to severe side effects that was also seen to be associated with the use of it [2]. It was seen that people after receiving ADI therapy were suffering from hyperuricemia, gastrointestinal toxicity, and also local rash at the site of injection [2]. Also, it was also seen that the effects ADI-PEG treatment are reversible by using neutralizing antibodies present in the circulating serum [2]. To increase the effectiveness of ADI-PEG treatment combinational approaches were started to be studied [2]. It is now under the phase I clinical trial and one the most commonly studied combinational therapy is 5-Fluorouracil drug with ADI [2]. Till now it has been proved that when ADI-PEG is combined with Gemcitabine the development of pancreatic growth was inhibited [2]. Also, when it is combined with cisplatin its effect towards ASS (+) melanomas and HCC cell lines was increased and evidences of inhibition of breast cancer progression was seen when ADI-PEG was combined with Doxorubicin [2]. Although there has been success achieved in the combinational therapy with ADI-PEG more preclinical studies needed to be carried out.

2.7 Arginase –

Arginase also known as Arg amidohydrolase (EC 3.5.3.1) which belongs to the ureohydrolase class of enzymes is a metalloenzyme as it uses manganese to catalyse the conversion of ornithine and urea [16,2]. It was thought that arginase appeared first in bacteria and as evolution persisted it began to be found in vertebrates, plants, invertebrates, and yeast and this transfer of the arginase bacteria to these higher organisms was suggested to have occurred via mitochondria [16]. It is found that yeast and bacteria which are simpler organisms has only one form of arginase, i.e., arginase 2 expressed in the mitochondria but in higher organisms there were two types of arginase, i.e., arginase1 and arginase 2 that was seen to be expressed [16]. It was found that those animals that were involved in the metabolism of excess nitrogen into urea express arginase 1 in the cytosol of the liver and RBCs and isoform A2 was seen to be expressed in the mitochondria of kidney, retina and brain [16]. In humans, arginase 1 (322 amino acid residues) which is involved in the elimination of excess ammonia that is generated due to amino acid catabolism is present in the cytosol of the liver whereas arginase 2 (354

amino acid residues) which is involved in the nitric oxide and polyamines biosynthesis is present in the mitochondria of the kidney [2]. These two isoforms of arginase are although encoded by distinct genes present on separate chromosomes but was seen to share more than 50% of their amino acid residues with 100% homology in the areas that were too involved in the enzymatic function [17]. The structure of these two arginases when studied through crystallography also showed that both the arginase has 3 subunits that are identical with one active site which is present at the bottom of a deep cleft and binding of the manganese ions to the active site was essential for the activity of the enzyme [16,17]. Each subunit of the protein folding of the enzyme belongs to the α/β family and the folding consists of a parallel, eight stranded β-sheet that is flanked by numerous α-helices [17].

Arginase has been studied to have two functions which involves detoxification of ammonia and production of ornithine which is used in the polyamines and proline synthesis that play an essential role in growth, neuronal development, inflammation, tissue repair, wound healing and cell proliferation [16]. Proline and polyamines are synthesised through the activity of ornithine aminotransferase (OAT) and ornithine decarboxylase (ODC) respectively [16].

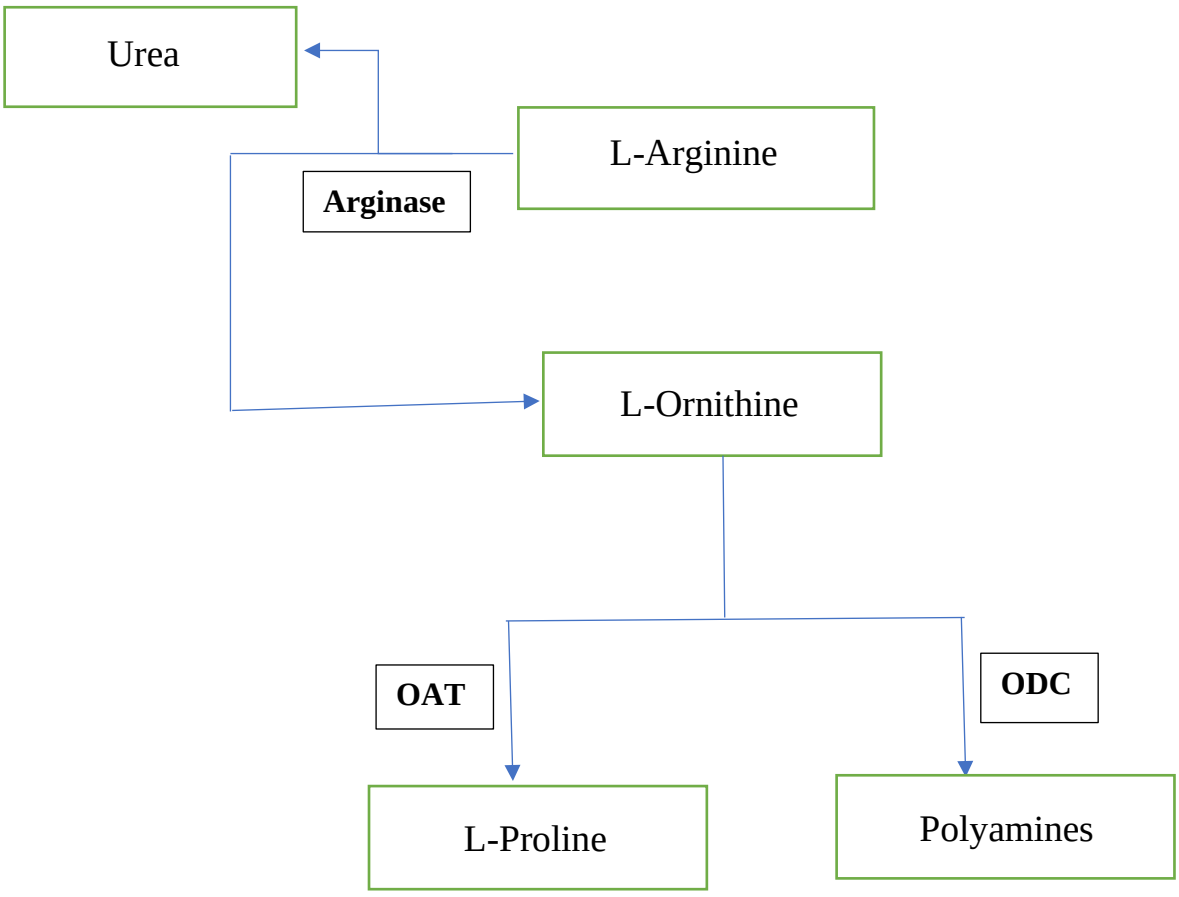


Fig.5: Production of Urea, proline and polyamines from arginine through arginase

The L-citrulline formed can be converted back to L-arginine through the enzymes ASS and ASL as seen in the synthesis of arginine which is a part of the urea cycle. Any defect in these enzymes involved in the urea cycle can result in a defective function of urea cycle which can ultimately result in the accumulation of toxic ammonia that can result in seizures, mental disorders and death if left untreated [16]. Those patients who have defects in the activity of enzyme Arginase1 can be treated by liver transplantation if severe and also can be treated by supplementing amino acids in their diet as well as decreasing the daily protein intake but there are no present literature that discusses the effect that occur due to the defects in the enzyme arginase 2 [16].

The study of arginase began after arginase was purified from ox liver by precipitation and it was seen that the arginase that was purified was able to decrease 77% tumor growth [2]. But there was some problem associated with the arginase, i.e., the arginase had high Km value and so high dose was required [2]. To improve this shortcoming the arginase was PEGylated which not only improved the catalytic efficacy but had also proved to be helpful in both ASS (-) and OCT (-) cancer cells [2]. Recombinant human arginase (rhArg) is recently developed for arginine deprivation therapy and is currently under investigation clinically. Till now, rhArg has been proved to be effective in the treatment of cancer cells that are OCT (-) [16]. But there was certain toxic effect that was seen to be associated with rhArg and theses short comings were overcome when the rhArg was PEGylated and the Mn ion was replaced by Co [2]. The Mn was replaced by Co ion because by replacing it with Co ion the activity of enzyme was 10-fold increased as its substrate binding capacity was increased [2]. This rhArg has been widely studied on different cancer cell lines like prostrate, HCC, breast, etc and after proving effective it is in phase I clinical trial. There are some researches that have been going on, on this rhArg like attempts are being made to increase the life-time and efficacy and for this they are fusing the enzyme with the Fc region of IgG [2].

Why is Arginase better than ADI?

Although ADI has been approved by FDA but there are many clinical studies that have shown demerits in its use. Firstly, as ADI is mainly seen to be expressed in the bacterial system so when it is administered it is recognised as foreign molecule in the body and the body will start forming antibodies against it to neutralise it which will ultimately decrease its efficacy [2]. Secondly, the ADI enzyme is seen to release citrulline and ammonia as a by-product and it may be possible that ammonia can turn toxic in the long-term treatment [2]. Then the third major,

shortcomings associated with ADI is that it is not effective in cells that are expressing ASS, because ASS can convert back citrulline to Arginine [2]. These drawbacks can be overcome when we use arginase enzyme for the deprivation therapy like as arginase is also present in human so, it is not recognised as foreign so there is no problem of immunogenicity and also the end products of arginase are non-toxic and it is also seen to be effective in cancer cells expressing ASS.

2.8 *Bacillus megaterium* -

Bacillus megaterium which is one of the widely used and studied model organism in the field of biotechnology and microbiology is a aerobic, gram-positive, spore forming bacteria [8,7]. It is reported to be found in many different but is considered as a commonly known soil bacterium [7]. It was first described in the year 1884 by Anton De Bary and later Ford and Lawrence defined this species of *Bacillus* in detail [7]. *Bacillus megaterium* because of its ability to grow at a wide temperature range i.e., 3 ℃ to 45 ℃ and due to its stable plasmid, secretion ability and large size has become an ideal organism to be used in the field of research in biotechnology and microbiology [8,7]. It is considered as the big beast because of its large cell which have a cell length of 4µm and a diameter of 1.5µm with whole genome size of 5.7 Mbp [18,19]. It has the ability to grow on cheap substrates and also do not produce any endotoxins that are associated with the outer membrane and also do not possess any recombinant gene product that are protease degrading [19]. *Bacillus megaterium* has been used in many aspects like in production of several α- and β- amylases (these amylases are used in baking industries in reactions involved in starch modifications), in producing HIV antigen intracellularly by Abbott Laboratories [19].

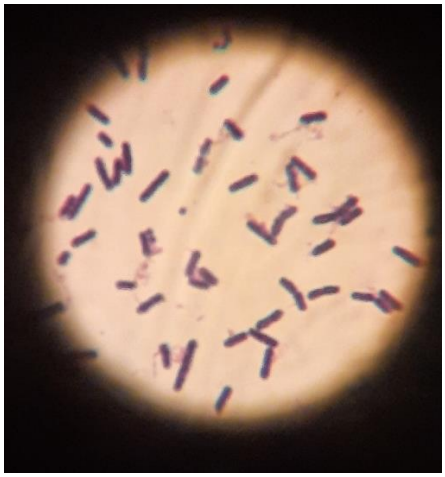


Fig. 6: Culture of *Bacillus megaterium* under Compound microscope

2.9 DNA Isolation from Bacterial cultures –

DNA which is considered as the genetic material is made up of two polynucleotide chains and the chains consists of four nucleotide subunits [20]. These two chains are held to each other by hydrogen bonds and the nucleotides are made up of nitrogen-containing base namely adenine, guanine, cytosine and thymine and a five-carbon sugar to which one phosphate groups are attached [20]. The nucleotides form the backbone as they are covalently linked with sugars and phosphates [20]. DNA is considered as the genetic material because it encodes the information on the basis of the order of the nucleotides present along each DNA strands [20].

DNA as known is a macromolecular structure and is composed of a repeating polymer which are formed by the nucleotides [21]. These polymers are composed of a pentose sugar (2-deoxyribose) and nitrogenous bases (purines and pyrimidines) and are called as the basic building blocks of nucleic acids [21]. First, a phosphate group is attached via ester linkage to the 5' end of the nucleoside and a nucleotide or nucleoside phosphate is formed. Then two nucleotides of adjacent sugars are joined together by means of phosphodiester bond (bond is formed between phosphate of one nucleotide and the 3' hydroxyl group of another nucleotide) and a long chain of polynucleotide molecules are formed with free hydroxyl group at both 5' and 3' end [21]. These two chains of polynucleotide molecules are then attached to each other by means of weak forces like H-bond between complementary bases and thus forms the two DNA strands [21]. These DNA strands contains regions which codes for a single RNA or protein and this region of DNA strands are called as the gene and the complete set of gene in a cell is called as the genome [21].

The DNA isolation or extraction methods are protocols used to isolate purified DNA from different organisms and was first done in 1869 by Friedrich Miescher [22]. One of the most efficient methods of isolating good quality DNA by preventing it from mechanical shearing is using phenol-chloroform isolation protocol. Firstly, the cell membrane present is needed to disrupted which is done by heat-boiling method in this case then a extraction buffer is prepared which contains components like Tris-HCl, EDTA, SDS, NaCl. The EDTA acts as the chelating agent that sequester the $Mg2+$ ions which are used by the enzymes that are involved in degrading the DNA and the Tris-HCl maintains the desired pH of the solution that is required [22]. The SDS solubilizes the membrane proteins and lipids by destroying the conformation of proteins by removing its negative ions and the NaCl helps in preventing the denaturation of DNA and remove the protein that remain attached to the DNA. After the cell membrane is

disrupted the protein contaminants are needed to be removed and this done by using phenol-chloroform solution. The phenol which is non-polar breaks the bonds between amino acids which leads the protein denaturation and as protein has both polar and non-polar groups it remains dissolved in the phenol whereas DNA being polar it is insoluble in phenol. The chloroform helps in increasing the efficiency of denaturation of protein by phenol and separates the aqueous and organic phase and keep the DNA dissolved in the aqueous phase. The phenol chloroform solutions also contain isoamyl alcohol which reduces the foaming occurring in the interphase. Finally, to precipitate the DNA from the aqueous phase isopropanol is used as it removes the hydration shell of phosphate and water. The isolated DNA is also needed to analyse for its purity and quantity and it is done by measuring the absorbance at 260 and 280nm [21]. As the one absorbance unit of DNA is equal to 50 µg/cm^3, so the concentration of DNA is calculated by:

$$[DNA] = 50 \times A_{260}\ \mu g/cm^3\ [21].$$

The purity of the DNA is determined by the ratio of A_{260}/A_{280} [21]. If the ratio is approximately 1,8 then it is considered as pure DNA and if the ratio is < 1.8 then there is considered to have protein contamination and if the ratio is >1.8 then there is considered to have RNA contamination [21].

2.10 Plasmid Isolation from cultures-

Plasmids that are found in many different microorganisms of domains *Archaea, Bacteria,* etc. are known as the linear or double-stranded circular extrachromosomal DNA [23,24]. The plasmids vary in size from 1kb to greater than 200kb and have a narrow host range [24]. It behaves as an accessory genetic material and replicate and inherit independently of the bacterial chromosome [24]. Plasmids was first described in 1952 by Joshua Lederberg who defined it as the extrachromosomal genetic element but it was first termed as episome in 1958 by Jacob and Wollman which was later renamed to plasmids in the year 1969 by Hayes [24]. Plasmids can be used for many purposes in genetic engineering artificial modification which results in the formation of cloning vectors [21].

Cloning vectors are designed by artificially modifying the natural plasmid which ultimately contains the following features [21]:

i) Vectors are smaller in size than natural plasmids and has increase efficiency of uptake by bacteria with resistance towards shearing.

ii) It contains a bacterial origin of DNA replication to ensure that it will be replicated independently in the host.

iii) It also contains genes that codes for resistance against antibiotics like tetracycline and kanamycin which helps in selection of cells that contain these artificial plasmids or cloning vectors.

iv) These vectors also contain single restriction enzyme sites of a number of different restriction enzymes that allows to cut the plasmid and make it linear which can be used for inserting the foreign DNA in the plasmid.

The cloning vector used in this project is pET 28a vector of size 5369 bp which is generally used for cloning and expression of genes in *E. coli* and have the following features [25]:

i) The bacterial origin of replication id pBR322 ori.

ii) It contains a Kanamycin resistance gene.

iii) The promoter present is a T7 bacteriophage promoter with an adjacent sequence of lac operator.

iv) It contains a translation initiation region which is mediated by the Shine-Dalgarno sequence.

v) It has a multiple cloning site where the gene to be expressed is cloned and this region has coding sequence for the poly-histidine purification tag (His_6) and thrombin protease recognition site.

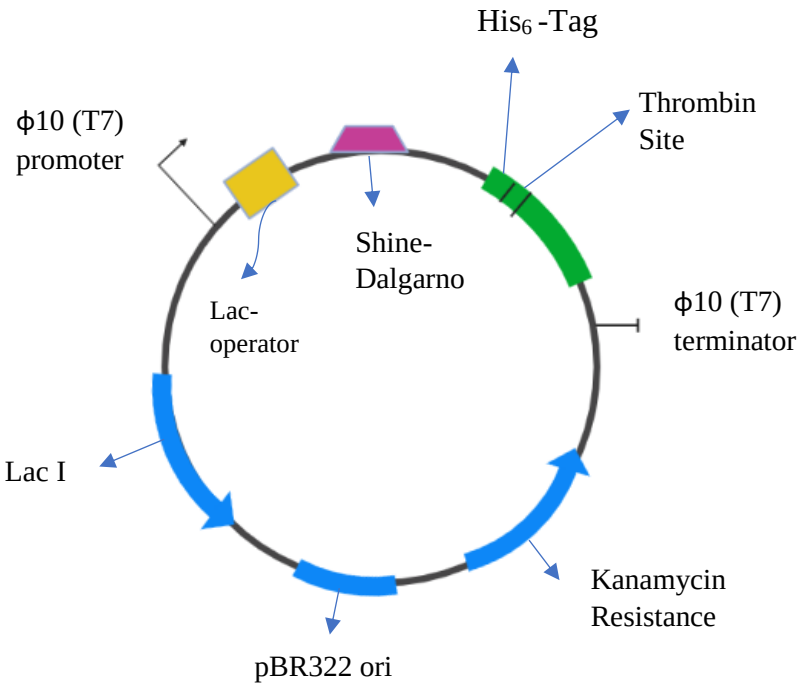


Fig. 7: Schematic diagram of pET28a vector (Created with Biorender.com)

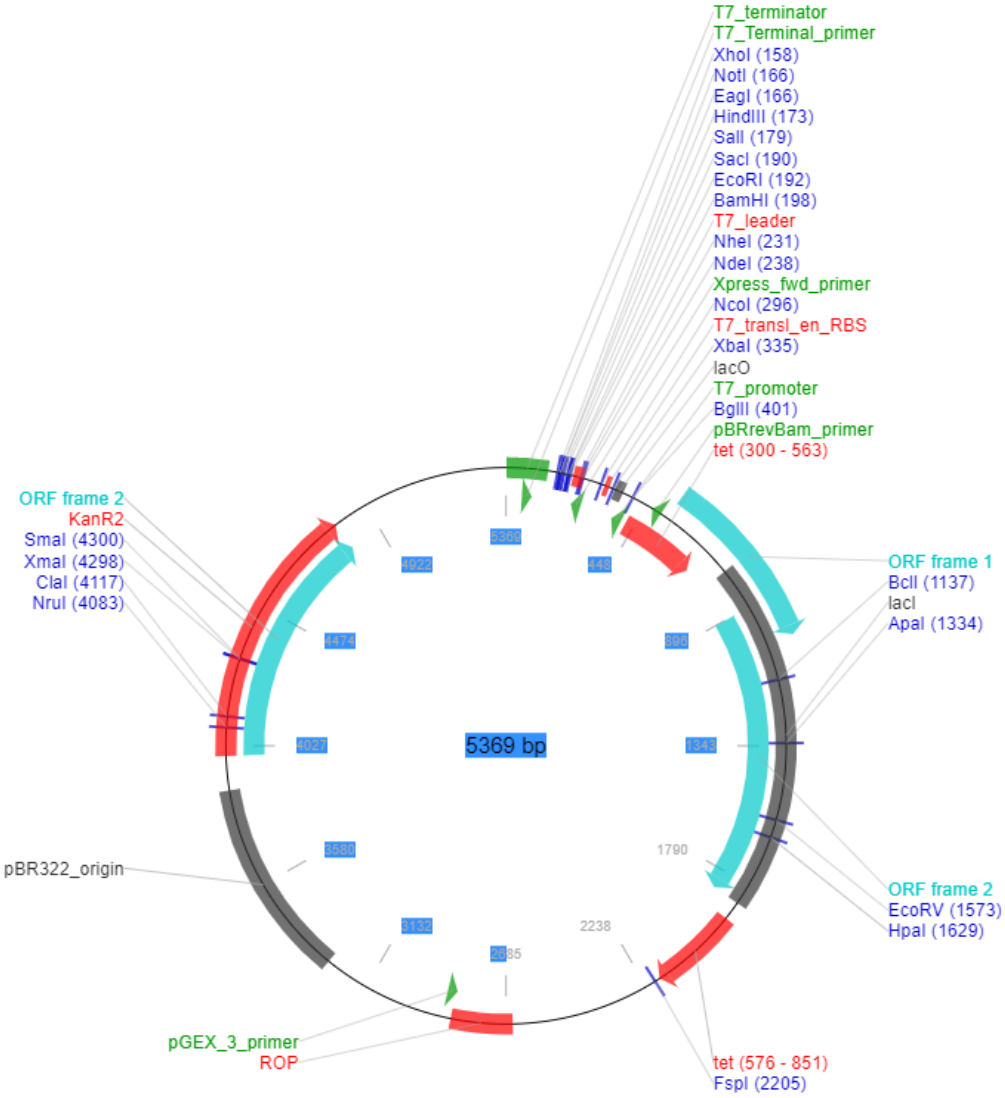


Fig.8: Design of pET28a vector [26]

2.11 Agarose Gel Electrophoresis –

Agarose gel electrophoresis which was first studied in 1964 is a highly effective and simple method which is used to separate and analyse DNA fragments of various sizes [27,28]. This study of electrophoresis began first to measure the mobility in free solution using the moving boundary method [28]. The gel matrices used for separation was made up of either agar, polyacrylamide, agarose, composite agarose-acrylamide, etc [28]. In agarose gel electrophoresis the gel used for the analysis and separation of DNA fragments is agarose. Agarose that is isolated from the seaweed of genera *Gelidium* and *Gracilaria* is an alternating copolymer of 1,3-linked β-D-galactose and 1,4-linked 3,6-anhydro-α-L-galactose [28,29]. In solution, at high temperature the agarose molecules have a random coil structure and upon cooling, the chains of agarose form helical fiber bundle and these fiber bundles are held together by noncovalent hydrogen bonds [28]. But the gelation of agarose occurs at a very lower temperature, the temperature at which the fiber bundles form more hydrogen bonds and gets linked together to form junction zones [28]. This technique has revolutionized the separation of DNA fragments which was previously done using sucrose density gradient centrifugation [29]. This technique works under the principle that when DNA is loaded into the wells of the gel and provided an electric field, the DNA fragments which have a negative charge migrates towards the positive charge of the anode and as these DNA molecules have a uniform mass/charge ratio they gets separated on the basis of their size, i.e., those fragments which have smaller charge migrates faster and those of lower size migrates slower [29]. This migration rate of different DNA fragments can be determined by the agarose concentration, voltage applied, size of DNA molecule, type of agarose, DNA conformation and the electrophoresis buffer used [29]. Once the DNA molecules are separated on the basis of their size it can be visualised under UV light in a GelDoc system either by staining the gel with Ethidium bromide dye or previously adding ethidium bromide to the agarose solution.

Fig.9: GelDoc use to visualize the gel

Agarose gel (%)	DNA segments of size that can be separated (in bp)
0.5	2000-50000
0.6	1000-20000
0.7	800-12000
0.8	800-10000
0.9	600-10000
1.0	400-8000
1.2	300-7000
1.5	200-3000
2.0	100-2000
3.0	25-1000
4.0	10-500
5.0	10-300

Table 1: Percentage of agarose gels required to separate different sizes of DNA segments [30]

2.12 Polymerase Chain Reaction-

Polymerase Chain Reaction or PCR which was developed in the year 1980s by Kary Mullis is a revolutionary method that is used to amplify a specific fragment of gene from the whole segment of template DNA [31]. The main components that are used in a PCR reaction are a template, DNA polymerase enzyme, free nucleotide bases and primers [32]. The template contains the desired region of gene fragment that is needed to be amplified, primers are short oligonucleotide sequences that is designed manually or computationally, DNA polymerase which is a thermostable enzyme that is able to synthesise complementary strand of DNA and are complementary to the 3' end of each target sequence [32]. The most commonly used DNA polymerase is Taq polymerase that is isolated from a bacterium which is thermostable and is found in hot springs [32]. There are three steps involved in the process of PCR: Denaturation, Annealing and Extension [21]. These three steps are repeated several 30-40 times and each repetition is then termed as a cycle [21]. Denaturation occurs at a higher temperature of around 90-94 degree Celsius, where the H-bonds between the ds DNA gets denatured and gets converted into ss DNA that exposes the region that is needed to be amplified [21]. Then

annealing of the Primers occur at 40-60 degree Celsius, in this step the primers bind to the 3' end of each template DNA strand [32,21]. This step of annealing prevents the re-hybridization of the separated strands [32]. Then comes the third step, i.e., extension which occurs at a temperature range of 72-74 degree Celsius [21]. In this step the Taq polymerase starts adding the free nucleotides present in the solution and synthesise a complementary strand [21,30].

The PCR reaction mixture contains the following components [21]:

i) Template DNA
ii) Forward and Reverse primers
iii) Nuclease free water
iv) Mater mix

The master mix contains the following components [21] –

i) DNA polymerase- It is involved in the synthesis of the complementary DNA strands and is usually thermostable.
ii) dNTPs – These free nucleotides are used by the enzyme DNA polymerase to synthesise the new DNA strand.
iii) $MgCl_2$ – It acts as a cofactor and plays the role in enhancing the enzymatic activity of DNA polymerase. The Mg^{2+} ion in $MgCl_2$ binds to the α-phosphate group of a dNTP and this facilitates the removal of β and α- phosphates, which results in the formation of dNMP. The dNMP formed than with 3'OH group of the adjacent nucleotide forms a phosphodiester bond. It also facilitates the binding of primers to the specific location and reduces the electrostatic repulsion present between two DNA strands.
iv) Taq Buffer- It contains 10mM Tris HCl, 50mM KCl, and 1.5mM $MgCl_2$. Tris HCl maintains the pH of the reaction to prevent DNA degradation and KCl neutralizes the charge present in the backbone of the DNA.

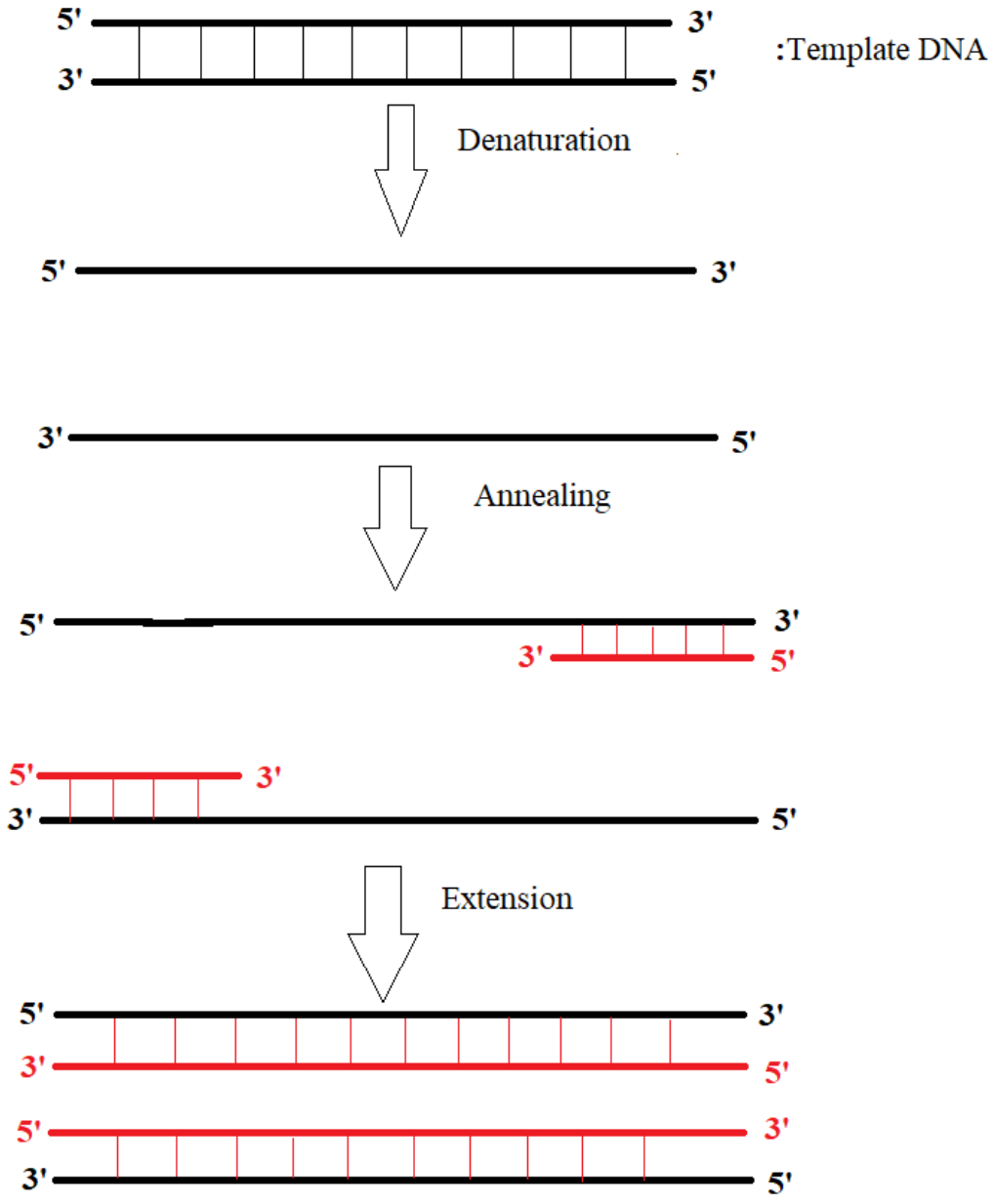


Fig. 10: Steps of PCR

2.13 Cloning and Expression -

A.C.Y. Chang, R.B. Helling, S.N. Cohen and H.W. Boyer in November 1973 reported in a paper entitled "Construction of Biologically Functional Bacterial Plasmids In Vitro" that individual genes can be isolated from DNA molecules by degrading the DNA molecules enzymatically, cloning it in plasmid vectors and then the recombinant DNA that is formed is introduced in the bacteria [33]. In the cloning process, first the closed circular plasmid DNA are cleaved using the restriction enzymes and then the foreign DNA is ligated in the plasmid DNA [24]. Then the product that is formed after ligation is transformed in an appropriate strain of *E. coli* [24]. The transformation of *E. coli* cells is screened by hybridization or by PCR [24]. The DNA fragments that is generated after digestion with restriction can form blunt-ended DNA fragments or DNA fragments with protruding ends [24]. The annealing of the protruding ends to the vector is easy than the annealing of the blunt end fragments [24]. The blunt ended fragments are needed to be cloned in a linearised plasmid which also have blunt ends and the optimal conditions that is required to clone blunt ended fragments are absence of polyamines, increase concentration of ligase, less concentration of ATP, and increase concentration of blunt-ended termini [24].

After the gene of interest is cloned in the vector it is needed to be expressed in *E.coli* cells but the plasmid does not enter the cells on its own, it requires assistance so that it reaches the correct location and can be expressed and replicated [24]. There are two methods used for transformation of cells, chemical and physical methods [24]. Transformation by chemical treatment is done using mixture of salt solutions as Mandel and Higa in 1970 showed that when the bacteria when treated with ice-cold solutions of $CaCl_2$ and then immediately heated to 37 or 42℃ take up the foreign DNA cloned in plasmid inside the cells [24]. This procedure was seen to generate $10^5 – 10^6$ transformed colonies of *E. coli* [24]. Transformation by physical methods includes using electrical charges [24]. These electrical charges when applied to the cells destabilizes the membrane and transient membrane pores are formed through which DNA molecules enters into the cell [24]. This method of transformation is known as electroporation and is one of the easiest, efficient and fastest method for transforming bacterial cells with a transformation efficiencies of 10^{10} transformants/µg of DNA [24]. After the cells are transformed then the gene is expressed in the bacterial cells.

Chapter 3 –

MATERIALS AND METHODS

Chapter 3 – MATERIALS AND METHODS

Materials –

- Cultures – Cultures of different bacteria (*E. coli, B. megaterium, L. rhamnosus, C. violaceum, P. aeruginosa* and *L. helveticus*) was obtained from the culture bank of JUIT, culture of recombinant *E. coli* $DH_5\alpha$ was obtained from IIT Delhi.
- Media – Nutrient Broth, Nutrient Agar and Luria-Bertani Broth was used of Himedia.
- Chemicals – Glycerol (SRL, India), KCl (MERCK, U.S), $MgSO_4$ (HIMEDIA, India), KH_2PO_4 (MERCK, U.S), $FeSO_4$ (Loba Chemie, India), $ZnSO_4$ (HIMEDIA, India), L-arginine (SRL, India), Phenol red (Qualigens, India), Crystal violet (Loba Chemie, India), Gram's iodine (Loba Chemie, India), Ethanol, Safranin (HIMEDIA, India), SDS (Bio-Rad, California), Tris base (MERCK, U.S), HCl (Fisher Scientific, U.S), EDTA (HIMEDIA, India), NaCl (HIMEDIA, India), Potassium acetate (SRL, India), Phenol (HIMEDIA, India), Chloroform (HIMEDIA, India), Isoamyl alcohol (HIMEDIA, India), Isopropanol(HIMEDIA, India), Tris-Cl (HIMEDIA, India), Agarose (HIMEDIA, India), Glacial acetic acid (HIMEDIA, India), Ethidium bromide, Kanamycin, Glucose (Qualigens, India), NaOH (SRL, India), K_2HPO_4(MERCK, U.S), $MnSO_4.H_2O$ (HIMEDIA, India), Ninhydrin, L-ornithine (HIMEDIA, India), FCR reagent (SRL, India), Acetone (MERCK, U.S),
- Instruments – Incubator (Macflow engineering), LAF hood (Microsil, India), Autoclave (Relitech, India), Centrifuge (Eppendorf, Germany), Gel electrophoresis unit, Thermocycler (Veriti, USA), GelDoc (Bio-Rad, California), Multiskan spectrophotometer (Multiskan GO, U.S), Microscope (Labovision).
- Others – Petri plates, Test tubes, Test tube stand, Conical flask, Measuring Cylinder (Tarsons), PCR tubes, Eppendorf tubes, Falcons, Spirit lamp, Slides, Reagent bottles, Nuclease free water, Distilled water, Forward and backward primers (of Eurofins), RNase A (Thermo Fisher Scientific),1kb DNA ladder (BioLabs), Loading dye (Thermo Fisher Scientific), Taq 2X Green Master mix (Thermo Fisher Scientific).

Methodology –

3.1 Culture Revival - Firstly, the culture of *Bacillus megaterium* that was stored in glycerol stock was revived in nutrient broth media. A 100mL of nutrient broth was prepared first by dissolving 1.3g of nutrient broth in 100mL distilled water and then after distributing

10mL in each test tubes, the test tubes were autoclaved. After the media was autoclaved, it was kept in incubator at 37°C for overnight to check for any contamination. Then the next day, a 100µL of *Bacillus megaterium* culture from glycerol stock was inoculated in a 10mL nutrient broth inside the LAF hood and then incubated at 37°C for overnight. In the same way, the culture of recombinant *E. coli* $DH_5\alpha$ strain was also revived in nutrient broth media after incubating at 37°C for overnight.

3.2 Subculturing – Nutrient Agar plates were prepared by dissolving 3.36g of nutrient agar in 120mL distilled water and then the media was autoclaved. After autoclaving the media was poured in autoclavable petri plates under LAF hood and then when the plates were solidified, they were kept in the incubator at 37°C for overnight to check for any contamination. Then the next day, the plates were streaked (quadrant streaking) using the culture grown in nutrient broth inside the LAF hood and kept in the incubator at 37°C for overnight. In the same way, the culture of recombinant *E. coli* $DH_5\alpha$ strain was also subcultured in nutrient agar plates containing kanamycin after incubating at 37°C for overnight.

3.3 Primary Culturing – Firstly, a 100mL of LB Broth was prepared by dissolving 2g of LB media in 100mL distilled water and then the media was autoclaved. After autoclaving the media, it was kept in the incubator at 37°C for overnight to check for any contamination. Then, the next day the broth was inoculated with pure colonies from the agar plates inside LAF hood and then kept in the incubator shaker at 37°C for overnight. In the same way, the culture of recombinant *E. coli* $DH_5\alpha$ strain was also cultured in LB broth media containing kanamycin after incubating at 37°C for overnight.

3.4 Secondary Culturing – Firstly, a 100mL of LB Broth was prepared by dissolving 2g of LB media in 100mL of distilled water and then distributed 10mL in each tube and the media was autoclaved. After autoclaving, the media was kept in the incubator at 37°C for overnight to check for any contamination. Then the next day 100µL culture from the primary culture was inoculated in the LB broth inside the LAF and kept in the incubator at 37°C for overnight. In the same way, the culture of recombinant *E. coli* $DH_5\alpha$ strain was also cultured in LB broth media containing kanamycin after incubating at 37°C for overnight.

3.5 Glycerol Stock Preparation – Glycerol stock was prepared of both the *B. megaterium* and recombinant *E. coli* $DH_5\alpha$ strain culture. Firstly, 50mL of glycerol was dissolved in 50mL dH_2O to prepare 50% glycerol and then it was autoclaved. After autoclaving, inside the LAF 500 µL culture was added to 500 µL 50% glycerol in autoclaved tubes.

3.6 Plate Assay for Screening – Plate Assay was done for 7 bacterial cultures, i.e., *B. subtilis, E. coli, B. megaterium, L. rhamnosus, C. violaceum, P. aeruginosa* and *L. helveticus,* to identify if these cultures have the arginase gene. It was done by preparing minimal media plates of composition as described by Nadaf. P., Ankalabasappa. V., and Kulkarni. G. A., in their paper "Isolation, screening and characterization of L-arginase producing soil bacteria". The composition of minimal media was [34]:

- KCl – 0.5%
- KH_2PO_4- 1.0%
- $MgSO_4$ – 0.5%
- $ZnSO_4$ – 0.1%
- $FeSO_4$ – 0.1%
- L-arginine – 1.0%
- Phenol red – 2%
- Agar- 1.3%

100mL of minimal media was prepared of the given composition and then the media was autoclaved. After autoclaving the media, inside the LAF the media was poured in autoclaved petri plates and kept for solidifying, when the plates solidified, they were kept in the incubator at 37°C for overnight to check for any contamination. Then the next day simple streaking was done using the primary cultures of the 7 different bacteria in the plates and again kept in the incubator at 37°C for overnight.

3.7 Purity Check by Gram Staining – To check if the culture of *B. megaterium* is a pure culture, the gram staining method was performed [35]. Firstly, sample from LB broth was taken and was spread om a glass slide. Then the sample was allowed to dry completely. After the sample was dried it was stained with crystal violet for 1min. It is the primary stain which stains both gram positive and gram-negative bacteria by binding to their negatively charged bacterial cell wall structures. Then the slide was washed with water to remove the excessive stain. After washing the sample was again stained with grams iodine for 1min, which is a mordant. It fixes the crystal violet stain by forming a crystal violet-iodine complex. After 1 min, the slide was again washed with water and then with few drops of ethanol. Ethanol removes the crystal violet stain from gram negative bacteria. Then the sample was stained with few drops of safranin for 30sec. Safranin is the secondary stain which counterstains the gram-negative

bacteria as it is a positively charged molecules that binds to the negative bacterial cell wall structures. After staining with safranin, the slide was washed again with water and then the slide was observed under microscope.

3.8 DNA extraction protocol – To isolate the bacterial DNA from the *Bacillus megaterium* culture the protocol described in "Research Journal of Pharmaceutical, Biological and Chemical Sciences" was used [36].

Firstly, a media of 100mL of LB broth was prepared and autoclaved and kept in the incubator at 37℃ for overnight to check for any contamination and then the next day from the agar plates pure colonies were inoculated in the LB media and kept for overnight at 37℃ incubator shaker. After there is a sufficient growth achieved in the media 20mL culture was taken in centrifuge tubes and centrifuged at 8000rpm for 5min. This step was repeated 3times to ensure proper pellet was formed. Then the supernatant was discarded and pellet was collected and to the pellet 650µL of SDS lysis buffer was added and vortexed properly so that the pellet is mixed in the lysis buffer completely. Then the tubes were kept in water bath at 80℃ for approximately 2hours by periodically mixing the contents by inverting. Then after the solution viscous which means the cells are lysed to it 100 µL of 5mM potassium acetate solution was added and kept in ice for 30min. After 30mins the solution was centrifuged at 12000rpm for 5min. Then the supernatant was collected in another centrifuge tube and to it equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) solution was added and the solution was mixed well. And then centrifuged at 12000 rpm for 5mins. After 5min again the supernatant was collected and then to it equal volume of Chloroform: Isoamyl alcohol (24:1) was added and mixed well. Then again, the mixture was centrifuged at 12000rpm for 5min. The supernatant was collected and to the walls equal volume of chilled isopropanol was added and then kept at -20℃ for overnight.

The next day the mixture was centrifuged at 12000 rpm for 5min and the pellets will be seen attached to the walls of the tubes. The pellet is then washed with 70% ethanol and then centrifuged at 12000 rpm for 2min. After centrifugation, the supernatant is discarded and the pellet is allowed to dry completely. Then the pellet is dissolved in 40 µL of TE buffer and stored at -20℃. Then the quality and concentration of DNA is assessed spectrophotometrically by taking OD at 260 and 280nm

Composition of different components used in the protocol:

- Extraction buffer composition [37]–
  - ✓ 1M Tris HCl (pH 7.5): Tris HCl maintains the pH of the solution.

- ✓ 0.5M EDTA (pH 8): EDTA acts as the chelating agent and sequester the metal ions that are required by the enzymes involved in nuclease activity and inhibits the nuclease activity of those enzymes.
- ✓ 10% SDS: SDS is an anionic detergent which solubilizes the proteins and lipids of the membrane. It destroys the protein conformation by removing the negative ions from the proteins and as the protein conformation is destructed the structure of proteins are lost and finally the cell membrane is disrupted.
- ✓ 5M NaCl: It prevents DNA from denaturation and removes the protein bound to the DNA and keep the proteins dissolved in the aqueous layer.
- ✓ ddH_2O

- 5mM Potassium acetate solution – It is prepared by dissolving 0.024g of potassium acetate in 50mL distilled H_2O. Potassium acetate solution is used for precipitation of the cellular debris.
- Phenol: Chloroform: Isoamyl alcohol solution – P:C: I solution is prepared of ratio 25:24:1. Here, phenol is used for protein denaturation and for separating DNA from proteins. Phenol is a non-polar solvent so DNA is insoluble in phenol whereas as proteins has both polar and non-polar end, so they are soluble in it. Also, it is known that protein folding into its secondary and tertiary structures relies on the amino acid polarity, so when phenol is added the bonds between amino acid break which ultimately denatures the protein.

  Chloroform increases the efficiency of phenol to denature the proteins and also allows proper separation of organic and aqueous phase. It also keeps DNA separated and protected in the aqueous phase.

  Isoamyl alcohol reduces the foaming between the interphase.
- Preparation of TE buffer – TE buffer is prepared by adding Tris HCl in 0.5M EDTA. TE buffer helps in solubilizing the DNA by protecting it from denaturation.

3.9 Agarose Gel Electrophoresis – To visualise the DNA fragments 0.8% of agarose gel is used [38]. Firstly, 0.8g of agarose is weighed and then dissolved in 100mL of TAE buffer. The solution is then heated to completely dissolve the agarose. Then, EtBr is added to the solution to a concentration of 0.5µg/mL and allowed the solution to cool. In the meantime, the gel tray is prepared by tapping the open edges of the tray to create

a mould. Then placing the comb in the gel mould the agarose solution is poured and then allowed the gel to solidify. After the gel is set properly the comb is removed and the taps are removed and then the gel tray was kept in electrophoresis unit, in a way that the wells are at the anode terminal. In the electrophoresis unit TAE buffer was added so that the wells are properly covered with the buffer. Then DNA samples were prepared. To prepare the DNA samples, in a parafilm to 3µL of DNA 1µL of loading dye was added and mixed properly. Then the 4 µL sample was loaded carefully to the wells of the gel. After loading the samples, the power supply was turn on and the samples start moving from the anode to the cathode.

Preparation of TAE buffer: TAE buffer is used as the running buffer and is prepared by adding Tris base in glacial acetic acid and then adding 0.5M EDTA to it. The solution is properly mixed and then the volume is maintained using dH_2O to it. This gives the 10X stock solution of TAE buffer which is then made into 1X working solution.

3.10 Designing Primers with restriction enzyme sites – To design the specific primers first the gene sequence of *Bacillus megaterium* rocF gene of strain ATCC 14581 was procured first. Then the gene sequence was pasted in the NEB cutter and in the NEB cutter 0 cutters was selected which gives the list of restriction enzymes that do not cut the gene sequence. From the list two restriction enzymes which do not cut the gene sequence were selected and forward and reverse primers were designed using them [39]. To design the forward primer the first 21bp of the sequence was selected and then at the 5'end of the sequence the R.E site of NdeI (CATATG) was added. Prior to the R.E site 3 codons were added and thus the 5'-3' forward primer was designed successfully. Then to design the reverse primer first 3 codons were written and then following it the R.E site of EcoRI was added. Then the last 20bp of the gene sequence was selected and transcribed and then was written after the R.E site in 3'-5' direction. This gave the 5'-3' reverse primer. After the primers are designed their Tm has been calculated by using the formula 2(A+T) +4(G+C).

3.11 Plasmid DNA isolation by alkaline lysis method with SDS – Plasmid DNA was isolated using the alkaline lysis method with SDS protocol as described in the Volume 2 of the book "Molecular Cloning – A Laboratory Manual" by Sambrook. J. and Russell. W. D., from *E. coli* DH5α strain [24].

First, the culture was revived from the glycerol stock in a NB media containing kanamycin and then was streaked in nutrient agar plates containing kanamycin. Then, one pure colony from the agar plate was inoculated in a 25mL LB media containing kanamycin and was

incubated at 37℃ for overnight. Then, the next day 15mL culture was taken and was centrifuged at 4000rpm for 10min at 4℃. The supernatant was removed and then the pellet was allowed to dry completely. After the pellet was dried completely 2.5mL of STE was added to it and was again centrifuged at 4000rpm for 10min. The pellet formed was then resuspended in 200µL of ice-cold Alkaline lysis solution I and was mixed thoroughly by vortexing. To the 200µL solution 400µL of freshly prepared alkaline lysis solution II was added and the contents was mixed by inverting 5-6times. Then the tube was stored on ice and to the solution 300µL of ice-cold alkaline lysis solution III was added. The content was then properly mixed by inverting the tube for several times and then the tube was stored on ice for 5min. After 5min, the tube was centrifuged at max speed for 5min at 4℃. After centrifugation, the supernatant was transferred to a fresh tube and to it 600µL of phenol: chloroform (1:1) was added. After adding phenol: chloroform the contents were again mixed properly by vortexing and then was centrifuged for 2min at 4℃.

The upper aqueous layer formed after centrifugation was transferred to a fresh tube and to it 600µL of isopropanol was added at room temperature. The solution was mixed by vortexing and then was kept at room temperature for 30min. After 30min. the solution was centrifuged at max speed for 5min at room temperature and then the pellet was collected and was allowed to dry. After the pellet was dried completely, it was washed with 1mL of 70% ethanol. After washing with ethanol, the pellet was allowed to dry completely and then the pellet was dissolved in 100µL of TE buffer containing RNase A and then was stored at -20℃.

Composition of different components used in the protocol [24]:

- Alkaline Lysis Solution I- Alkaline lysis solution I was prepared by adding 50mM glucose, 25mM Tris-Cl (pH 8.0) and 10mM EDTA (pH 8.0). The solution after preparation was autoclaved and then was stored at 4degree Celsius. Glucose helps in preventing the immediate osmotic lysis of the bacteria and also prevent the DNA from shearing. Tris-Cl acts as buffer for solution II and it also prevent irreversible denaturation of covalently closed circular DNA. EDTA acts as chelating agent and disrupts the outer membrane by chelating the Ca^{2+} and Mg^{2+} ion.
- Alkaline Lysis Solution II- It was prepared by adding 0.2N NaOH and 1%SDS. This solution denatures the protein, chromosomal and linear DNA.
- Alkaline Lysis Solution III- It was prepared by adding 5M potassium acetate, glacial acetic acid and dH_2O. Potassium acetate helps in precipitation of cell

debris and glacial acetic acid neutralizes the pH and allows the DNA strands to renature.

- STE – It was prepared by adding 10mM Tris-Cl (pH 8.0), 0.1M NaCl and 1mM EDTA (pH 8.0).

3.12 Polymerase Chain Reaction – PCR reaction mixture of 20µL is prepared first in sterile PCR tubes by adding the components in the following order:

Table 2: Components of PCR reaction in order:

Components		Volume of each component (µL)
i)	Nuclease-free water	8.2
i)	Taq 2X Green Mater mix (2X)	10
ii)	Forward Primer (10pM)	0.4
iv)	Reverse Primer (10pM)	0.4
v)	Template DNA (9.4ng)	1

The reaction is prepared by keeping the tubes in ice. After the reaction is prepared, the thermocycler is switched on, and the following reaction condition is set with different annealing conditions:

Table 3: Reaction conditions in which PCR was performed [36]

1st Reaction condition			
Steps	Temperature	Time	Total No. of cycles
Initial Denaturation	94℃	5min	1
Denaturation	94℃	45sec	35
Annealing	52℃,55℃,57℃,59℃,61℃,64℃	45sec	
Extension	72℃	2min	
Final Extension	72℃	5min	1
2nd Reaction condition			
Steps	Temperature	Time	Total No. of cycles

Initial Denaturation	94℃	5min	1
Denaturation	94℃	45sec	35
Annealing	56℃,57℃,58℃,59℃,60℃,62℃	45sec	
Extension	72℃	2min	
Final Extension	72℃	5min	1
3rd Reaction condition			
Steps	Temperature	Time	Total No. of cycles
Initial Denaturation	94℃	5min	1
Denaturation	94℃	45sec	35
Annealing	56℃,59℃,61℃,63℃,65℃,67℃	45sec	
Extension	72℃	2min	
Final Extension	72℃	5min	1
4th Reaction condition			
Steps	Temperature	Time	Total No. of cycles
Initial Denaturation	94℃	3min	1
Denaturation	94℃	30sec	35
Annealing	50℃,52℃,54℃,56℃,58℃,60℃	35sec	
Extension	72℃	35min	
Final Extension	72℃	4min	1

After the PCR was performed to check if the template DNA has been amplified agarose gel electrophoresis is performed in 1.2% gel.

3.13 Enzyme Assay – To perform the enzyme activity firstly, 200mL of minimal media was prepared of the following composition [34]:

i. $(NH_4)_2SO_4$: 0.75g
ii. KH_2PO_4: 0.75g
iii. $MgSO_4.7H_2O$: 0.15g
iv. Arginine: 0.375g
v. NaCl: 0.375g

vi. Glucose: 1g

The minimal media was prepared and autoclaved and was kept at 37℃ incubator to check for any contamination. The next day, the media was inoculated using 5mL culture of *Bacillus megaterium* from nutrient broth and was kept in the incubator shaker for overnight at 37℃. After there was sufficient growth achieved in the media, 50mL of culture was taken in a falcon tube and was centrifuged. After centrifugation the supernatant (will be referred as supernatant1) was stored in another falcon that will be used for determining the extracellular enzyme activity and the pellet was washed 2-3times with 50mM phosphate buffer of pH 7.2. After washing the pellet with phosphate buffer, it was dissolved in 5mL phosphate buffer and was sonicate for 10cycles with 10sec on and 20sec off. After sonication, it was again centrifuged and the pellet was discarded while the supernatant (will be referred as supernatant2) was used for determining the intracellular enzyme activity. Then both the supernatant 1&2 was used for performing the activity assay. For activity assay, the protocol mentioned in the paper entitle "Expression, Purification and Characterization of Arginase from *Helicobacter pylori* in Its Apo Form" published by Z. Jinyong, Z. Xiaoli, W. Chao, L. Dongshui, G. Gang, M. Xuhu, Z. Ying, W. Dacheng, L. Defeng, Z. Quanming was used [40]. For performing the activity assay 25µL of enzyme (both intracellular and extracellular) was added to 25µL of 10mM $MnSO_4$. The solution was then heated at 55℃ for 30min and after 30min, 200µL of 10mM L-arginine dissolved in phosphate buffer was added. The solution was again incubated at 37℃ for 1hr and then after 1hr 750µL of acetic acid was added to stop the reaction. Then 250µL of 4mg/mL ninhydrin was added to generate the colour and was incubated at 95℃ for 1hr. After 1hr OD was taken at 515nm using multiskan spectrophotometer.

This method for performing the enzyme activity was performed for four different test samples.

- Test 1 – Performing the enzyme assay using both extracellular and intracellular enzyme to check whether the enzyme is intracellular or extracellular.
- Test 2 – Performing the enzyme assay with the enzyme which showed the maximum activity in test 1 after replacing the 25µL $MnSo_4$ with water (i.e., without metal ion).
- Test 3 – Performing the enzyme assay with the enzyme which showed the maximum activity in test 1 after replacing the 25µL $MnSo_4$ with water (i.e., without metal ion) and removing the half and hour incubation at 55℃.
- Test 4 – Performing the enzyme assay by increasing both the amount of enzyme and $MnSO_4$ to 100µL.

Table 4: Enzyme Assay for Arginase

Tests	Amount of enzyme (µL)	Amount of 10mM $MnSO_4$ (µL)		Amount of 10mM L-arginine (µL)					
Blank	0	25	Heated at 55°C for 30min	200					
Control 1	25 (Extracellular enzyme)	25	Heated at 55°C for 30min	0					
Control 2	25 (Intracellular enzyme)	25	Heated at 55°C for 30min	0	INCUBATED AT 37°C FOR 1Hr	Added 750µL Of Acetic acid	Added 250µL Of 4mg/mL ninhydrin	INCUBATED AT 95°C FOR 1Hr	Taken OD At 515nm
Test 1	25	25	Heated at 55°C for 30min	200					
Test 2	25	0 (Added 25µL water instead)	Heated at 55°C for 30min	200					
Test 3	25	0 (Added 25µL water instead)	----------	200					
Test 4	100	100	Heated at 55°C for 30min	200					

The standard curve was prepared according to the following table and was plotted with absorbance at 515nm on "Y axis" and concentration of L-ornithine on "X-axis".

Table 5:Standard curve preparation table of ornithine

Conc. Of L-ornithine (µM)	Ornithine Volume (µL)	Phosphate buffer Volume (µL)	Added 750 µL Of Acetic acid	Added 250 µL Of Ninhydrin	INCUBATED AT 95°C	Taken OD At 515nm
0	0	250				
20	50	200				
40	100	150				
60	150	100				
80	200	50				
100	250	0				

After the graph was plotted, and the value of unknown was found enzyme activity was calculated using the following formula:

$$\textbf{Enzyme Activity} = \frac{\mu \boldsymbol{M\ of\ product\ formed}}{\boldsymbol{Incubation\ time \times Volume\ of\ enzyme}} \times \boldsymbol{Vol.of\ Reaction}$$

3.14 Protein Estimation – For protein estimation, Folin-lowry method was used and the amount of protein was estimated spectrophotometrically by taking OD at 660nm. Firstly, 1mL of sample was taken and to it 5mL of solution C was added. The solution was then vortexed and incubated for 10min at room temperature. Then, to it 0.5mL FCR reagent was added and vortexed and incubated in dark for 30min at room temperature. After 30min, OD was taken at 660nm using multiskan spectrophotometer. The standard curve was prepared according to the following table and was plotted with absorbance at 660nm on "Y axis" and concentration of BSA (mg/mL) on "X-axis".

Composition of the different solutions used are:

- Solution C – For preparation of Solution C, solution A and B is required to be prepared first. Solution A was prepared by dissolving 2% Sodium carbonate in 0.1N NaOH, i.e., 2g in 100mL NaOH and solution B was prepared by dissolving 0.5% copper sulphate

in 1% sodium potassium tartarate. Then after A and B solutions are prepared 50mL of solution A was added to 1mL of solution B which ultimately gave the solution C.

- FCR – FCR is prepared by dilution in the ratio of 1:1, i.e., if 100mL of 10mL FCR is being prepared 5mL of FCR will be added in 5mL of dH_2O.

Table 6: Standard curve preparation table of BSA.

Conc. Of BSA (mg/mL)	Volume of BSA (µL)	Volume of dH_2O (µL)	Added 5mL of Solution C	Vortexed and Incubated for 10min at room temperature	Added 0.5mL of FCR reagent	Vortexed and Incubated for 30min at dark in room temperature	Taken OD At 660nm
0	0	1000					
0.2	200	800					
0.4	400	600					
0.6	600	400					
0.8	800	200					
1	1000	0					

After the graph was plotted, and the value of unknown was found protein estimation was calculated using the following formula:

$$\boldsymbol{Protein\ Estimation\ = \frac{1}{slope} \times OD\ at\ 660nm}$$

After both the enzyme activity and protein estimation was calculated specific activity of the enzyme was calculate by using the following formula:

$$\boldsymbol{Specific\ Activity\ = \frac{Enzyme\ activity}{Protein\ Estimation}}$$

Chapter 4 –

RESULTS AND DISCUSSIONS

Chapter 4 – RESULTS AND DISCUSSIONS

4.1 Culture Revival – The culture of *Bacillus megaterium* was revived successfully in nutrient broth media after incubating at 37℃ for overnight. In the same way, the culture of plasmid DH5α strain was also revived from glycerol stock successfully in nutrient broth media containing kanamycin for providing antibiotic resistance, so that only recombined *E. coli* cells grow after incubating at 37℃ for overnight.

4.2 Subculturing – The culture of *B. megaterium* after revival was subcultured in nutrient agar plates by quadrant streaking and pure colonies were obtained after incubating at 37℃ for overnight. The culture of plasmid DH5α strain was also subcultured in nutrient agar plates by quadrant streaking containing kanamycin for providing antibiotic resistance, so that only recombined *E. coli* cells grow and pure colonies were obtained after incubating at 37℃ for overnight.

Fig. 11: Subculturing of *B. megaterium* culture in nutrient agar plates.

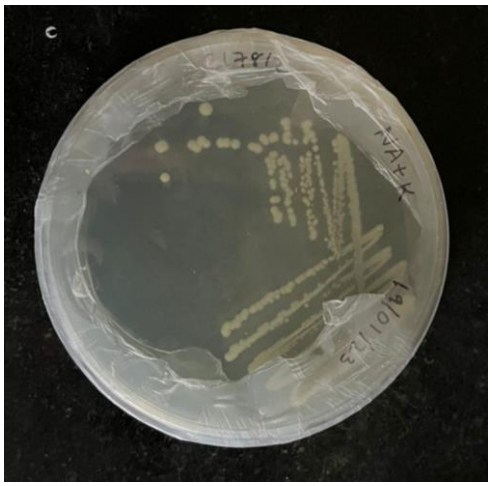


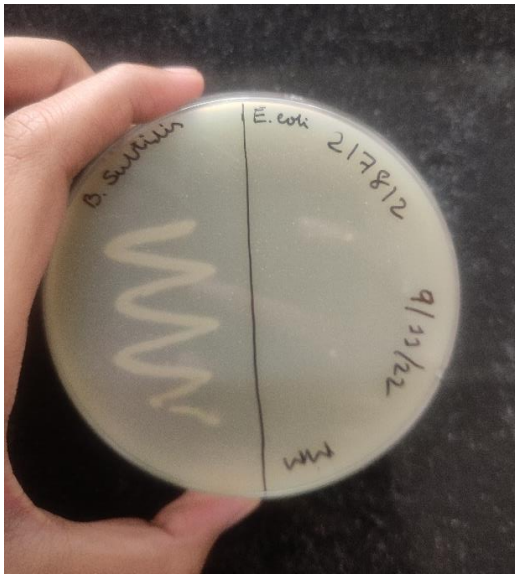
Fig.12: Subculturing of *E. coli* DH5α strain culture in nutrient agar plates containing kanamycin.

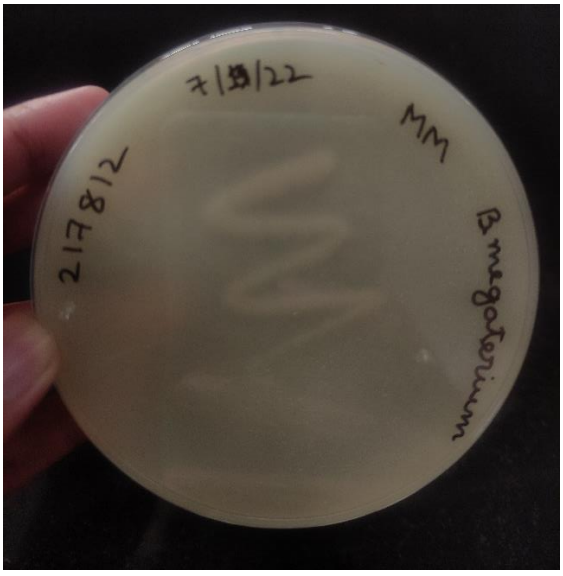
4.3 Primary Culturing – The pure colonies from the nutrient agar plates of *B. megaterium* was used for primary culturing in LB broth media and was successfully cultured after incubating at 37℃ for overnight and in the same way, pure colonies from the nutrient agar plates of *E. coli* was used for primary culturing in LB broth media containing kanamycin for providing antibiotic resistance, and was successfully cultured after incubating at 37℃ for overnight.

4.4 Secondary Culturing - To increase the growth of *B. megaterium* it was again cultured in LB broth by inoculating culture from the primary culture. The culture was successfully grown in the LB broth after incubating at 37℃ for overnight. Also, to increase the growth of *E. coli* it was also cultured again in LB broth containing kanamycin by inoculating culture from the primary culture. The culture was successfully grown in the LB broth after incubating at 37℃ for overnight.

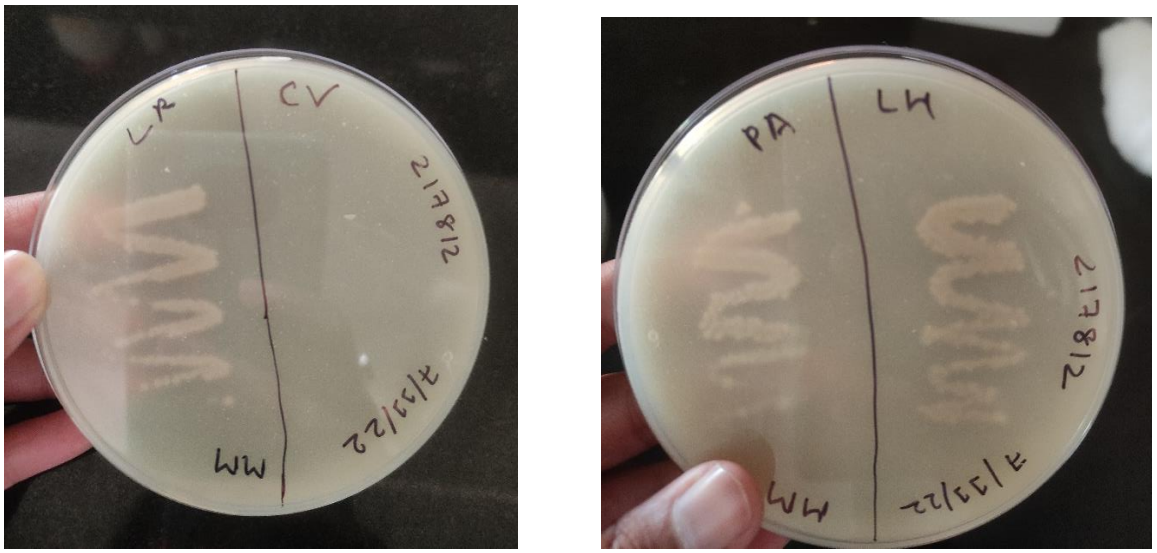
4.5 Glycerol Stock Preparation – For both the cultures of *B. megaterium* and *E. coli* DH5α strain glycerol stock was prepared so that the culture can be stored and used for future use.

4.6 Plate Assay for Screening – From the 7 cultures that were *B. subtilis, E. coli, B. megaterium, L. rhamnosus, C. violaceum, P. aeruginosa* and *L. helveticus*, positive results was obtained in the following 5 cultures, i.e., *B. subtilis, B. megaterium, L. rhamnosus, P. aeruginosa* and *L. helveticus* which proved that these five cultures contain the arginase gene so they were able to utilise the arginine present in the minimal media and grow. This screening experiment helped us to confirm experimentally that the culture of *B. megaterium* contains the gene which codes for arginase and also there are other cultures which have the arginase coding gene present.





1. *B. subtilis*
2. *E. coli*
3. *B. megaterium*



4. *L. rhamnosus*
5. *C. violaceum*
6. *P. aeruginosa*
7. *L. helveticus*

Fig.13: Plate Assay of seven different bacterial cultures

4.7 Purity Check by Gram Staining – Gram Staining was performed to check the purity of the culture and to check if the bacteria is gram positive or gram negative. As the bacteria appeared to be rod-shaped this proved that the culture is pure and is of the genus *Bacillus*. This also showed that it is a gram-positive bacterium because the cultures did not lose the violet color after washing with ethanol.

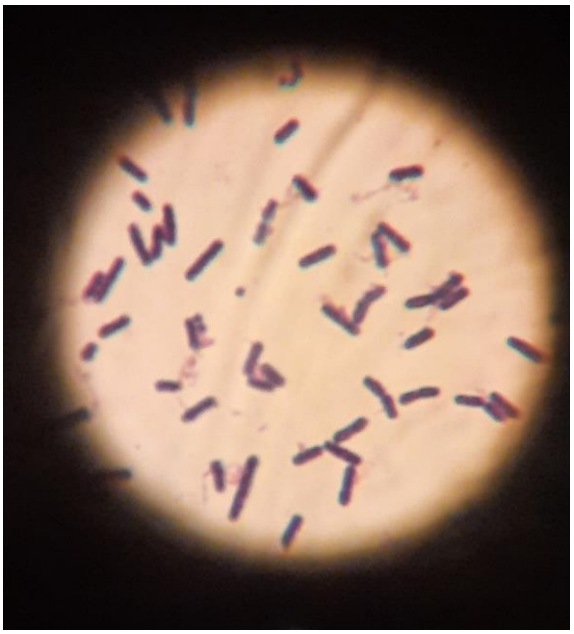


Fig.14: Gram Staining result of *B. megaterium* culture

4.8 DNA extraction – To isolate the bacterial DNA from the *Bacillus megaterium* culture the protocol described in "Research Journal of Pharmaceutical, Biological and Chemical Sciences" was used and then agarose gel electrophoresis was performed to visualise the isolated DNA. A 1kb ladder was run along with the samples of DNA to confirm the DNA isolation. The fig. shows the DNA bands and the quality of DNA can be accessed by comparing them with the highest band of the ladder, i.e., the DNA bands which are seen at the top can be said as the good quality DNA bands and the bands below the top bands are the medium quality band of maybe size 25kb.

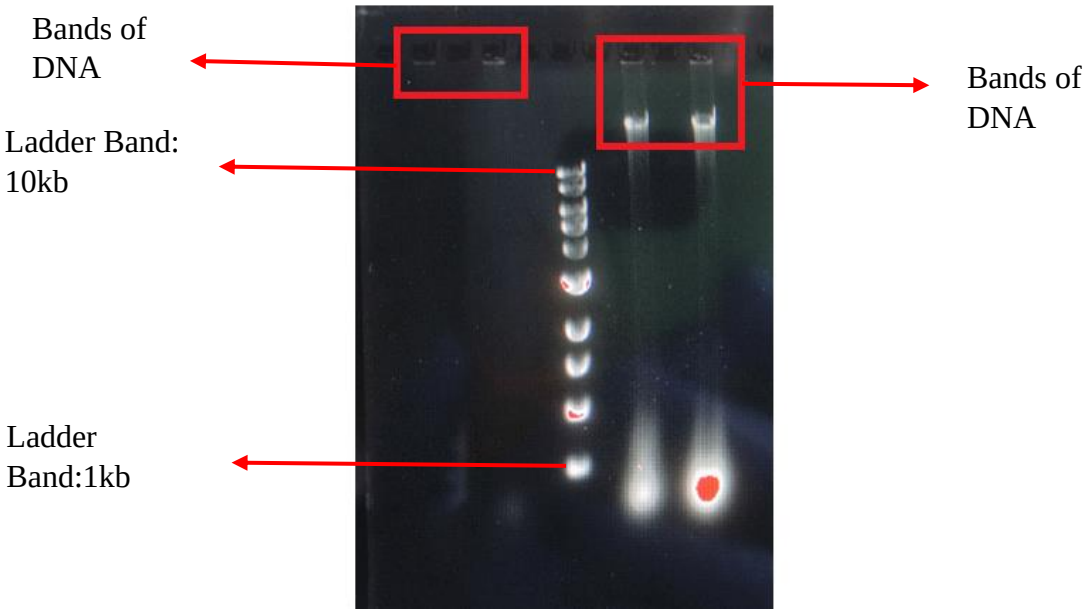


Fig.15: DNA isolation from *B. megaterium* culture

The isolated DNA is analysed for its purity and quantity and it is done by measuring the absorbance at 260 and 280nm. As the one absorbance unit of DNA is equal to 50 µg/cm^3, so the concentration of DNA is calculated by:

$$[DNA] = 50 \times A_{260}\ \mu g/cm^3$$

The ODs obtained at 260nm and 280nm after measuring spectrophotometrically are 0.188 and 0.140 respectively. Therefore, the concentration of DNA was calculated to be 9.4ng/µL. The purity of the DNA is determined by the ratio of A_{260}/A_{280}. If the ratio is approximately 1,8 then it is considered as pure DNA and if the ratio is < 1.8 then there is considered to have protein contamination and if the ratio is >1.8 then there is considered to have RNA contamination. The

ratio between A_{260}/A_{280} was obtained to be 1.34, this indicated that the DNA sample has protein contamination in it.

4.9 Primer Designing - To design the specific primers first the protein sequence and gene sequence of *Bacillus megaterium* rocF gene of strain ATCC 14581 was procured first.

- Protein sequence of *Bacillus megaterium* rocF gene of strain ATCC 14581 procured from UniProtKB

MNKLSIIGVPMDLGQTRRGVDMGPSAIRYAGVIERIENIGYSVEDKGNIEIALAE RVHSDENTNLKNLKAVADASERLAQTVSDVITNKRFPLVLGGDHSIAIGTLAGVS RHYKNLGVIWYDAHGDLNTADTSPSGNIHGMPLAASIGIGDDALTRIGGYTPKV KPENIVIIGARSLDEGEKELIKEKGIKVYTMHEIDRMGMTKVMEETILYLREKT DGVHLSLDLDGLDPHDAPGVGTPVIGGISYRESHLAMEMLAESQLITSAEFVEV NPILDERNKTATVAVALMGSLLGEKLV

- Gene sequence of *Bacillus megaterium* rocF gene of strain ATCC 14581procured from GenBank

ATGAATAAGCTTTCAATTATTGGAGTACCAATGGATTTAGGTCAAACTCGCC GAGGGGTCGATATGGGGCCAAGTGCGATACGATATGCAGGGGTTATTGAAC GCATCGAGAATATTGGATATAGTGTAGAAGATAAAGGGAATATTGAAATTGC CTTAGCTGAACGAGTACATAGTGATGAAAATACAAACTTAAAGAATTTAAAA GCTGTAGCAGATGCTAGCGAGAGGCTTGCTCAGACCGTAAGTGATGTTATT ACAAATAAAAGGTTTCCTTTAGTGCTAGGGGGAGATCACAGCATCGCGATC GGAACACTAGCAGGAGTAAGTCGTCATTATAAAAACTTGGGCGTGATTTGG TATGATGCACACGGAGATTTGAATACAGCAGATACGTCTCCTTCAGGAAATA TTCACGGTATGCCATTGGCGGCAAGTATAGGTATAGGAGACGACGCATTGA CTCGAATTGGCGGTTATACACCTAAAGTCAAGCCTGAAAATATTGTGATTAT CGGAGCACGTTCTTTAGATGAAGGAGAAAAAGAGTTAATAAAGGAAAAAGG GATTAAAGTGTATACGATGCATGAGATAGATCGAATGGGTATGACAAAAGT AATGGAAGAAACAATTTTATATTTGCGTGAAAAAACAGATGGCGTTCATCTA TCACTCGATTTAGATGGATTGGATCCACATGATGCACCTGGAGTAGGAACG CCGGTCATCGGTGGAATAAGCTACAGAGAAAGTCATTTAGCTATGGAGAT GTTAGCAGAATCTCAGCTAATCACTTCTGCTGAGTTTGTGGAAGTGAATCCT ATTTTAGATGAACGGAATAAAACCGCTACAGTAGCTGTTGCATTAATGGGCT CACTTTTAGGAGAGAAATTAGTATAA

After the gene sequence was procured forward and backward primers containing restriction enzyme sites were designed manually. The restriction enzymes that do not cut the gene sequence were selected from the NEB cutter. Here, NdeI (CATATG) and EcoRI (GAATTC) was selected as they do not have any cut site in the gene sequence

- Designed Primers with their calculated Tm values:
  - ✓ **Forward Primer** – ATA CAT ATG AAT AAG CTTT CAA TTA TTGG

  Calculated Tm value is – 56.78℃
  - ✓ **Backward Primer** – AAT GAA TTC TTA TAC TAA TTT CTC TCC TAA AAG

  Calculated Tm value is – 59.54℃

4.10 Plasmid DNA Isolation –

Plasmid DNA was isolated successfully using the alkaline lysis method with SDS protocol as described in the Volume 2 of the book "Molecular Cloning – A Laboratory Manual" by Sambrook. J. and Russell. W. D., from *E. coli* DH5α strain. To visualise the plasmid DNA, agarose gel electrophoresis was performed which photo is shown below. The gel when seen in GelDoc showed bands which confirmed that plasmid DNA was successfully isolated.

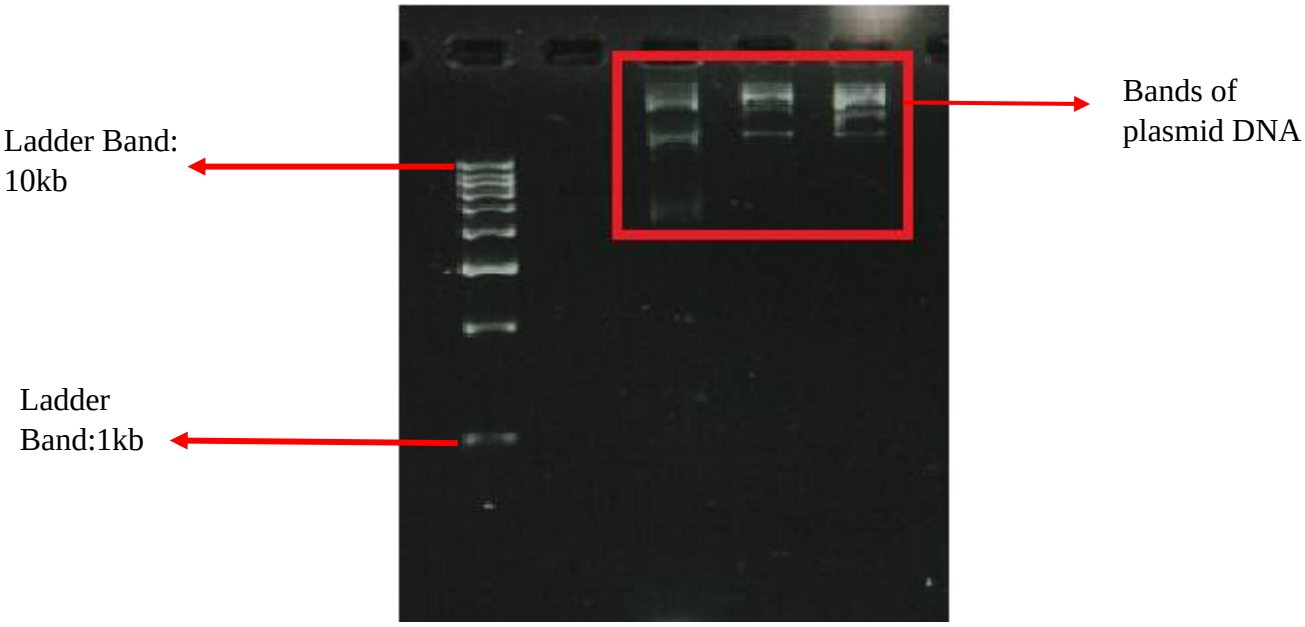


Fig.16: Plasmid DNA isolation from culture *E. coli* DH5α strain

4.11 Polymerase Chain Reaction – Gradient PCR was performed with different annealing temperature as mentioned in the methodology section and to visualize the product agarose gel electrophoresis was performed but there were no significant bands seen which confirmed that there was no amplification occurred. This might be due to many reasons so several troubleshooting steps were performed. First, the primers specification was checked if they were designed correctly, it was done by running BLAST and it was shown that the primers perfectly bind to the gene sequence. It was also stated in a ThermoFisher Scientific trouble shooting guide if there is no amplification the annealing temperature should be lowered by 5℃ from the lowest melting temperature of the primer and the gradient should be set at a difference of 2-3℃ but amplification was still failed [41]. The mastermix and other components used in the reaction was also changed if they were causing the problem but it also has not shown any prominent results. Also, as primer dimers were seen to be formed the template DNA quantity was also reduced but there was no amplification seen. After several attempts, when the DNA was not amplified, a new culture order was placed of *Bacillus megaterium* (Strain 14581).

4.12 Enzyme Assay – First, the standard curve of ornithine was prepared using the methodology as described in the paper entitle "Expression, Purification and Characterization of Arginase from *Helicobacter pylori* in Its Apo Form" published by Z. Jinyong, Z. Xiaoli, W. Chao, L. Dongshui, G. Gang, M. Xuhu, Z. Ying, W. Dacheng, L. Defeng, Z. Quanming.

Table 8: Standard Curve readings of ornithine:

Conc. Of L-ornithine (µM)	**OD at 515nm**
0	0
20	0.154
40	0.247
60	0.361
80	0.451
100	0.614

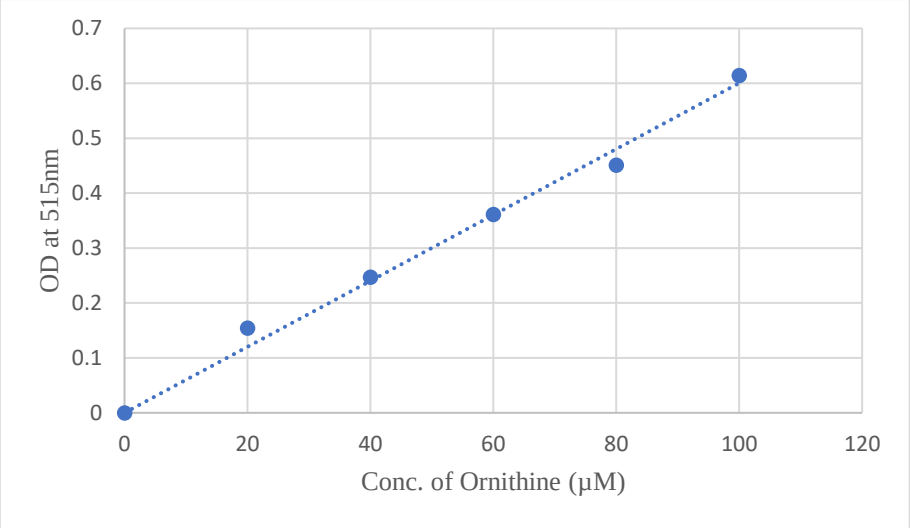


Fig.17: Standard curve L-ornithine

Table 9: Readings of Enzyme Assay:

Tests	**OD at 515nm**
Blank	0.076
Control 1	0.300
Control 2	0.081
Test 1A (extracellular enzyme)	0.045
Test 1B (intracellular enzyme)	0.021

From this table, we can infer that arginase is an extracellular enzyme as its absorbance at 515nm is found to be maximum in case of extracellular enzyme than the intracellular ones.

Tests	**OD at 515nm**
Blank	0.076
Control 1	0.300
Control 2	0.076
Test 2	0.036
Test 3	0.080
Test 4	0.058

Calculated Enzyme Activity:

- Test 1A – 3.84 U/mL
- Test 2 – 2.82 U/mL
- Test 3 – 7.82 U/mL
- Test 4 – 1.53 U/mL

As, the arginase was found as the extracellular enzyme from the initial tests, so extracellular enzyme was taken to check for other parameters. As arginase is a metalloenzyme so from the enzyme assay it is proved that metal ion is required for its activity because when the source of metal ion, i.e., $MnSO_4$ in this case, is removed the enzyme activity decreased from 3.84U/mL to 2.82 U/mL. But when the metal ion concentration was removed excluding the incubation step of half and hour and directly adding the substrate source, i.e., arginine to it the enzyme activity although increased to 7.82U/mL. It may be because, as the substrate was added directly to enzyme without any incubation time the chances of enzyme-substrate interaction increased. It has also been seen that when the volume of enzyme is increased along with increasing the volume of $MnSO_4$ but keeping the volume of substrate same the enzyme activity also decreased, it may be because of substrate depletion. Because, when the metal ion and enzyme volume is increased but the arginine volume is kept same, the concentration of substrate per unit volume gets decreased, which can ultimately result in the depletion of substrate, i.e., as the volume of enzyme is increased its requirement of substrate also increased, and in this the enzyme may not have found enough substrate molecules to bind to and hence cannot catalyse the reaction further, which results in decreasing enzyme activity to 1.53 U/mL

4.13 Protein Estimation – First, the standard curve of BSA was prepared using Folin-Lowry method.

Table 10: Standard Curve readings of BSA:

Conc. Of BSA (mg/mL)	OD at 660nm
0	0
0.2	0.265
0.4	0.421
0.6	0.617
0.8	0.777
1	0.878

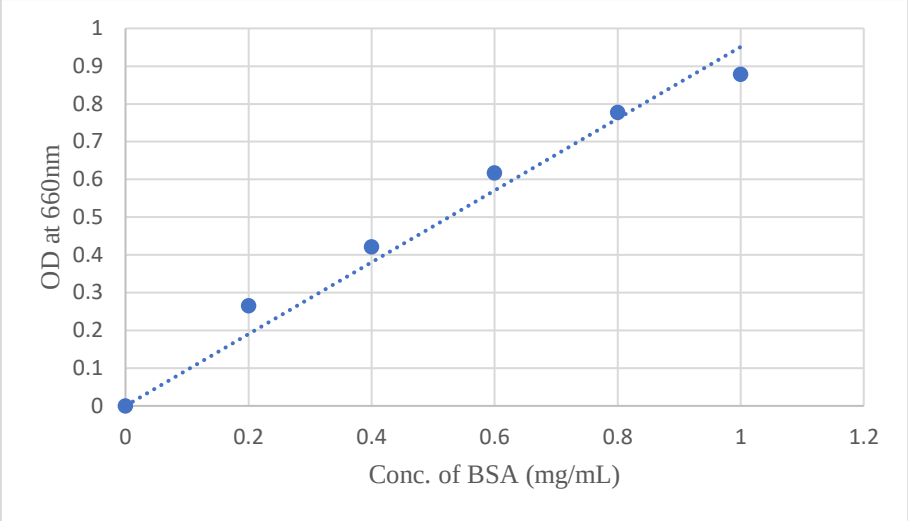


Fig. 18: Standard curve BSA

Table 11: Readings of Protein Estimation:

Tests	**OD at 660nm**
Extracellular enzyme	0.267

Calculated Protein Estimation – 1.089 mg/mL

Calculated Specific Activity – 3.52 U/mg

The protein concentration of the enzyme arginase was estimated to be 1.089 mg/mL by Folin-lowry method and the specific activity of arginase was also calculated which was found to be 3.52 U/mg.

Chapter 5 –

CONCLUSION

Chapter 5 – CONCLUSION

As the techniques to treat cancers are very expensive, so with the increasing research in the field of cancer a new treatment method was identified that is depletion of semi essential amino acids that are needed by cancerous cells. There were several cancer cells that were identified to have an important feature of arginine auxotrophy, for which those cancer cell lines were dependent on the extracellular nutritional pool to fulfil their nutrient demand. Enzymatic degradation of arginine was identified to be one of the most efficient approaches to deprive arginine, and among the enzymes involved in arginine degradation arginase was one of the enzyme which is a metalloenzyme belonging to the ureohydrolase class was found to have several advantages over others. So, in this project we have focussed on the *Bacillus megaterium* arginase which is unexplored till date.

In this project, we isolated DNA from the culture of *Bacillus megaterium* and tried performing PCR using it as the template DNA. Also, we characterized the arginase using enzyme assay and protein estimation methods. From the enzyme assay it has been found that arginase is an extracellular enzyme as its absorbance at 515nm is found to be maximum in case of extracellular enzyme than the intracellular ones. Also, as arginase is a metalloenzyme so from the enzyme assay it is proved that metal ion is required for its activity because when the source of metal ion, i.e., $MnSO_4$ in this case, is removed the enzyme activity decreased from 3.84U/mL to 2.82 U/mL. But when the metal ion concentration was removed excluding the incubation step of half and hour and directly adding the substrate source, i.e., arginine to it the enzyme activity although increased to 7.82U/mL. It may be because, as the substrate was added directly to enzyme without any incubation time the chances of enzyme-substrate interaction increased. It has also been seen that when the volume of enzyme is increased along with increasing the volume of $MnSO_4$ but keeping the volume of substrate same the enzyme activity also decreased, it may be because of substrate depletion. Because, when the metal ion and enzyme volume is increased but the arginine volume is kept same, the concentration of substrate per unit volume gets decreased, which can ultimately result in the depletion of substrate, i.e., as the volume of enzyme is increased its requirement of substrate also increased, and in this the enzyme may not have found enough substrate molecules to bind to and hence cannot catalyse the reaction further, which results in decreasing enzyme activity to 1.53 U/mL. The protein concentration of the enzyme arginase was estimated to be 1.089 mg/mL by Folin-lowry method and the specific activity of arginase was also calculated which was found to be 3.52 U/mg.

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