"EXTRACTION OF THE ENZYME ASPARAGINASE FROM

Mycobacterium smegmatis"

Dissertation/project report submitted for partial fulfillment of the requirement for the degree of

BACHELOR OF TECHNOLOGY

IN BIOTECHNOLOGY

Under the supervision of

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DECLARATION

We hereby declare that the work reported in the B.Tech. thesis entitled "**Extraction of the enzyme asparaginase from *Mycobacterium smegmatis***" submitted at **Jaypee University of Information Technology, Waknaghat** is an authentic record of our work carried out under the supervision of **Dr. Saurabh Bansal**. We have not submitted this work elsewhere for any other degree or diploma.

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CERTIFICATE

This is to certify that the work reported in the B.Tech. thesis entitled "**Extraction of the enzyme asparaginase from *Mycobacterium smegmatis***", submitted by **Apoorv Sharma** and **Pallavi Ganate** at **Jaypee University of Information Technology, Waknaghat** is a bonafide record of their original work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.

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ABSTRACT

Mycobacterium smegmatis is a non-tuberculous, non-pathogenic, rapidly growing environmental species of mycobacterium which is present ubiquitously in soil and water. Readily cultivable in most synthetic or complex laboratory media and afast grower, it is used as an attractive model organism and a surrogate host for genetic analysis of the worldwide pathogen *Mycobacterium tuberculosis*.

L-asparaginase (LA) catalyzes the degradation of asparagine, an essential amino acid for leukemic cells, into ammonia and aspartate. Owing to its ability to inhibit protein biosynthesis in lymphoblasts, LA is used to treat acute lymphoblastic leukemia (ALL). Different isozymes of this enzyme have been isolated from a wide range of organisms, including plants and terrestrial and marine microorganisms. Owing to the huge medical importance, there has been an increased market demand of L- asparaginase. Hence a lot of other potential sources are being tried and tested apart from the conventional sources such as *Escherichia coli* and *Erwinia chrysanthemi.*

In this study here, we are trying to explore *M. smegmatis* as another potential candidate for the production of L- asparaginase owing to the similarity it shares with L- asparaginase from *Pyrococcus furiosus* (another organism that produces thermostable L- asparaginase).

OBJECTIVES

- To isolate the pure colony of ***Mycobacterium Smegmatis MC^{2}155.***
- To establish the presence of the enzyme asparaginase in *M. smegmatis.*
- To design primers
- To isolate genomic DNA
- Cloning

CHAPTER 1

INTODUCTION

The enzyme asparaginase has garnered a lot of attention lately owing to its usage in both, the medical industry as well as the food industry. Used as an anti-cancerous substance and as an agent to reduce the formation of acrylamide in food products, asparaginase production has seen a hike in the recent years. Asparaginase earned the approval for medical usage in 1978 in the United States. It is also holds a position in WHO's list of Essential medicines.

The enzyme asparaginase works on the amino acid asparagine and converts it in to aspartate by the process of hydrolysis. This amino acid asparagine is synthesized by the normal cells but the tumor cells, especially the acute lymphoblastic leukemia cells, lack the ability to synthesize this non-essential amino acid namely asparagine. So the tumor cells demand an increased supply of asparagine. Hence they rely on the asparagine that is being circulated around.

If asparaginase is administered to these Acute Lymphoblastic Leukemia cells (ALL cells), the asparaginase, acting as a catalyst, would hydrolyze and convert L-asparagine to aspartic acid and ammonia. This would leave no amino acid asparagine for protein production in the tumor cells, hence leading to their suffocation and consequent cell death.

This was a breakthrough achievement, which was safe and effective, to treat Acute Lymphoblastic Leukemia. Asparaginase also medicates and treats Acute Myeloid Leukemia and Non-Hodgkin's Lymphoma.

The more commonly and more frequently applied usage of the enzyme asparaginase is in the food processing industry.

Acrylamide is a carcinogen. And acrylamide is a by-product in most of the starchy, fried and bakery food products. Asparagine combines with starch, mainly glucose, and leads to the formation of acrylamide.

If asparaginase is administered, asparagine would be broken down and acrylamide production can be hindered. This is a simple yet effective method to combat the unwanted production of the supposed carcinogen, acrylamide.

The enzyme asparaginase is usually, more oftenly derived from the bacterial species of *Escherichia coli* and *Erwinia chrysanthemi.*

But to find out other alternative sources for the enzyme, asparaginase from *Mycobacterium smegmatis* is being studied in this research.

Mycobacterial species can be divided into rapid and slow growers. ***Mycobacterium smegmatis*** is a saprophytic fast grower that shares the house keeping genes and characteristics of long-term survival with those of pathogenic mycobacteria. *M. smegmatis* is thus used as a model for the study of mycobacterial biology and the long-term persistence. However, the fundamental mechanisms of the long-term survival are mostly unknown.

M. smegmatis is useful for the research analysis of other Mycobacterium species in laboratory experiments. *M. smegmatis* is commonly used in work on the Mycobacterium genus due to it being a "fast grower" and non-pathogenic. *M. smegmatis* is a simple model that is easy to work with, i.e., with a fast doubling time and only requires a biosafety level 1 laboratory. The time and heavy infrastructure needed to work with pathogenic species prompted researchers to use *M. smegmatis* as a model for Mycobacterial species. This species shares more than 2000 homologous genes with *M. tuberculosis* and shares the same peculiar cell wall structure of *M. tuberculosis* and other mycobacterial species. It is also capable of oxidizing carbon monoxide aerobically, as is *M. tuberculosis.*

The discovery of plasmids, phages, and mobile genetic elements has enabled the construction of dedicated gene-inactivation and gene reporter systems. The *M. smegmatis* mc^2155 strain is hyper transformable, and is now the work-horse of mycobacterial genetics. Furthermore, it is readily cultivatable in most synthetic or complex laboratory media, where it can form visible colonies in 3–5 days. These properties make it a very attractive model organism for *M. tuberculosis* and other

mycobacterial pathogens. *M. smegmatis* mc^2155 is also used for the cultivation of mycobacteriophage. [5]

CHAPTER 2

REVIEW OF LITERATURE

2.1 The enzyme: ASPARAGINASE

All enzymes are proteins (and in some cases RNA molecules) with varied functions. They catalyze reactions used in normal development, help in the maintenance of the cell, and work as defense against diseases. Enzymes can operate intracellularly, extracellularly, or even on the surface of a cell membrane. Several hundred different enzymes have been identified, and many of them have been characterized to a considerable degree. Quite a few of these enzymes have future of particular interest and command the attention of loyal bands of investigators. However, only a relatively small number have moved to the center stage and turned into the objects of extensive investigations. One of such enzymes is **L-asparaginase.** (Fig. 2.1)

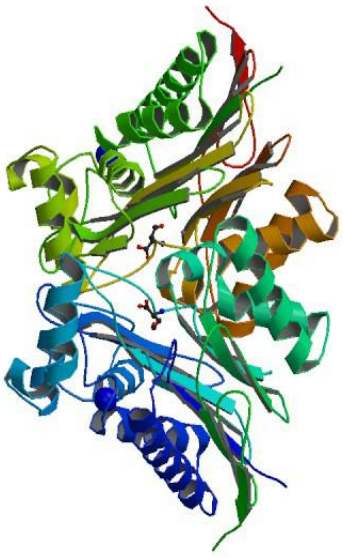

Fig.2.1 3-D structure of L-asparaginase [12]

- **Molar mass:** 31,731.9 g/mol
- **Formula:** $C_{1377}H_{2208}N_{382}O_{442}S_{17}$
- **Biological half-life:** 39-49 hours (IM), 8-30 hours (IV) [For asparaginase sold under trade name of Elsapar, others]

Table 2.1 Characteristics of the enzyme Asparaginase

L-Asparaginases hydrolyzes L-asparagine to L-aspartate and ammonia (Fig.2.2). Both the substrate and the product of this enzymatic reaction play important roles in a number of metabolic processes in all organisms, from bacteria to mammals. In plants, L-asparagine is the most abundant metabolite for storage and transport of nitrogen that is utilized in protein biosynthesis. In the human body, L-aspartate plays an important role as a precursor of ornithine in the urea cycle and in transamination reactions forming oxaloacetate in the gluconeogenic pathway leading to glucose. Initially, the interest in asparaginases was instigated by their antitumor activity [11]

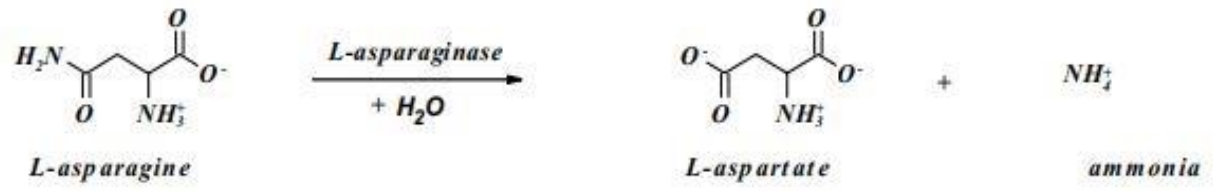

Fig.2.2 Asparaginase reaction [11]

The enzyme asparaginase is present in plants, animals and various living organisms.

It has also famously been reported that the very enzyme catalyses the hydrolysis of asparagine and converts it into aspartic acid and ammonia (via the process of hydrolyzing the amide group present in the side chain of the molecule of asparagine).

2.2 Use of Asparaginase as a Drug in the Medical Industry

Two of the bacterial enzymes, the periplasmic proteins, known as type II asparaginases, from *Escherichia coli* (EcAII) and *Erwinia chrysanthemi* (ErA), have been in clinical use in the treatment of acute lymphoblastic leukemia and some other tumors for more than 30 years.In plants, asparaginase has a hand in energy utilization because asparagine stores nitrogen in most plants. In animals, asparaginase mitigates the chances of cancer as it breaksdown asparagine. Both normal and leukemic cells require the amino acid L-asparagine, for their metabolic needs. Normal cells can synthesize L-asparagine for their growth by utilizing transaminase enzyme that converts oxaloacetate into an intermediate aspartate, which later on transfers an amino group from glutamate to oxaloacetate producing α-ketoglutarate and aspartate. Finally, in healthy cells, aspartate is converted to asparagine by enzyme asparagine synthetase. Neoplastic cells lack the ability to synthesize the asparagine due to the absence of L-asparaginase synthetase enzyme, hence are dependent on the exogenous supply of asparagine for their existence and reproduction. Consequently, as a result, provision of L-asparaginase to tumor cells drains all circulating asparagine, which leads to starvation of cancer cells and ultimately they die off.

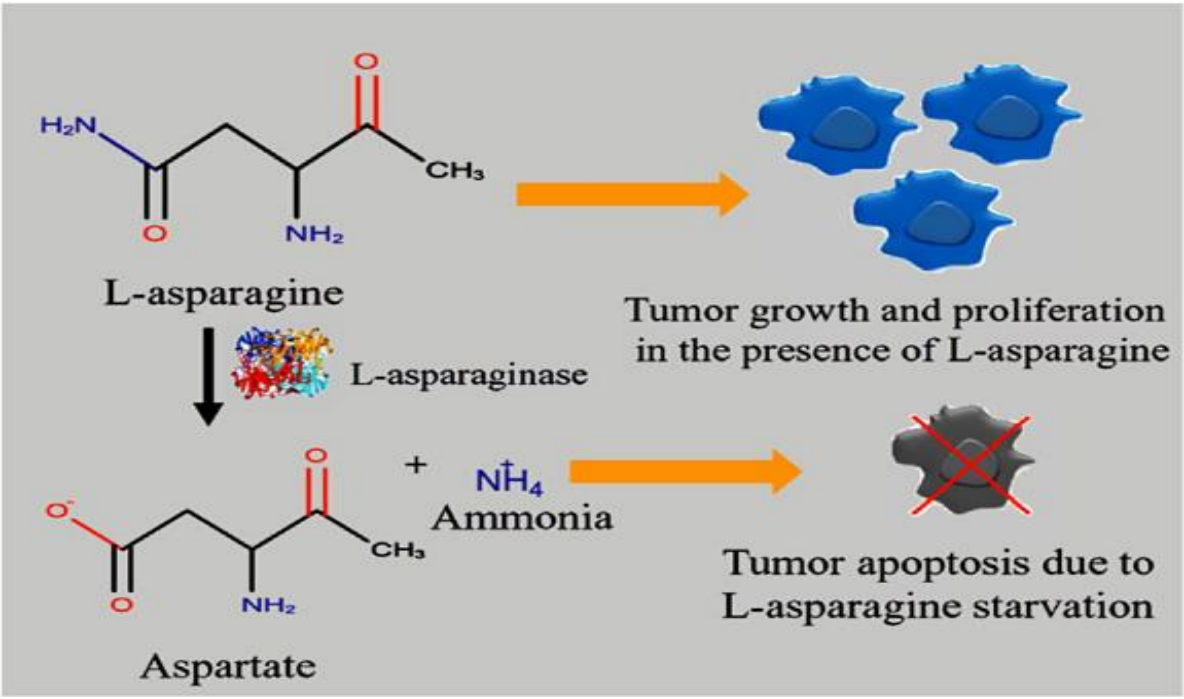

Fig. 2.3 Effect of asparaginase on normal and tumor cells [4]

It is used in the treatment of acute lymphoblastic leukemia, lymphosarcoma, acute myeloblastic leukemia, chronic lymphocytic leukemia, Hodgkin's disease, melanosarcoma, and non-Hodgkin's Lymphoma. This enzyme may be produced by various sources like plants, animals, bacteria, and fungus. But commercially they are produced from *Escherichia coli* and *Erwinia chrysanthemi*. Due to high medical importance and huge market demand of L-asparaginase, industries are in constant search of better producing strains, and methods of its production. [16, 17]

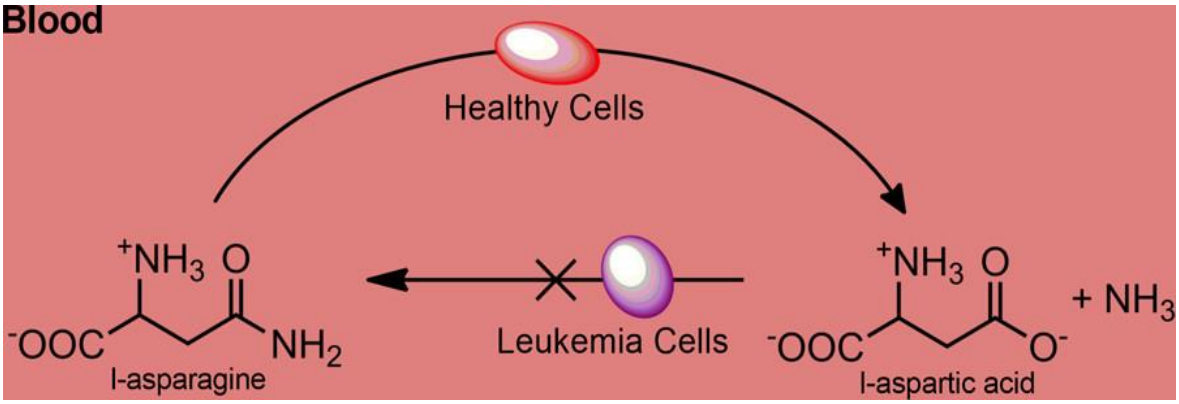

Fig2.4 Asparaginase chemotherapeutic action. Asparaginase catalyzes the hydrolysis of l-asparagine to l-aspartic acid. Since leukemia cells are unable to produce its own endogenous l-asparagine in a sufficient amount, the reduction of blood-circulating l-asparagine induces them to starve without affecting healthy cells. [15]

2.3 The use of Asparaginase as a food-processing aid in the food industry

Recent advances in food technology have demonstrated that fried and baked food (particularly fried potato) contains a significant amount of acrylamide (a carcinogen) formed by the reaction of asparagine with reducing sugars. The reaction is termed as Maillard Reaction. (Fig.2.4). [10]

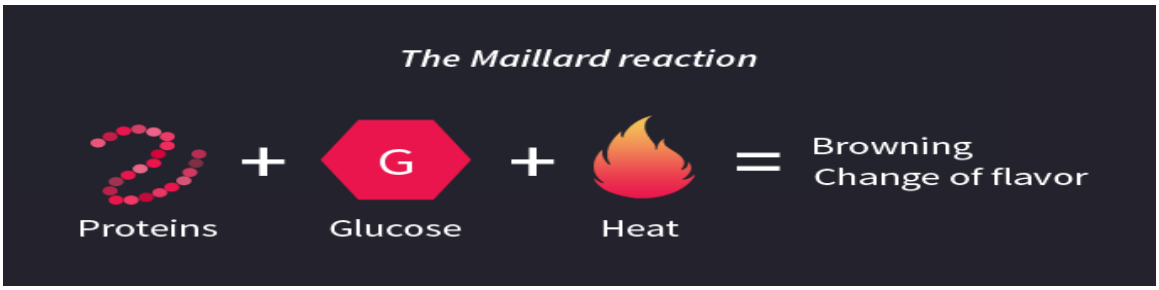

Fig.2.5 Maillard reaction

The use of asparaginase to convert asparagine to aspartic acid may provide a means to reduce acrylamide formation, while maintaining sensory quality.

Strategies proposed for reducing the formation of the suspected carcinogen acrylamide in cooked foods often rely on a reduction in the extent of the Maillard reaction, in which acrylamide is formed from the reaction between asparagine and reducing sugars. However, the Maillard reaction also provides desirable sensory attributes of cooked foods. Mitigation procedures that modify the Maillard reaction may negatively affect flavour and colour. The use of asparaginase to convert asparagine to aspartic acid may provide a means to reduce acrylamide formation, while maintaining sensory quality.

Asparaginase is a powerful tool for the food industry and it is likely that its use will increase. However, the potential adverse effects of asparaginase treatment on sensory properties of cooked foods are still being studied.

Asparagine and reducing sugars take part in a conjugation reaction resulting in the formation of N-glycosylasparagine, which as a result of high temperature treatment will form a decarboxylated Schiff base. The decarboxylated Schiff base may decompose directly to form acrylamide or may hydrolyse to form 3-aminopropionamide. 3-Aminopropionamide is also believed to be an important precursor of acrylamide [23, 24]

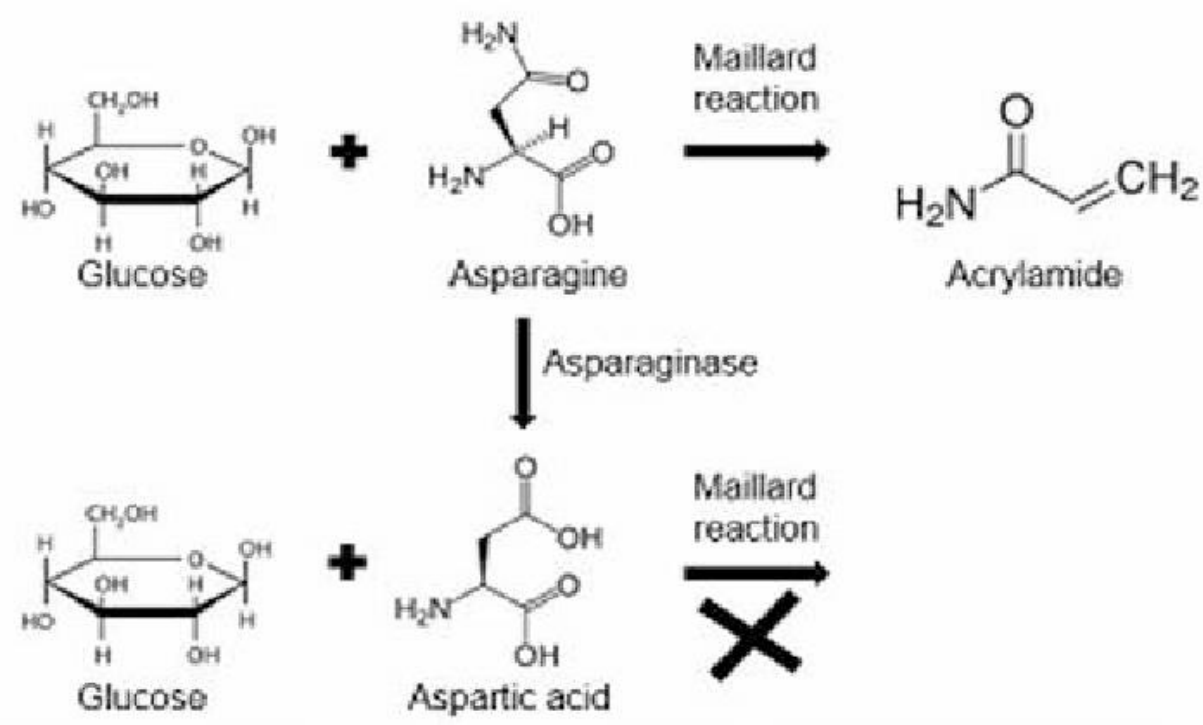

Fig.2.6 Asparaginase affect on Maillard reaction [2]

A pretreatment of potato slices and bread dough with asparaginase before frying or baking prevents acrylamide formation. In this regard, asparaginases from *Aspergillus oryzae* and *A. niger* are in use in baking industries. These enzymes work optimally at 40-60°C and pH 6.0-7.0. Since baking temperatures often go up to 120°C, it is desirable to have enzymes that are stable and active over a wide range of temperature and pH. Therefore, L-asparaginases from various sources (bacterial, fungal, plant, and animal) are under constant research. [13]

For both therapeutic and industrial applications, stability emerges as a key factor determining applicability of L-asparaginase.

Commercially circulating asparaginases in the market include Colapase, Crasnitin, Crisantas, Pasum, Kidrolse, Elspar, Erwinaze, PEG- asparaginase and Pegasparagasum.

2.4 Side effects of Asparaginase

To date, asparaginases from *Escherichia coli* and *Erwinia chrysanthemi* have been used for clinical purposes. However, these enzymes have an associated glutaminase activity, which causes side effects. Further, these enzymes have low stability and a reduced half life in the blood, requiring multiple dose administration for effective treatment. [18]

Asparaginase is used in the treatment of acute lymphoblastic leukemia and lymphoblastic lymphoma in children. It has minimal bone marrow toxicity. Its major side effects are anaphylaxis, pancreatitis, diabetes, coagulation abnormalities, and thrombosis, especially intracranial. [19, 20]

Overall toxicity of asparaginase is low. The most frequent side effects were coagulation abnormalities, which were more frequent in the *E. coli* asparaginase, when compared to asparaginase from *E. chrysanthemi.* [21]

2.5 *Mycobacterium smegmatis*

Mycobacterium smegmatis is an acid-fast bacterial species in the phylum *Actinobacteria* and was first reported by Lustgarten in 1884. It is also known as *Mycobacterium paratuberculosis smegmatis, Bacterium smegmatis, Bacillus smegmatis.*

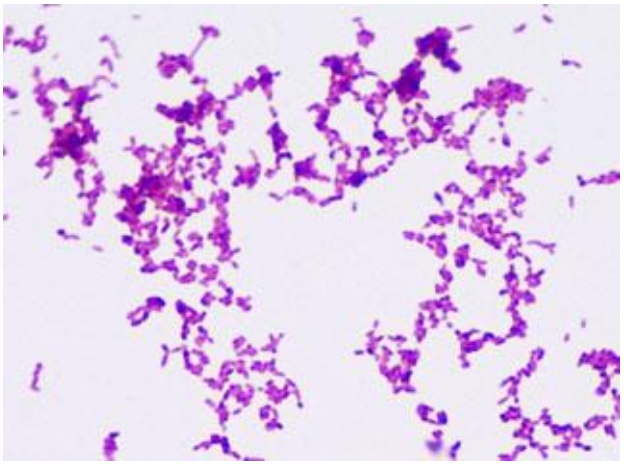

Fig. 2.7 Acid-fast stain showing positive *Mycobacterium smegmatis* results @ 1000xTM [22]

2.5.1 Source:

M. smegmatis are extensively present near water bodies and mostly in soil and plants. They live in aggregate layers of cells that are attached to each other in a community called a biofilm [25].

Kingdom: Bacteria
Phylum: Actinobacteria
Class: Actinobacteridae
Order: Actinomycetales
Suborder: Corynebacterineae
Family: Mycobacteriaceae
Genus: *Mycobacterium*
Species: *smegmatis*

Table 2.2 Classification of *Mycobacterium smegmatis*

2.5.2 Morphology:

M. smegmatis is a gram positive, bacillus shaped bacterium with 3.0 to 5.0 µm length and thick cell wall. It can be stained by Ziehl-Neelsen method and a uramine-rhodamine fluorescent method. When bacteria is grown on accessible nutrients, it is finely wrinkled and creamy white in color. After 48 hours of growth color of bacteria changes from creamy white to nonpigmented creamy yellow [26]. It also forms waxy structure because of high amount of unique Gram-positive cell wall coated with mycolic acids. The bacteria varies in textures as some are smooth, flat and glistening or coarsely folded, others may be finely wrinkled. *Mycobacterium smegmatis* are immotile and endospores are also not formed.

2.5.3 Pathology:

M. smegmatis is classified as a saprophytic species as it is non-pathogenic in nature but in rare cases may cause disease and is not dependent on living in an animal, unlike some pathogenic *Mycobacterium* [27].

2.5.4 Genome Structure:

The genome of *M. smegmatis* is 6,988,209 nucleotides long with 67% guanine cytosine content and 33% adenosine thymine content and is therefore classified as a high GC content gram-positive bacteria. 90% of the genome represents the coding regions that encode for total 6716 proteins. *M. smegmatis* is a slow growing bacteria as it contains only one copy of the ribosomal RNA (rRNA) genes unlike fast growing bacteria such as *Escherichia coli* which has two copies of the rRNA genes. *M. smegmatis* doesn't need so many copies of the genes because it does not require the high production of proteins as it grows slow, while *Escherichia coli* does as it grows fastly. The genome of *M. smegmatis* was sequenced by the J. Craig Venter Institute in November 29, 2006 [28].

2.5.5 Metabolism:

M. smegmatis is an obligate aerobic organism which requires oxygen for the aerobic respiration and undergo oxidative phosphorylation to yield the highest amount of energy. It may donate its final electrons in aerobic respiration to oxygen using one of the three terminal oxidases [29]. It does not undergo anaerobic respiration but in case of infection some virulent *M. smegmatis* may undergo anaerobic respiration. It is a chemolithotrophic organism as carbon monoxide is its inorganic carbon source during aerobic respiration [31]. The bacteria may also use methanol for its sole source of carbon and energy. For the production of mycolic acids of cell wall, it requires unique fatty acid biosynthesis [30].

2.5.6 Ecology:

Bio films of *M. smegmatis* uses stigmasterol as a carbon source from plants. The bacteria metabolizes stigmasterol to an androstenedione which is a potent androgen. As *M. smegmatis* is usually found around a large body of water therefore it secretes more androstenedione. This potent androgen in the water causes female mosquito fish to form male anatomical sex organs therefore, in this way *M. smegmatis* contributes to the environment [32].

2.6 Rationale behind selecting *Mycobacterium smegmatis*

The table below (table2.3) shows the percentage of similarity between some asparaginase producing organisms (obtained after performing BLAST).

Pyrococcus furiosus (an organism that produces thermostable L-asparaginase) [7] showed 50% similarity to *Mycobacterium smegmatis.*
This deemed the organism fit for the ongoing experiment.

	Streptococcus thermophilus	*Escherichia coli I*	*Escherichia coli II*	*Erwinia chrysanthemi*	*Pyrococcus horikoshii*	*Pyrococcus furiosus*	*Mycobacterium smegmatis*
Mycobacterium smegmatis	31%	28%	34%	33%	31%	50%	-
Streptococcus thermophilus	-	33%	28%	30%	35%	53%	31%
Escherichia coli I	33%	-	28%	31%	28%	29%	28%
Escherichia coli II	28%	28%	-	47%	21%	X	34%
Erwinia chrysanthemi	30%	31%	47%	-	25%	50%	33%
Pyrococcus horikoshii	35%	28%	21%	25%	-	88%	31%
Pyrococcus furiosus	53%	29%	X	50%	88%	-	50%

Table2.3 Percentage similarity between Asparaginase producing organisms (after BLAST)

2.7 Primer Designing

Primers are short oligonucleotide sequences synthetically synthesized that are complementary to a certain specific DNA region. They are generally used to amplify a specific DNA/gene region or to identify the sequence of DNA/gene complementary to that of the primer. Primer designing requires finding an equilibrium between –

- Specificity - frequency with which a primer binds to non-specific regions
- Efficiency - how close a primer pair is able to amplify a product to the theoretical optimum of a twofold increase of product for each PCR cycle

Factors affecting primer designing are-

2.7.1 Primer Length

Oligonucleotides between 18 and 24 bases tend to be very sequence specific. Short oligonucleotides of 15 bases or less tend to bind non-specifically. Longer primers, on the other hand, are not preferred because of increased chances of secondary structure formation within the primer.

2.7.2 Terminal Nucleotide in the PCR Primer

The 3'-terminal position in the primer is essential for controlling mispriming. Generally G/C is preferred as the terminal nucleotide as it promotes more strength to primer binding.

Also, primer complementarity should be avoided, particularly at 3’ end because it causes unwanted primer dimer formation which hampers desired amplification.

2.7.3 Rational GC content and T_m

PCR primers should maintain a reasonable GC content. G+C content higher than 40% and T_m greater than 50˚C with similar or nearby values with difference not greater that 5˚C is preferred. [6]

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Bacterial Strain used:

Mycobacterium smegmatisMC^2 155

3.1.2 Media used:

- LBGT (Luria Broth with Glycerol and Tween 80)
- NA (Nutrient Agar)
- NAT (Nutrient Agar with Tween 80)
- Czapek Dox media
- LB (Luria Broth)

3.1.3 Apparatus used:

- Autoclave
- Laminar Air Flow
- pH meter
- Weighing balance
- Centrifuge
- Nanodrop
- Vortex
- -80°C storage freezer
- Incubator
- Gel electroproresis apparatus
- Gel doc
- Water baths

3.2 METHODOLOGY

1. Growth of *Mycobacterium smegmatisMC^2 155*

⬇

2. Screening by NaOH method

⬇

3. Acid-fast staining

⬇

4. Streaking of the organism

⬇

5. Enzyme assay

⬇

6. Primer designing

⬇

7. Genomic DNA isolation

⬇

8. Plasmid isolation

3.2.1 GROWTH OF *Mycobacterium Smegmatis MC²155*

- LBGT was freshly prepared and 10 ml of media was added to each test tube.
- *M. Smegmatis* was revived by inoculating it in test tube.
- Inoculated test tubes were incubated at a temperature of 37°C for a period of 48-72 hrs.
- Results were observed.

3.2.2 SCREENING BY NaOH METHOD

- 4%NaOH was prepared in autoclaved water.
- From 10 ml LBGT tube in which culture was grown take 1 ml .
- To this add 4%NaOH sol and then vortex it.
- Incubate it for 1 hr at 37°C.
- 1 ml of tween normal saline and vortex it.
- Centrifuge at 3000 rpm for 25 min.
- Add 500µl of TNS to LBGT medium.

3.2.3 ACID FAST STAINING

- Bacterial smear was prepared on a clean glass slide.
- Smear was allowed to air dry and was heat fixed using a burner.
- Carbol fuchsin dye was added to the glass slide and the glass slide
- Glass slide was placed on hot plate for 4-5 minutes (at a temperature of about 80°C).
- Do not over heat heat until vapors start to form.
- Allow it to cool and then washed off the extra stain with water.
- Cover the smear with acid alcohol (decoloriser) and keep it for 20 seconds.
- Wash well with clean water.
- Cover the smear with malachite green and keep it for 20 seconds.
- Wash off with clean water.
- Allow the smear to air dry and view under microscope.

3.2.4 STREAKING OF *M. smegmatis*

- NAT plates were made.
- Inoculation loop was heat sterilized and then inoculated from the media onto the NAT plates.
- Plates were incubated at a temperature of 37°C for 2-3 days.
- Results were observed on the third day.

3.2.5 ENZYME ASSAY [1, 3]

- Samples of *E.coli* (for setting as standard) and *M. smegmatis* were procured.

- They were checked for viability by growing them on Nutrient Agar, NA (for *E. coli*) and Nutrient Agar Tween, NAT (for *M. smegmatis*).
- Incubation at 37°C, for 24 hrs. (*E. coli*) and for 48 hrs. (*M. smegmatis*) was carried out.
- A single colony was picked up from both the plates and inoculated separately in Nutrient Broth, NB (*E.coli*) and in Luria Broth Glycerol Tween, LBGT (*M. smegmatis*).
- These primary broth cultures were incubated at 37°C for 24 hrs. (*E. coli*) and for 48 hrs. (*M. smegmatis*).
- Secondary broth cultures were prepared by the same method.
- A modified Czapek Dox medium was prepared. This broth of Czapek Dox was inoculated with the secondary broth cultures of both the organisms, separately.
- The change in colour and the subsequent results were noted.

3.2.6 PRIMER DESIGNING

- Two restriction sites from MCS of pET28a vectorwere chosen in a manner so that they are not present in our gene encoding asparaginase enzyme.
- EcoRI and NdeI were finalized for designing the forward primer and reverse primer.
- High specificity and affinity were the factors taken care of, while primer designing.

ORGANISM: ***Mycobacterium smegmatis***

PROTEIN SEQUENCE:

>tr|A0QX50|A0QX50_MYCS2 L-asparaginase OS=Mycobacterium smegmatis (strain ATCC 700084/ mc (2)155) GN=MSMEG_3173 PE=4 SV=1

MNPSAQVVLITTGGTISTSTDNAGVRRPTRRGAQLTAALTAPTAPTVSVVDLMTT
DSSHL

TPSDWDAIREAVAKATDGPGGPDGVVITHGTDSLEETALWLELTHTGDTPVVITG
AQRSS

DAPEADGPRNLADAIAVAASPDARGLGVLVCFAGTVRAPLGLHKRHTQELDAFS
GTALGT

VADGTVELTEDKTRPVLGALSAAAAPRVDIVAGYPGADAAALDACVAAGARGV
VLEAMGS

GNLGAALTEGVERHLRAGVEIVVSTRVPGGVIAPDYGPGRRLADAGAVVAPRLRP
PQARV

LLMAALAAQRPVREVFSSWG

DNA SEQUENCE:

Mycobacterium smegmatis (strain ATCC 700084 / mc (2)155)

ATGAACCCGTCCGCACAAGTCGTCCTCATCACCACCGGTGGCACCATATCCAC
GAGCACCGACAACGCGGGCGTCCGGCGCCCGACCAGGCGCGGAGCGCAGCTG
ACGGCGGCTTTGACCGCCCCGACGGCGCCCACGGTGAGTGTGGTCGATCTCAT
GACCACCGACAGCTCACACCTGACCCCGTCGGACTGGGACGCGATCCGTGAGG

CCGTCGCGAAGGCCACCGACGGCCCCGGCGGGCCCGACGGTGTGGTGATCAC
GCACGGCACCGACAGCCTGGAGGAGACCGCGCTGTGGCTCGAGCTCACCCAC
ACCGGTGACACCCCGGTCGTGATCACGGGCGCGCAGCGCAGCTCGGACGCGCC
CGAGGCCGACGGGCCGCGCAATCTCGCCGACGCGATCGCGGTCGCGGCGTCCC
CGGACGCCCGCGGACTCGGCGTGCTGGTGTGCTTCGCCGGCACGGTGCGCGCA
CCGCTCGGGTTGCACAAGCGGCACACGCAGGAACTCGACGCGTTCTCGGGCAC
GGCGCTCGGCACGGTGGCCGACGGCACTGTGGAGCTCACCGAGGACAAGACG
CGACCTGTGCTCGGTGCGCTGTCGGCGGCCGCGGCACCGCGGGTCGACATCGT
GGCCGGGTATCCCGGCGCCGATGCCGCGGCGCTCGACGCGTGCGTCGCGGCCG
GGGCGCGCGGGGTGGTGCTTGAGGCCATGGGGTCGGGCAACCTCGGCGCGGC
GCTCACCGAGGGTGTCGAACGCCATCTGCGTGCCGGTGTCGAGATCGTGGTGT
CGACGCGGGTGCCGGGCGGCGTCATCGCACCGGACTACGGACCGGGACGCCG
GCTGGCCGACGCGGGCGCCGTGGTGGCGCCTCGTCTGCGCCCGCCGCAGGCGC
GGGTGCTGCTGATGGCCGCGCTCGCCGCGCAGCGGCCCGTGCGCGAGGTTTTC
TCCTCATGGGGTTGA

RESTRICTION ENZYME SITES which do not cleave in between:

EcoR I

5′. . . G|AATTC . . . 3′

3′. . . CTTAA|G . . . 5′

Nde I

Therefore PRIMERS FOR CLONING:

Forward: 5'- GGAATTCCATATGAACCCGTCCGCACAAG -3'

Reverse: 5'- CGCGGATCCTCAACCCCATGAGGAGAAAA -3'

Primers	**Length (gene specific)**	**Tm (˚C)**	**GC content (%)**
Forward primer	**29**	**68.1**	**51.7**
Reverse primer	**33**	**73.2**	**57.6**

Table 3.1 Properties of the primers designed

3.2.7 GENOMIC DNA ISOLATION

- Pellet the culture in 50 ml tarson tube at 7000 rpm for 15 minutes.
- Supernatant was removed and 2 ml of TES buffer was added to each tube.
- Incubate at a temperature of 80°C for 1hr.
- 2 mg/ml of lysozyme was added to each tube.
- Tarson was incubated at a temperature of 37°C for 1 hr.
- 1.5% SDS and 100µg/ml of protinase K was added to each tarson.
- Tarson was incubated at a temperature of 50°C for 1 hr.
- 1 ml buffer phenol was added to each tarson.
- Tarson was centrifuged at 12000 rpm for 15 min.
- Chloroform:Isoamyl alcohol was added in each tube (24:1).
- Tarson was centrifuged at 12000 rpm for 15 min.
- Aqueous layer was transferred to a fresh tube.
- Again chloroform:isoamy alcohol was added and the tarson was centrifuged at 12000 rpm for 10 min.
- Aqueous layer was transferred to a fresh tube.
- Equal volume of chilled ispropanol was added to each tarson.
- Tarson was incubated at 4°C overnight.
- Tarson was centrifuged at 12000rpm for 10 min.
- 70% of ethanol was added to each tube.
- Tarson was centrifuged at 12000 rpm for 10 min.
- Supernant was discarded and 100 µl of autoclave distilled water was added to each tarson.
- Tarson was placed in ice for 2 hrs for peoper suspension of DNA.
- 0.8% gel was prepared.
- The isolated DNA was loaded and the gel was run.
- The gel was then visualized.

3.2.8 PLASMID ISOLATION

Plasmid isolation of: pET28a vector

Plasmid isolation from: *E.coli* DH5α

Plasmid isolation by: Alkaline lysis method

- 2ml fresh *E.coli* culture was transferred to a microcentrifuge tube.
- Sample was centrifuged @ 8000rpm for 7 minutes.
- Supernatant was discarded.
- 100 µl of ALS I solution (50 mM glucose, 25 mM tris Cl pH 8, 10 mM EDTA pH 8) was added to the pellet and vortexed.
- Sample was incubated at RT for 5 minutes.
- 200 µl of ALS II solution (0.2N NaOH, 1% SDS) was added. The solution was mixed by inverting the tube.
- The sample was again incubated at RT for 5 minutes.
- 500 µl of ALS III solution (3M potassium acetate pH 5.5) was added and mixed by inversion of the tube.
- The sample was kept in ice for 5 minutes.
- Equal volumes of phenol: chloroform (24:1) was added to the contents of tube.
- Sample was centrifuged at 10,000 rpm, 4˚C for 5 minutes.
- Supernatant was collected in a fresh microcentrifuge tube.
- Equal volumes of isopropanol were added to the supernatant and mix properly.
- Solution was incubated for 30 minutes and then centrifuged at 4,000 rpm, 4˚C for 30 minutes.
- The pellet after removing the supernatant was twice washed with ethanol.
- Pellet was then air-dried to remove ethanol completely.
- Dried pellet was resuspended in 40 µl TE buffer and stored at -20˚C.

CHAPTER 4

RESULTS AND DISCUSSIONS

1. *Mycobacterium smegmatis MC^{2}155* was grown in LBGT first and then Acid-fast staining was done to screen out pure colonies.

   Pure culture isolates of *M. smegmatis* were obtained on NAT media plate, after streaking and their 30% glycerol stocks were prepared and stored at -80˚C.

Fig 4.1 *M. smegmatis* streaked on NAT plates

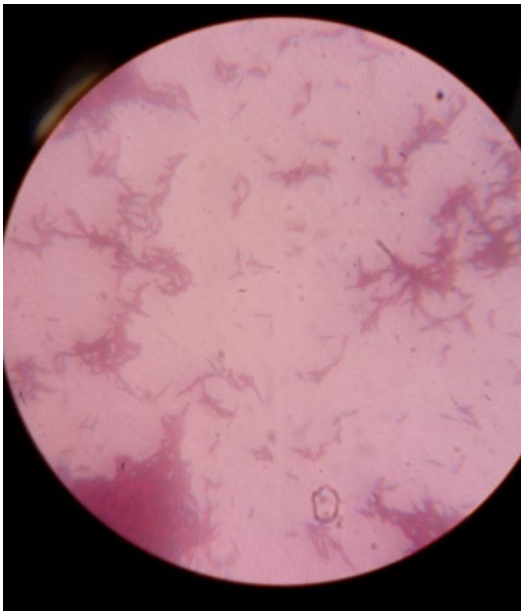

Fig. 4.2 Acid fast staining of *M.smegmatis* from LBGT (100x)

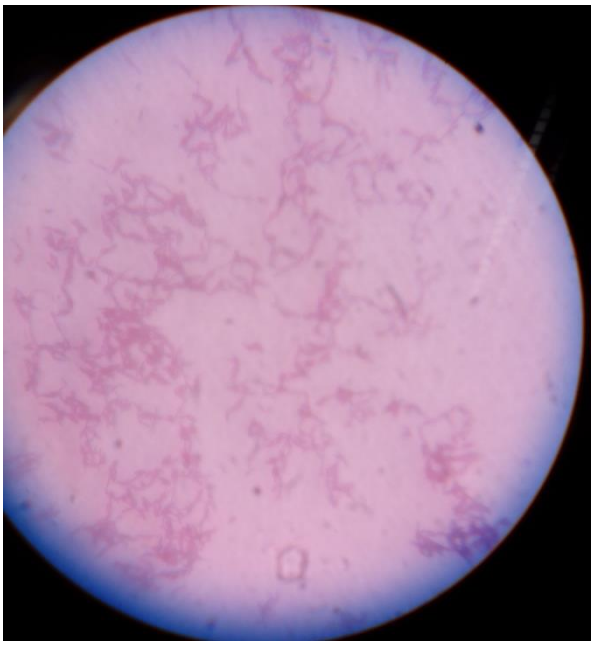

Fig 4.3 Acid-fast staining of *M.smegmatis* from NAT plates (100x)

2. Enzyme plate assay:

- The indicator used in Capek Dox medium was Bromothymol Blue, BTB, a dye which functions in the pH range of 6.0 – 7.6
- The colour gradation being from yellow (at acidic) to green (at neutral) to blue (at basic) (Fig.4.4).
- When the L-asparagine in the medium is broken down by the asparaginase of the organisms, L- aspartate and ammonia are released.
- This increases the pH of the medium (originally set at 5.5), leading to the change in colour of the medium.
- Originally, the uninoculated Czapek Dox medium was yellow in colour (Fig.4.5).
- After inoculation with the two separate organisms, the *E.coli* showed a dark green colour (Fig.4.6), whereas the *M. smegmatis* displayed an olive green colour (Fig.4.7).

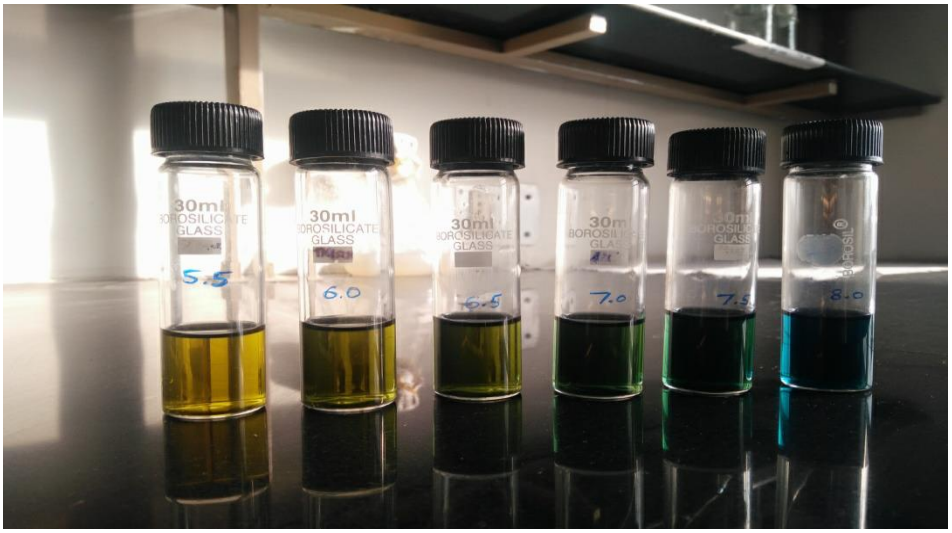

Fig. 4.4 The gradual colour changes in the BTB dye at various pH ranges

Fig. 4.5 Uninoculated Czapek Dox medium

Fig. 4.6 *E. coli* after the assay

Fig. 4.7 *M. smegmatis* after the assay

3. Genomic DNA isolation was successfully carried out.

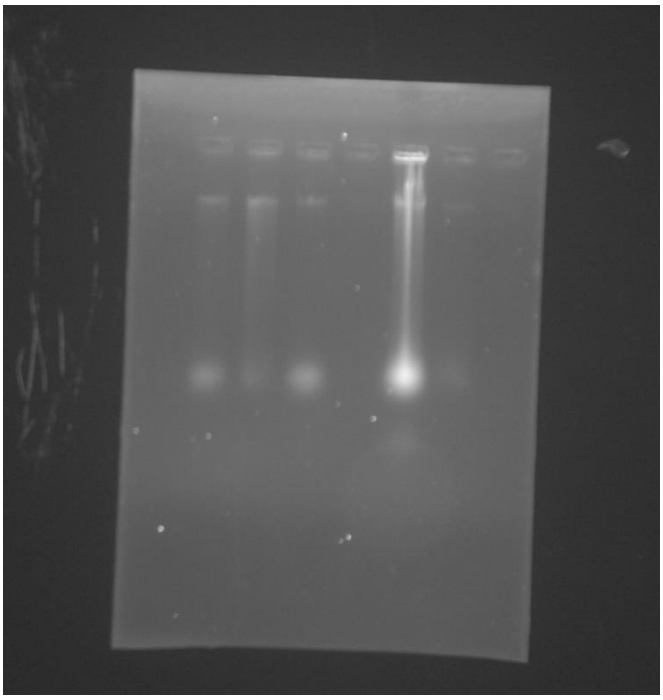

Fig. 4.8 Genomic DNA isolated and visualized on 0.8% agarose gel stained with EtBr

4. Quantification of the isolated genomic DNA from *M. smegmatis* was carried out using the nanodrop. Since sample 4 gave the brightest bands (inspite of disintegration of DNA), it was further calculated.

According to the formula, to calculate the concentration of DNA :-

OD of DNA @260nm X dilution factor X 50 =

1.288 X 1 X 50 ng/µl = **64.4 ng/µl**

SAMPLE	OD at 260nm
Blank	0.063
Sample I	0.447
Sample II	0.479
Sample III	0.512
Sample IV	1.288

Table 4.1 ODs taken at 260 nm for various samples

5. pET28a vector was also isolated from *E. coli* DH5α strain.

LANES 1 2 3 4

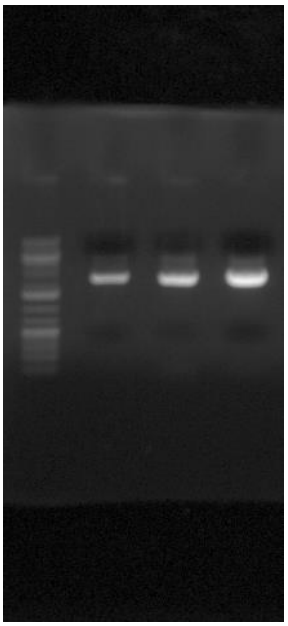

Fig.4.9 Plasmid isolated and visualised on 1% agarose gel stained with EtBr.

Lane 1	DNA ladder of size 10 kb
Lane 2	5 kb plasmid observed
Lane 3	5 kb plasmid observed

Table 4.2 Plasmid gel description

CHAPTER 5

CONCLUSION

Initially, pure cultures of *M.smegmatis* were isolated and were successfully screened through Acid - fast staining. These cultures were grown in NAT plates first, then later subcultured to secondary (LBGT) media, whose composition stimulates the production of asparaginase enzyme.

Via the enzyme assay mentioned, the presence of the enzyme asparaginase was tested.

DNA of mycobacterium growing under such conditions was isolated .

Furthermore, pET28a vector was also isolated from its host – *E. coli* DH5α strain successfully.

CHAPTER 6

REFERNECES

1. Mahajan, R. V., Saran, S., Saxena, R. K., & Srivastava, A. K. (2013). A rapid, efficient and sensitive plate assay for detection and screening of l-asparaginase-producing microorganisms. *FEMS microbiology letters*, *341*(2), 122-126.

2. Batool, T., Makky, E. A., Jalal, M., & Yusoff, M. M. (2016). A comprehensive review on L-asparaginase and its applications. *Applied biochemistry and biotechnology*, *178*(5), 900-923.

3. Gulati, R., Saxena, R. K., & Gupta, R. (1997). A rapid plate assay for screening l-asparaginase producing micro-organisms. *Letters in applied microbiology*, *24*(1), 23-26.

4. Vimal, A., & Kumar, A. (2017). Biotechnological production and practical application of L-asparaginase enzyme. *Biotechnology & genetic engineering reviews*, *33*(1), 40.

5. Bashiri, G., & Baker, E. N. (2015). Production of recombinant proteins in Mycobacterium smegmatis for structural and functional studies. *Protein Science*, *24*(1), 1-10.

6. Dieffenbach, C. W., Lowe, T. M., & Dveksler, G. S. (1993). General concepts for PCR primer design. *PCR Methods Appl*, *3*(3), S30-S37.

7. Bansal, S., Gnaneswari, D., Mishra, P., & Kundu, B. (2010). Structural stability and functional analysis of L-asparaginase from Pyrococcus furiosus. *Biochemistry (Moscow)*, *75*(3), 375-381.

8. Ott, J. L. (1960). Asparaginase from mycobacteria. *Journal of bacteriology*, *80*(3), 355.

9. Salazar, L., Fsihi, H., Rossi, E., Riccardi, G., Rios, C., Cole, S. T., & Takiff, H. E. (1996). Organization of the origins of replication of the chromosomes of Mycobacterium smegmatis, Mycobacterium leprae and Mycobacterium tuberculosis and isolation of a functional origin from M. smegmatis. *Molecular microbiology*, *20*(2), 283-293.

10. Anese, M., Quarta, B., & Frias, J. (2011). Modelling the effect of asparaginase in reducing acrylamide formation in biscuits. *Food chemistry*, *126*(2), 435-440.

11. Borek10, D., & Jaskólski, M. (2001). Sequence analysis of enzymes with asparaginase activity. *Acta Biochim Pol*, *48*, 893-902.

12. https://www.rcsb.org/structure/4O0H

13. Amrein, T. M., Schönbächler, B., Escher, F., & Amadò, R. (2004). Acrylamide in gingerbread: Critical factors for formation and possible ways for reduction. *Journal of Agricultural and Food Chemistry*, *52*(13), 4282-4288.

14. Xu, F., Oruna-Concha, M. J., & Elmore, J. S. (2016). The use of asparaginase to reduce acrylamide levels in cooked food. *Food chemistry*, *210*, 163-171.

15. Ramirez-Paz, J., Ortiz-Andrade, B. M., Griebenow, K., & Díaz-Vázquez, L. (2017). Show Yourself, Asparaginase: An Enzymatic Reaction Explained through a Hands-On Interactive Activity. *Journal of Chemical Education*, *94*(6), 722-725.

16. Mashburn, L. & Wriston, J. (1963) Tumor inhibitory effect of L-asparaginase. Biochem. Biophys. Res. Commun. 12, 50–55.

17. Broome, J. (1961) Evidence that the L-asparaginase activity of guinea pig serum is responsible for its antilymphoma effects. Nature 191, 1114–1115.

18. Duval, M., Suciu, S., Ferster, A., Rialland, X., Nelken, B., Lutz, P., & Otten, J. (2002). Comparison of Escherichia coli–asparaginase withErwinia-asparaginase in the treatment of childhood lymphoid malignancies: results of a randomized European Organisation for Research and Treatment of Cancer—Children's Leukemia Group phase 3 trial. *Blood*, *99*(8), 2734-2739.

19. Priest, J. R., Ramsay, N. K., Steinherz, P. G., Tubergen, D. G., Cairo, M. S., Sitarz, A. L., ... & Cich, J. A. (1982). A syndrome of thrombosis and hemorrhage complicating L-asparaginase therapy for childhood acute lymphoblastic leukemia. *The Journal of pediatrics*, *100*(6), 984-989.

20. Sahu, S., Saika, S., Pai, S. K., & Advani, S. H. (1998). L-asparaginase (Leunase) induced pancreatitis in childhood acute lymphoblastic leukemia. *Pediatric hematology and oncology*, *15*(6), 533-538.

21. Dellinger, C. T., & Miale, T. D. (1976). Comparison of anaphylactic reactions to asparaginase derived from Escherichia coli and from Erwinia culturs. *Cancer*, *38*(4), 1843-1846.

22. http://www.scienceprofonline.com/science-image-libr/sci-image-libr-mycobacterium-bacteria.html

23. Granvogl, M., & Schieberle, P. (2006). Thermally generated 3-aminopropionamide as a transient intermediate in the formation of acrylamide. *Journal of Agricultural and Food Chemistry*, *54*(16), 5933-5938.

24. Hedegaard, R. V., Frandsen, H., & Skibsted, L. H. (2008). Kinetics of formation of acrylamide and Schiff base intermediates from asparagine and glucose. *Food chemistry*, *108*(3), 917-925.

25. Brown-Elliott, B. A., & Wallace, R. J. (2002). Clinical and taxonomic status of pathogenic nonpigmented or late-pigmenting rapidly growing mycobacteria. *Clinical microbiology reviews*, *15*(4), 716-746.

26. Gordon R., Smith M., *"RAPIDLY GROWING, ACID FAST BACTERIA I. Species' Descriptions of Mycobacterium phlei Lehmann and Neumann and Mycobacterium smegmatis (Trevisan) Lehmann and Neumann"*, Journal of Bacteriology, vol. 66, pp. 41-48, Nov. 1953.

27. Branca A., Baglioni C., "*Evidence that types I and II interferons have different receptors*", Nature, vol. 294, pp. 768-770, Dec. 1981.

28. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genome&cmd=Retrieve&dopt=Overview&list_uids=20087.

29. Megehee, J. A., & Lundrigan, M. D. (2007). Temporal expression of Mycobacterium smegmatis respiratory terminal oxidases. *Canadian journal of microbiology*, *53*(3), 459-463.

30. Weber, I., Fritz, C., Ruttkowski, S., Kreft, A., & Bange, F. C. (2000). Anaerobic nitrate reductase (narGHJI) activity of Mycobacterium bovis BCG in vitro and its contribution to virulence in immunodeficient mice. *Molecular microbiology*, *35*(5), 1017-1025.

31. Park, S. W., Hwang, E. H., Park, H., Kim, J. A., Heo, J., Lee, K. H., ... & Kim, Y. M. (2003). Growth of mycobacteria on carbon monoxide and methanol. *Journal of bacteriology*, *185*(1), 142-147.

32. McLachlan, J. A. (2001). Environmental signaling: what embryos and evolution teach us about endocrine disrupting chemicals. *Endocrine Reviews*, *22*(3), 319-341.

CHAPTER 7

APPENDIX

7.1 Bacteriological Media

All the media were prepared in distilled water and autoclaved at 15 pounds per square inch.

7.1.1 LBGT (100ml)

- Luria broth - 2.3 g
- Glycerol(0.5%) - 500µl
- Tween 80 (0.15%) – 150 µl

7.1.2 NAT (100ml)

- Nutrient agar – 1.5 g
- Tween 80 (0.05%) – 50 µl

7.1.3 Modified Czapek Dox media (pH 5.5) (100ml)

- Na_2HP0_4 - 0.6 g
- KH_2PO_4 – 0.2 G
- NaCl – 0.05 g
- L – asparagines – 2 g
- Glycerol – 0.2 g
- $MgSO_4.7H_2O$ – 0.02 g
- $CaCl_2.2H_2O$ – 0.0005 g

7.1.4 LB (100ml)

- Luria broth – 2.3 g

7.1.5 NA (50ml)

- Nutrient agar – 1.2 g

7.2 Reagents and Buffers

7.2.1 TES Buffer

- Tris Cl – 10mM(pH 8)
- EDTA – 1mM(pH 8)
- NaCl – 150 mM

7.2.2 TAE Buffer (50X for 1L)

- Tris base – 242g

- Acetic acid – 57.1ml
- 0.5M EDTA – 100ml (pH 8)

7.2.3 ALS I (pH 8)

- Glucose – 50 mM
- Tris Cl – 25 mM (pH 8)
- EDTA – 10 m M(pH 8)

7.2.4 ALS II

- NaCl – 0.2 N
- SDS – 1%

7.2.5 ALS III (pH 5.5)

- Potassium acetate – 5M
- Glacial acetic acid – 1.15 ml
- H_2O – 2.85 ml

7.2.6 Tween Normal Saline

- Tween 80 (0.1%) - 1ml
- NaCl (0.89%) - 8.9g

7.2.7 Carbol Fuchsin (primary stain)

- Basic Fuchsin - 3g
- Phenol - 5%
- Ethanol (96%) - 10ml

Mixed 10ml of Basic Fuchsin to 90ml of phenol and the solution was filtered through Whatman filter paper.

7.2.8 Acid alcohol (Decolourizer)

- HCL (conc.) - 3ml
- Ethanol (96%) - 97ml

7.2.4 Malachite Green solution (Counter stain)

- Malachite green - 0.25g