Production and characterization of L-methionine gamma lyase from different bacterial sources

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

Masters of Science in Biotechnology

By

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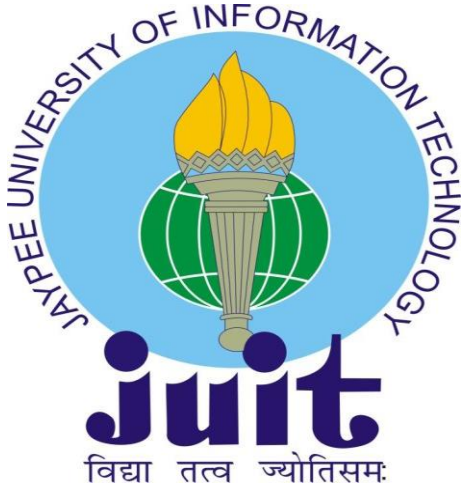

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SELF- DECLARATION

I hereby declare that the work reported in the M. Sc. Dissertation thesis entitled **"Production and characterization of L-methionine gamma lyase from different bacterial sources"** submitted to Jaypee University of Information Technology, Waknaghat, is an authentic record of my work carried out under the supervision of Dr Anil Kant (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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Date:

Place:

CERTIFICATE

This is to certify that the work reported in the M. Sc. Dissertation thesis entitled **"Production and characterization of L-methionine gamma lyase from different bacterial sources"** submitted by Shreya Mahajan (217803) 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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Abbreviations

i. MGL- Methionine gamma lyase

ii. MET- Methionine

iii. PLP- Pyridoxal 5 phosphate

iv. TNBC- Triple-Negative Breast Cancer

v. NCBI- National Center for Biotechnology Information

vi. MTCC- Microbial Type Culture Collection and Gene Bank

viii. VSCs- Volatile sulphur compounds

Abstract

The aim of this study was to both validate production of and characterize methionine gamma lyase from three different bacterial sources that are selected through literature survey and *in silico* search. Production of MGL was observed in all three, with *Pseudomonas lurida* producing the enzyme only intracellularly whereas *P. fragi* and *C. braakii* produced the enzyme both extracellularly and intracellularly. The MGL production was found to be maximum at pH 6 and 30°C in case of *P. lurida.* Whereas *P.* fragi and *C. braakii* showed maximum MGL production at 35°C and pH 7. Enzyme characterization revealed highest enzyme activity at 40°C and pH 6 for *P. lurida* whereas enzyme activity was maximum at 40°C and pH 7 in case of *P. fragi* and C. braakii. The Km value of MGL from *C. braakii* was observed to be highest (0.88 mM for intracellular and 0.926 for extracellular), while that from *P. lurida* was observed to be 2.11 mM and from *P. fragi* 1.11 mM and 1.08 mM for extracellular and intracellular enzymes respectively.

CHAPTER 1: INTRODUCTION

1.1 Introduction

According to the WHO website, cancer is one of the prime causes of death throughout the world, and accounts for almost 10 million deaths in 2020 alone with approximately 4 lakh children affected every year [1]. The disease in which some cells of the body start growing abnormally and eventually spread in the body is termed as cancer [2]. The most common cancer is breast, lung, prostate, colon, etc but these can vary in different countries [1]. In India, more than 8 lakh people have died in 2020 due to cancer. An estimate of 25% cancer deaths in Indian males occurs due to cancer of stomach, oral cavity and lungs; and 25% of cancer deaths in Indian females occurs due to cancer of breast, cervix and oral cavity [3]. This shows how cancer is the first and foremost health care issue that disrupts the social standards and imposes a huge clinical burden [4]. The cancer burden can be reduced by early diagnosis and proper treatment as each cancer type has a unique treatment regimen [1]. Treatment can include surgery, chemotherapy, biological therapy or hormonal therapy depending upon the type of cancer and the individual.

As cancer has a very high incidence in the world, many therapeutic strategies have been developed and improved for their application in cancer treatment [5]. One such strategy is the emerging enzyme-therapy. This utilizes the enzymes as they are being recognized superior to other drugs. Such enzymes just act on targeted molecules or sites and are highly specific. This feature is what makes the use of enzymes very reliable [21]. Many such enzymes are being utilized in cancer therapy. Few of them are arginase, methioninase, asparaginase, etc. The development of cancer modifies the nutrient intake of cancer cells [6]. One such modification is the dependency of cancer cells on methionine. It was observed that cancer cells had elevated dependency on methionine and this phenomenon got termed as ‘Hoffman effect’ [7]. Till date many cancer lines have been evaluated and found to have such dependency on methionine. Due to this property of cancer cells, therapies have been developed to target methionine in cancer cells as it was observed that the cancer cells got arrested in S/G2 phase of cell cycle when the cells were deprived of exogenous methionine (MET restriction). Normal cells easily survive in these conditions as these have an active methionine synthase in them which means that they can produce their own methionine and usually are dependent on homocysteine more than being dependent on methionine directly [21]. It is important to mention that such a strategy cannot be devised on every type of cancer as few cancer types have responded poorly towards such therapy.

Many enzymes can be utilized for the purpose of restricting the methionine synthesis in cancer cells and methioninase or methionine gamma lyase (MGL) is one of such enzymes. MGL along with other anticancer drugs has proved to have antitumor effects in mouse models of human tumours [8]. MGL can be found in many eubacteria like *Clostridium sporogenes, P. putida, aeromonas sp*., etc [10]. MGL from *Pseudomonas putida* has been therapeutically exploited as it depletes plasma methionine from the cancer cells and has given positive results. It has been seen that most microbial sources produce MGL intracellularly though there are few species which produce this enzyme extracellularly as well as intracellularly [22]. This provides a push to explore the MGL protein from the different species. Examining the protein producing ability of the species from the already established genera increases the chances of finding a species producing the protein of desired properties. That is why organisms of the previously explored genera can be utilized as this would increase the chances of finding protein with higher affinity towards methionine which is the desired target in cancer therapy.

1.2 Problem statement

As the increasing cases of cancer throughout the world need to be addressed, several attempts in different fields are being made to find an effective therapy for this disease. Due to diversity in the disease itself, only one type of therapy cannot be accepted for every type of cancer. That is why different types of cancer require different kinds of treatment. Even though much progress has been made in the past century, there is still room for a lot of research that could provide us with new and effective treatments. One such treatment is the methioninase therapy wherein the cancer cells are deprived of methionine with the help of MGL which eventually leads to their death. This happens due to the Hoffman effect.

MGL can be obtained from many sources but not all sources can be utilized at a commercial level. As sources utilized for MGL production are usually bacterial or fungal, more such sources can be tracked to find a source that can provide us with the enzyme of desired characteristics i.e., has high affinity towards the targeted substrate and can be utilized in the cancer therapy of various cancer types in a cost-efficient manner and can be tested for its efficiency in the same.

1.3 Methodology

The proposed methodology for this project is as follows:

Selection of different organisms was done based on the literature review and in-silico search using the 'Gene' section of the NCBI website. Once three different organisms have been selected, the cultures of all these were procured from MTCC, Chandigarh.

The next step was to make an optimum condition for different cultures, so that maximum amount of enzyme can be produced. Once optimum enzyme production was achieved, the enzymes were characterized for few parameters. This was done by performing enzyme assay at each step to know the enzyme activity of enzymes and at last the specific activity of each enzyme from different source was calculated (assays include- Nesslerization for getting total activity of enzyme and Folin-Lowry to get the protein concentration). In the end, Michaelis' constant (Km) was determined for each enzyme to know its affinity towards the desired substrate i.e., METHIONINE.

1.4 Objectives

1. Selection of target organisms for the production of L-MGL

2. Optimization of conditions for enzyme secretion in different organisms.

3. Characterization of the enzyme with respect to affinity towards methionine.

CHAPTER 2: REVIEW OF LITERATURE

2.1 MGL enzyme

MGL enzyme, also known as methioninase, is a PLP-dependent (Pyridoxal 5 phosphate) enzyme which is hydrolytic in nature. It is characterized as a member of the γ-family of PLP-dependent enzymes. It catalyses the *α, γ*-elimination of L-methionine and its derivatives like L-selenomethionine, L-homocysteine, etc. Such reactions give keto acid, thiols and ammonia as end products [8]. The protein sequence length of the enzyme typically lies between 200-600 amino acids long. Specificity of MGL is seen to vary amongst different organisms which could signify the fact that its physiological role is organism specific [8]. Many organisms have not been experimented with, for the characterization of this enzyme and only enzyme from *P. putida* has been studied in full length.

On analysis of MGL from *Pseudomonas putida* and reactions of PLP-dependent enzymes, the MGL elimination reaction can be demonstrated in four steps:

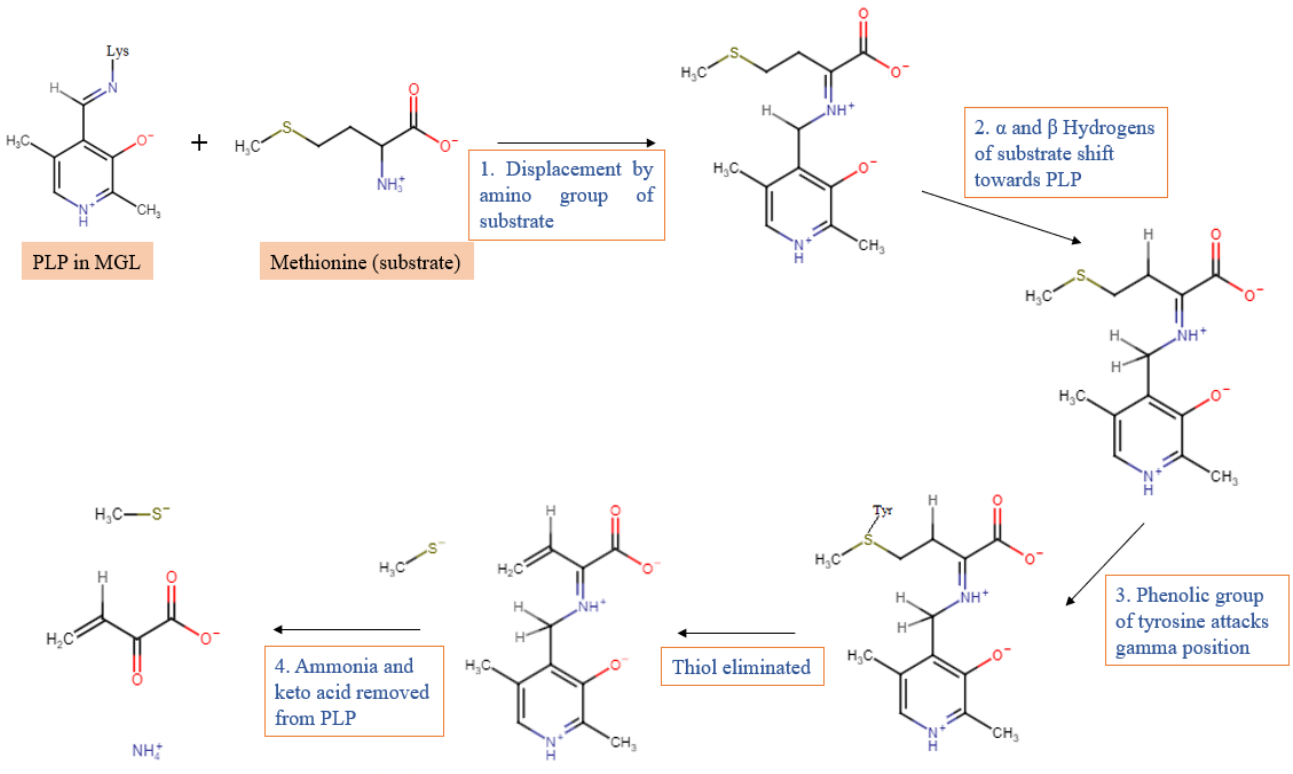


Fig 1: Mechanism of α, γ-elimination of methionine by methionine-gamma-lyase with steps mentioned on each arrow.

STEP 1: Schiff-base linkage forms between PLP and lysine residue of the enzyme and then is displaced by the amino group of the substrate.

STEP 2: α- and β-hydrogens of the substrate shift to PLP.

STEP 3: Adjacent tyrosine residue (phenolic group) in MGL attacks the γ-position of the substrate as an acid catalyst (confirmed by studies on mutant MGL of *P. putida*).

STEP 4: Thiol group gets eliminated from substrate; and ammonia & α-keto acid are released from PLP.

This enzyme has been observed as a tetramer. It is found in the cytosolic region of the cell and is usually secreted in the presence of methionine in the media. *Pseudomonas putida* is the most researched organism when it comes to MGL. A structural analysis of the enzyme from this source reveals six amino acids that are important and conserved, as these are found in the centre of substrate and quite close to PLP. Its three-dimensional structure has also been determined and available on Protein Data Bank [21].

L-MGL has been characterized as an intracellular enzyme in bacterial organisms and as an extracellular in fungal sources. It also plays an important role in the food industry as it imparts an aroma to many fermented foods which is due to the degradation of L-methionine to Volatile Sulphur Compounds (VSCs). Most common of these VSCs is the compound methanethiol, found in cheese and curd [24]. MGL has also proved beneficial for controlling obesity, in experiments involving mice on a high fat diet and recombinant MGL. Recombinant MGL developed from *Pseudomonas putida* proved to be efficient in preventing obesity in mice [25].

2.2 Methionine and Cancer

Uncontrolled growth that is rapid, compared to the normal cells is a distinct characteristic of tumour cells. Such cells, when malignant, require methionine in higher concentrations, so as to have high protein synthesis and DNA expression. Earlier, only 'Warburg effect' was known, which meant utilization of glucose gets increased in cancerous cells, but then Hoffman introduced the concept of increased dependency of cancerous cells on methionine which further opened up new horizons for treatment of different cancer types [12]. Methionine dependency of abnormal cells can be utilized for the arrest of cancer cells by the enzymatic depletion of methionine. It was observed that such depletion when

introduced in various carcinomas, gliomas, melanomas, etc resulted in the cell cycle of these cells getting arrested in late S/G2 phase [10].

Methionine gets converted to S-adenosylmethionine and participates in DNA methylation, which is associated with cancer. Higher levels of L-methionine in the body means higher bioavailability of S-adenosylmethionine which will donate methyl groups to DNA . This can eventually lead to DNA hypermethylation of regulatory regions which could include the tumour suppressors. A study has shown that high methionine diets increased the risk for prostate cancer [12].

As cancer cells possess enhanced transmethylation rate compared to normal cells this can be also accounted as additional basis for methionine dependency in cancer cells. Such dependency of cancer cells on methionine and its raised use has been termed as "Hoffman Effect" and was seen to be more consequential than the "Warburg Effect", which was the dependency of cancer cells on glucose. Thompson once stated metabolic reprogramming as a hallmark of cancer and listed six metabolic hallmarks. And according to Robert Hoffman 'altered methionine metabolism' could be the seventh hallmark [7].

2.3 MGL in cancer therapy

L-methionine dependency is known as a metabolic defect relating to cancer. Even if the patient is put on a 'Met-free' diet for a long term, the results of clinical studies highlight the deleterious effect of doing so. So other strategies need to be adapted for depletion of the same from the body [23]. It has been observed that cancer cell targeting drugs are not fully effective against them as cancer stem cells are capable of expelling out the drugs before the cells get destroyed, this causes relapse of the disease. That is why combinational therapy is recommended whenever we deal with such a disease. So, a therapeutic approach is proposed wherein methionine is depleted from tumours by treating them with MGL, isolated from a source. As of now, recombinant MGL from *P. putida* has been utilized for such purposes. It was reported that it inhibited the growth of human tumours. MGL either alone or in combinations with different chemotherapeutic agents has shown efficacy in mouse models of brain, lung and colon cancer [12]. It was also reported that diet low in methionine inhibited the metastasis of Triple-Negative Breast Cancer (TNBC) in mice [13].

MGL has also been introduced to the human system in the form of a fusion protein. A fusion protein having L-MGL linked with human annexin-V was injected in the bloodstream and this protein only bound to the vascular endothelial cells' marker of the tumour cells. After binding the fusion protein prevented methionine supplementation to tumour cells which resulted in the death of the tumour cells or their growth inhibition because of MET restriction. Fusion protein has an advantage that it can be delivered through the bloodstream and doesn't need to be delivered directly to the tumour cells [12]. There is one more mode of administration which is orally. Recombinant MGL when administered orally has shown efficacy in colon cancer, pancreatic cancer, malignant melanoma, etc. It has also shown promising results in inhibiting post-surgical recurrence of TNBC in mouse-models. The biggest advantage it has is that it is administered orally without toxicity [13].

At the initial stages of using L-methioninase in cancer therapy it was seen that its administration resulted in steady depletion of plasma methionine but it came with certain side effects. Its administration resulted in less food intake and weight loss. While administering in macaque monkeys resulted in frequent vomiting and decline in serum albumin and red cell values. So, to overcome such severe reactions to its administration, it was conjugated with polyethylene glycol and PEG-MGL was prepared. The effectiveness of PEG-MGL was enhanced by simultaneous administration of PLP (Pyridoxal 5 phosphate) and oleic acid. This PEG-MGL though had a decreased efficacy for methionine depletion, saw a significant decrease in its antigenicity. So even if MGL from prokaryotic origin showed immunogenic issues, such issues were overcome with the help of PEGylation. And it also helped in removing the PLP dependence of MGL by its protective effect which arises from the high-level of PEGylation [12].

2.4 MGL producing organisms

Onitake was the first person to report that there were some bacterial species that produced methanethiol in the presence of L-methionine and L-cysteine. This led to a flux of research in this area such that till date many bacterial species have been reported that possess the MGL protein and their protein has been characterized. Few examples of such bacteria can be Clostridium *sporogenes, Citrobacter freundii, Pseudomonas putida, brevibacterium linens,* etc. The enzyme efficiency of the enzyme from these sources was tested not just in wild type, but also as a recombinant protein and in a mutant form. MGL is produced not only

by bacteria but many other organisms also, few of them are- *Trichomonas vaginalis*- which is a protozoon, *Arabidopsis thaliana*-a model plant, *Ferroplasma acidarmanus*- an archaeon, *Aspergillus* sp.- a fungus and many more [8]. After bacteria, the most explored organisms for the production of MGL are the fungal species. Many fungal species that have been screened for the production of this enzyme are- *Cladosporium oxysporum*, *Fusarium nivale*, several species of *Aspergillus* [14], *Candida tropicalis* [15], etc. From these mentioned organisms few have been or can be utilized as a source for the manufacturing of the therapeutic enzyme at a clinical level because every source has the MGL enzyme of slightly different properties and only the MGL which provides better quality of the enzyme and higher affinity towards methionine, can be relied upon for its utilization in cancer therapy.

It was reported that nutrients greatly affected the production of MGL. El-Sayed et al. suggested that methionine was a mandatory inducer of the MGL protein but many studies on various organisms like *P. putida* and *G. candidum* showed that MGL can be produced independent of the presence or absence of L-methionine in the growth media. Determination of the quality of enzyme is based on Michaelis-Menten kinetic parameters. These kinetic parameters give an idea about the interaction and efficiency of an enzyme to convert the desired substrate into product [16]. There are several parameters defined under this equation and one of such is the Km value which signifies the substrate concentration at which the enzyme can reach the 50% of its maximum velocity potential in the state of full saturation. In other words, it defines the affinity of the enzyme towards a particular substrate. MGL from *Pseudomonas putida* has been utilized in many studies revolving around the anti-cancer effects of this enzyme because it has a very low Km value for L-methionine, which means that it can very efficiently convert the methionine present even in small quantities into ammonia, thiol and keto acid. This trait is what makes the enzyme from this source so desirable [17].

Even with so many organisms with the ability to produce this enzyme, only the enzyme from a few species has been analysed for obtaining its crystal structure. The MGL unit is observed to be a tetramer, as shown in Fig 2, which can be split up into two catalytic dimers. Each dimer contains two active sites which have amino acid residues from both subunits and two molecules of PLP are bound covalently to Lys210. This analysis was done on *Pseudomonas putida* [18].

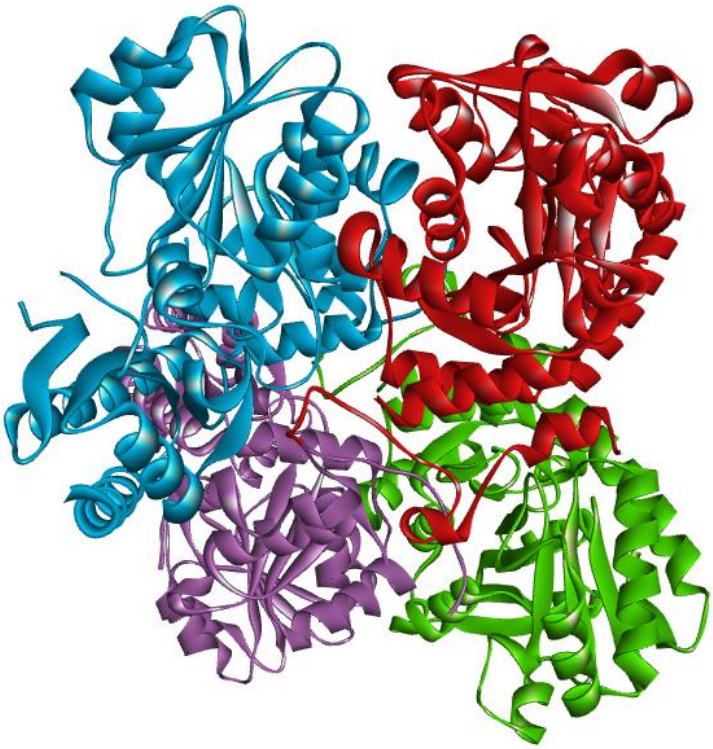


Fig 2: Crystal structure of PLP-dependent L-MGL from *Pseudomonas putida* with chain A represented by red colour, chain B by green, chain C by light blue and chain D by lavender colour. [PDB ID: 1GC0]

2.5 Assays for detection of MGL

Folin-Lowry Method for protein estimation: This is built on the biuret reaction and includes extra materials and stages to boost detection reactivity. In this reaction, the peptide has four nitrogen atoms that combine with copper to create a cuprous complex. When phosphomolybdic/ phosphotungstic acid, also called as the Folin-Ciocalteu reagent, is added, it engages in interactions with the cuprous ions and side chains of tyrosine, cysteine and tryptophan, so a blue-green hue is observed and the absorbance is taken between 650 nm and 750 nm. The range for protein detection is 5-100 µg. This method is insensitive to interfering substances and can detect protein even in complex mixtures. But as it is multi-step, it could be referred to as time-consuming, so accuracy could be affected sometimes.

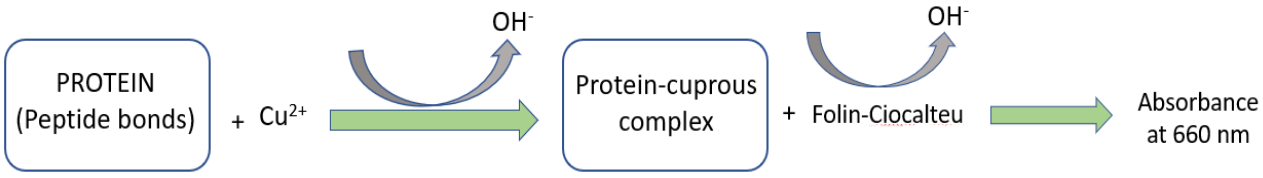


Fig 3: Chemical reaction of Folin-Lowry for estimation of protein content.

Nesslerization: The end product of the reaction between MGL and methionine yields- thiol, keto acid and ammonia. The released ammonia can be detected with the help of this method. The reaction between ammonium ions and Nessler's reagent results in the formation of a yellow to brown coloured precipitate of mercury ammonium iodide ($HgNH_2I_3$), which is directly proportional to the concentration of ammonium ions in the solution being tested. The intensity of the yellow to brown color is measured using a spectrophotometer at 420-480 nm, and the concentration of ammonium ions is determined by comparing the absorbance of the sample to a standard curve, prepared using known concentrations of ammonium sulphate [14]. This method provides us with the total activity of the enzyme.

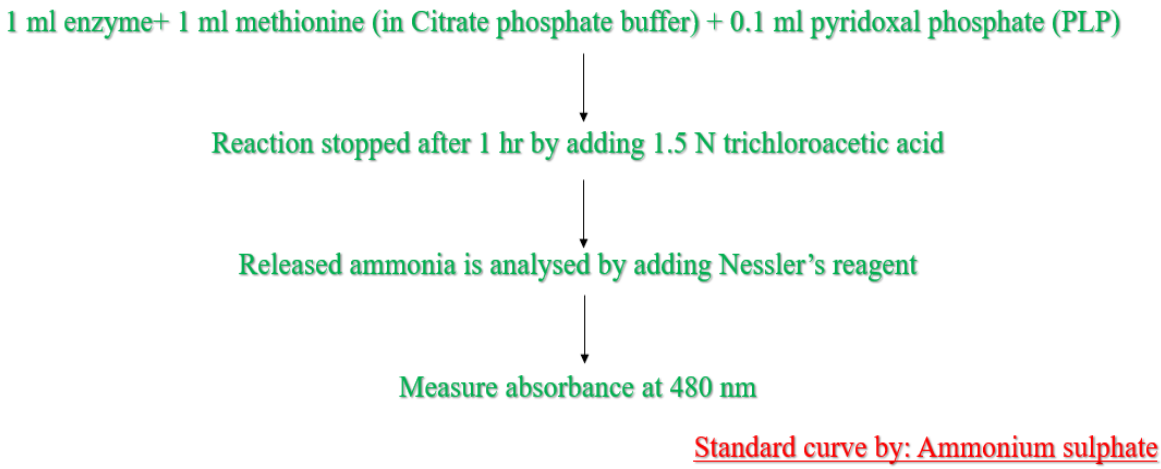


Fig 4: Procedure of nesslerization for assessing the total activity of the enzyme.

2.6 Michaelis Menten kinetics and Lineweaver Burk plot

Michaelis-Menten kinetics is considered as the simplest case of enzyme kinetics which can be applied to reactions of a substrate to a product. Such system can be represented as-

$$E + S \leftrightharpoons ES \rightarrow E + P$$

Where, S represents the substrate, E represents enzyme, ES represents the enzyme-substrate complex and P represents the product [26]. And various parameters of this system can be defined with the help of Michaelis Menten equation, which is represented is this form:

$$V_{\circ} = \frac{Vmax\ [S]}{Km + [S]}$$

Where, Vmax is maximum velocity achieved at maximum substrate concentration.

Km is substrate concentration at which the velocity is half of the Vmax.

[S] represents the substrate concentration.

The graph of Michaelis-Menten is plotted between the reaction velocity and substrate concentration and is a hyperbolic curve.

Another way to represent this curve is the Lineweaver Burk plot, which is the reciprocal of the Michaelis-Menten equation and can be written as-

$$\frac{1}{V_{\circ}} = \frac{Km}{Vmax\ [S]} + \frac{1}{Vmax}$$

The Lineweaver Burk plot is between the reciprocal of concentration of substrate and reciprocal of the rate of reaction. The graph is the linear line which is represented by y= mx + c. It is also called double reciprocal plot. One important thing about this plot is that the x-axis can go into negative values which means that it can extrapolate a hypothetical data where negative substrate concentration can exist [27].

Enzymes extracted from various sources have been characterized with respect to their affinity towards methionine, like- MGL from *Citrobacter freundii* exhibits a km value of 0.7 mM, MGL from *Pseudomonas putida* has a Km value of 1 mM, etc [15]. This value lets us know whether an enzyme has a good efficiency as an antitumor agent or not.

CHAPTER 3: MATERIALS & METHODOLOGY

3.1 Materials:

Following is the list of materials utilized throughout this study.

3.1.1 Biological Material: The organisms utilized in this study have been represented in table 1. Few characteristics of these organisms are also as follows:

Table 1: List of organisms procured from MTCC and utilized in this study.

S no.	Organism name	Company
1	*Pseudomonas fragi*	MTCC
2	*Pseudomonas lurida*	MTCC
3	*Citrobacter braakii*	MTCC

Pseudomonas fragi- It is Rod shaped, gram-negative and about 0.5-1 microns in breadth and 0.75- 4 microns in length. This usually occurs singly, in chains or in pairs. It belongs to the family- Pseudomonadaceae. It is motile and has a polar flagellum. Its characteristic growth in broth is visible as turbidity with sediment of thin pellicle. It is an aerobic bacterium and grows from 10ºC to 30ºC. It is sensitive to heat so no growth is seen at temperatures above 42ºC. Unlike other members of this genus, *P. fragi* is a non-pigmented member of this genus. [19].

Pseudomonas lurida- It is a motile, gram negative and rod-shaped bacteria. It forms whitish–yellow colonies on nutrient agar. It can grow at 4ºC, 15ºC and 30ºC with maximum growth observed at 30ºC. It belongs to the family Pseudomonadaceae [11].

The reason to choose these two species was to discover the properties of the enzyme from a genus whose one species has already been studied in detail for the same enzyme, i.e., *P. putida*. As it has been observed that many Pseudomonas sp. grow well on M9 minimal media, so the same can be utilized in this study.

Citrobacter braakii- It is gram-negative, straight rods, about ~1.0 μm × 2.0–6.0 μm. Its characteristics are defined similar to the characteristics of the whole genus. It occurs singly and also in pairs. It Belongs to the family Enterobacteriaceae. M9 minimal media can be utilized for its growth [11].

3.1.2 Chemicals: The list of chemicals utilized in this study is presented in Table 2.

Table 2: List of chemicals used in this study.

S.no.	Chemical	Manufacturer
1.	L-methionine	Acros organics, Loba Chemie
2.	Nessler's reagent	CDH, Himedia
3.	Citric acid anhydrous	Fisher Scientific
4.	Sodium phosphate dibasic dihydrate	Loba Chemie
5.	Pyridoxal-5'-phosphate anhydrous	Himedia
6.	Trichloroacetic acid	Loba Chemie
7.	Ammonium sulphate	Himedia
8.	Nutrient Agar	Himedia
9.	Nutrient Broth	Himedia
10.	Agar Agar	Himedia
11.	Sodium Chloride	Himedia
12.	Magnesium sulphate	Merck
13.	Gram's Iodine	Loba Chemie
14.	Safranin Red	Himedia

15.	Gram's Crystal violet	Loba Chemie
16.	Dipotassium Hydrogen Phosphate	Fisher Scientific
17.	Potassium Dihydrogen Phosphate	Merck
18.	Sodium Hydroxide	SRL
19.	Hydrochloric Acid–	Fisher Scientific
20.	Calcium Chloride	Loba Chemie
21.	Glucose	Fisher Scientific
22.	Ethanol	-
23.	Triton X-100	Sigma-aldrich
24.	HEPES Buffer	Fisher Scientific
25.	Tris Hydrochloride	Himedia
26.	EDTA	Merck
27.	Bovine Serum Albumin	Himedia
28.	Sodium Carbonate	Fisher Scientific
29.	Copper Sulphate	Himedia
30.	Sodium-Potassium Tartrate	Fisher Scientific
31.	Folin Ciocalteu Reagent	SRL

3.1.3 Instruments and Equipment: the list of equipment and instruments utilized in this study has been presented in Table 3.

Table 3: List of equipment and instruments used in this study.

INSTRUMENTS		
S. no.	**Instrument/ Equipment**	**Company**
1.	LAF	Microsil India
2.	Incubator	Mac engineering, Orbitek
3.	Centrifuge	Eppendorf
4.	Sonicator	Sonics
5.	Weighing Balance	Citizon
6.	pH meter	Eutech
7.	Water Bath	NSW India
8.	Autoclave	SANYO
9.	Freezer	Celfrost, Blue star
10.	Spectrophotometer	Multiskan GO
EQUIPMENTS		
1.	Micropipettes	Thermo scientific/Eppendorf
2.	Flasks	JSW, Borosil
3.	Reagent bottles	JSW, Borosil
4.	Microtips	AXYPET, Tarsons
5.	Falcon tubes	Tarsons, Genaxy
6.	Measuring cylinders	Tarsons

7.	Test tubes	JSW, Borosil
8.	Petri plates	Genaxy
9.	Microscope	Labovision
10.	Cryovials	Genaxy

3.2 Methodology:

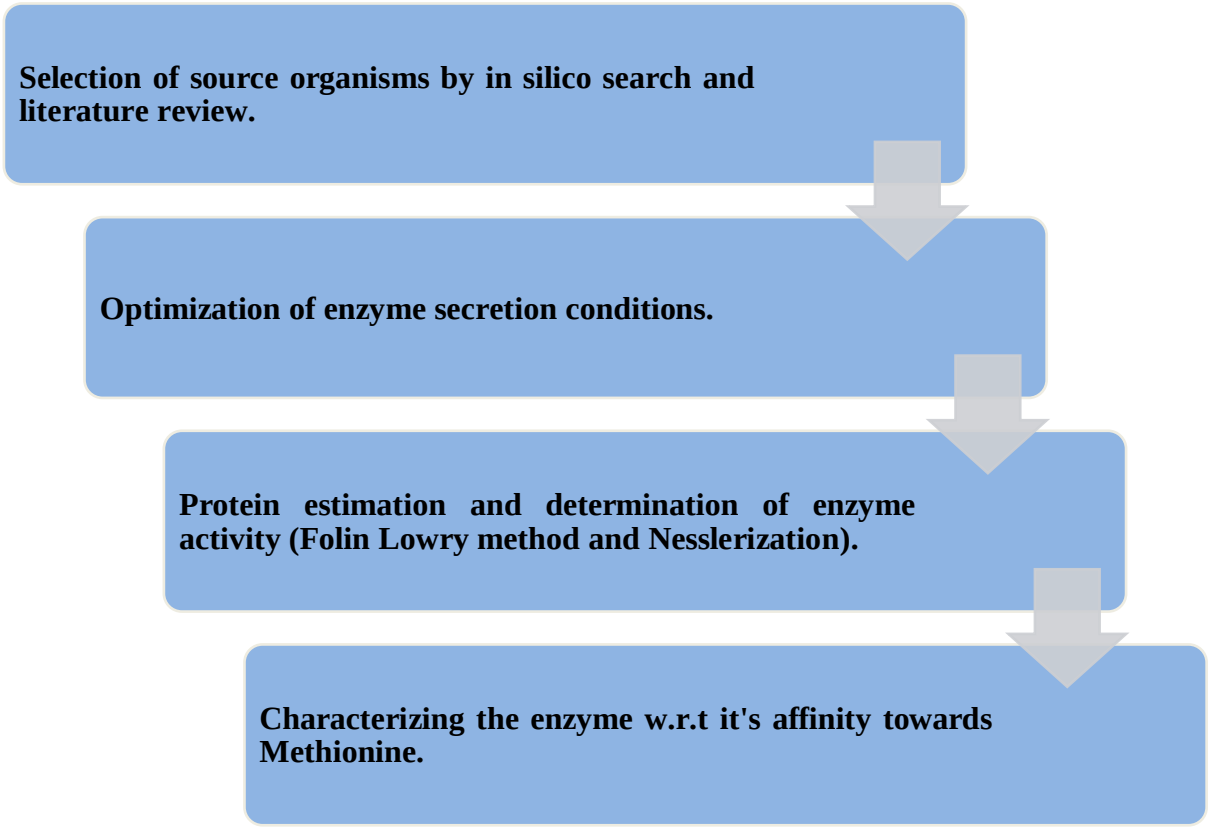


Fig 5: Flow chart representation of methodology of the experiment.

3.2.1 In-silico search for bacterial species: In-silico search was performed using the NCBI website. The 'Gene' option was selected on the "https://www.ncbi.nlm.nih.gov/" and the query- 'Methionine Gamma Lyase' was typed into the search box (Fig 6). From the available results, only bacterial species were considered and the results were tabulated.

These results were compared with the existing literature to filter out the strains that have not been utilized till date. The results obtained are then cross checked if their growth is possible in laboratory conditions. Then different bacterial species belonging to the genus with fully characterized enzymes were shortlisted to increase the chances of obtaining an enzyme with desired properties. Once all these parameters are fulfilled, the strains were searched on the MTCC website to check their availability and at the end, three strains were finalized to be investigated in this piece of work.

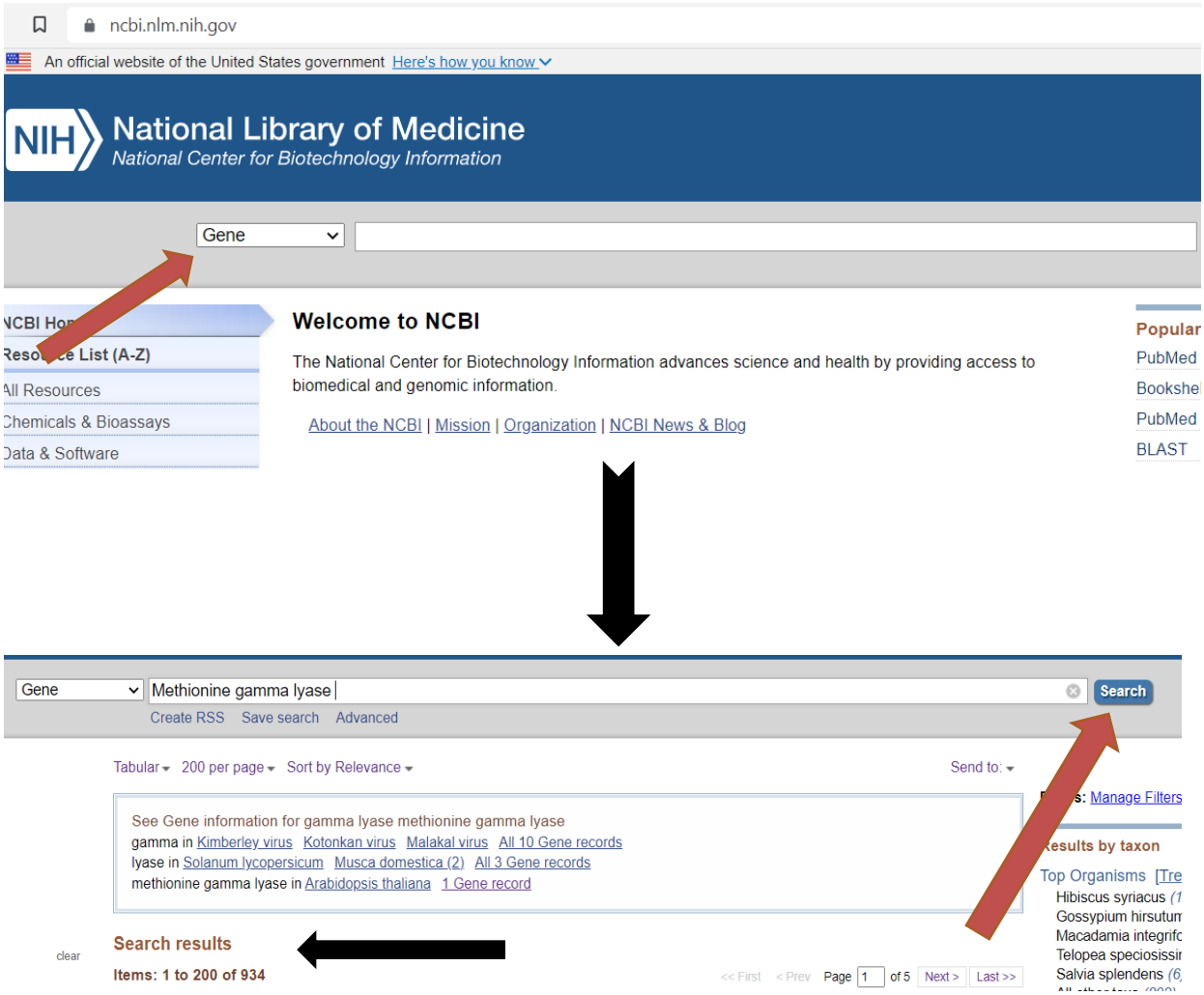


Fig 6: Snapshots of NCBI website showing the procedure for the selection of gene-option for searching methionine gamma lyase.

3.2.2 Revival of cultures: The cultures were received in a freeze-dried form and had to be revived in a media recommended by MTCC website. According to the website, all the three strains were to be revived in the same type of media, which was- Nutrient Agar/ Broth.

Step 1- Primary culturing: The glass vials were broken with the help of a clean cut using a filer. Once the vial was opened under sterilized conditions (In a LAF hood), an ample amount of culture was taken from the vial into a 250 ml flask, which contains 100 ml of autoclaved nutrient broth. This was done with the help of a sterilized inoculating loop. Once inoculation was performed, the cultures were kept at optimum temperatures recommended by the MTCC.

Table 4: The organisms utilized in this study and their recommended growth temperatures.

ORGANISM	**RECOMMENDED MEDIA**	**RECOMMENDED TEMPERATURE**
***Pseudomonas fragi* strain 4973**	Nutrient Broth	25ºC
***Pseudomonas lurida* strain NPRs3**	Nutrient Broth	25ºC
***Citrobacter braakii* strain 51113**	Nutrient Broth	37ºC

Step 2- Platting: Agar plates were prepared by pouring autoclaved nutrient agar into pre-sterilized petri plates. This was done to isolate pure colonies in case of any contamination in the freeze-dried sample. Several types of streaking can be performed to get pure colonies. Quadrant streaking was performed for this particular experimentation.

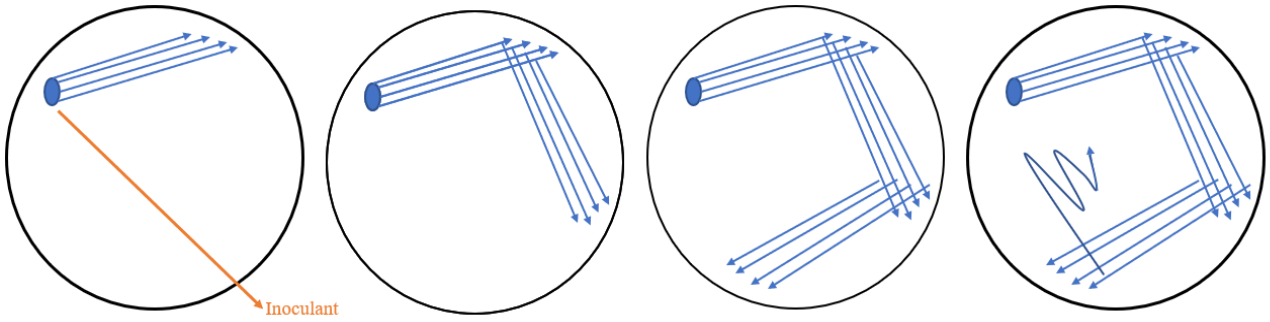


Fig 7: Inoculation procedure. (Quadrant streaking)

Step 3- Culturing of pure colonies: Once isolates were obtained on the agar plate, these were picked with the help of sterilized loop and inoculated into 50 ml nutrient broth prepared in 100 ml flasks.

Step 4- Preparation of glycerol stock: Glycerol stocks were prepared so that pure cultures could be revived for any future use. Cryo vials were autoclaved and 80% glycerol was prepared. 1 ml of glycerol was added with 1 ml of bacterial culture into the cryovials under a sterilized environment. The cryovials were kept at -80ºC.

3.2.3 Checking purity of the cultures: Once growth was obtained in the 100 ml flasks, the cultures were checked for their purity by gram's staining. The procedure was as follows-

Each culture was taken on a clean sterilized slide in a sterilized environment with the help of an inoculating loop.A smear was prepared. The smear was heat fixed with the help of flame from a spirit lamp. Once heat fixed, a few drops of gram's crystal violet were applied for 45 secs to 1 min and then the slide was washed with distilled water. After washing, gram's iodine was added for 45 secs and washed off with distilled water. Next step was to add a decolourizer (ethanol) to remove any excess stain. Once the colour gets washed off properly, counterstain- Safranin red was added and washed off after 45 seconds. The slide was dried and then observed under the microscope.

3.2.4 Optimization of enzyme production: The cultures were grown in M9 media and different parameters were tested to find the optimized conditions in which the bacteria produced the highest amount of enzyme. Three main parameters were tested- methionine concentration in the media, temperature and pH of the media.

For the first production setup, 5 ml of inoculant was added to 4 flasks containing 50 ml of M9 media each. Each flask had different concentrations of methionine- 0 g/L, 1 g/L, 2 g/L and 3 g/L. After 24 hrs of incubation, the crude enzyme was collected from each flask. Same procedure was followed to check the optimum temperature and pH conditions for enzyme production. While testing these parameters, both extracellular and intracellular enzymes were examined for the activity of MGL.

3.2.5 Cell lysis for intracellular enzyme: As the organisms were grown for 24 hrs, the cells were pellet out by centrifugation at 7000 rpm for 10 mins. The supernatant was separated and considered as the extracellular enzyme. For the intracellular enzyme, the cell pellet was washed with potassium phosphate buffer by centrifugation at 7000 rpm for 10 mins. After the wash, the cells were kept in a 10 ml lysis buffer for 30 mins at 4^{o}C. After incubation the cells were sonicated at the following settings-

Pulse: 10 secs off/ 10 secs on

Amplitude: 35%

Time: 2 mins

The lysed cells were centrifuged at 7000 rpm for 10 mins and the supernatant was separated and taken as the crude intracellular enzyme.

3.2.6 Optimization of enzyme activity assay: The conditions of the enzyme assay were optimized due to the formation of white precipitates that interfered with the Nesslerization method and provided us with inaccurate results. Several tests were performed for this purpose-

Test 1: One of the components of the lysis buffer was removed, i.e., EDTA. EDTA was removed as the literature suggested that it could interfere with the nesslerization process.

Test 2: Using masking components- It is believed that presence of certain metal ions can interfere with nesslerization and these metal ions can be engaged by using a masking component like- Rochelle salt (potassium sodium tartrate) that would make a complex with such ions. Few drops of 1% Rochelle salt were used prior to the addition of Nessler's reagent.

Test 3: Replacing Tris HCl with HEPES buffer: According to the literature, Tris HCl can form complexes with trichloroacetic acid used in the enzyme assay to stop the reaction. This could interfere with nesslerization. So, the lysis buffer was changed to HEPES buffer which was never reported to interfere with the ammonia assay, directly or indirectly.

Test 4: Reducing incubation time of the enzyme assay: The time of incubation was reduced from 60 mins to 30 mins.

Test 5: Reducing incubation time and enzyme volume: The time of incubation was reduced to 30 mins and enzyme volume was taken as 0.5 ml, rather than 1 ml.

Test 6: Pre-incubation of the cofactor PLP with the enzyme: A prior incubation of 0.5 ml enzyme with its cofactor was given for 10 mins and then the 1 ml substrate was added with an incubation time of 30 mins.

3.2.7 Calculation of total enzyme activity and specific activity: The ODs obtained from different assays were extrapolated on a standard graph to obtain the amount of product produced. This standard curve was obtained by measuring the OD of samples with known

concentration of ammonia, after their treatment with Nessler's reagent. Fig 8 depicts the procedure of enzymatic assay performed on the samples to calculate the enzyme activity.

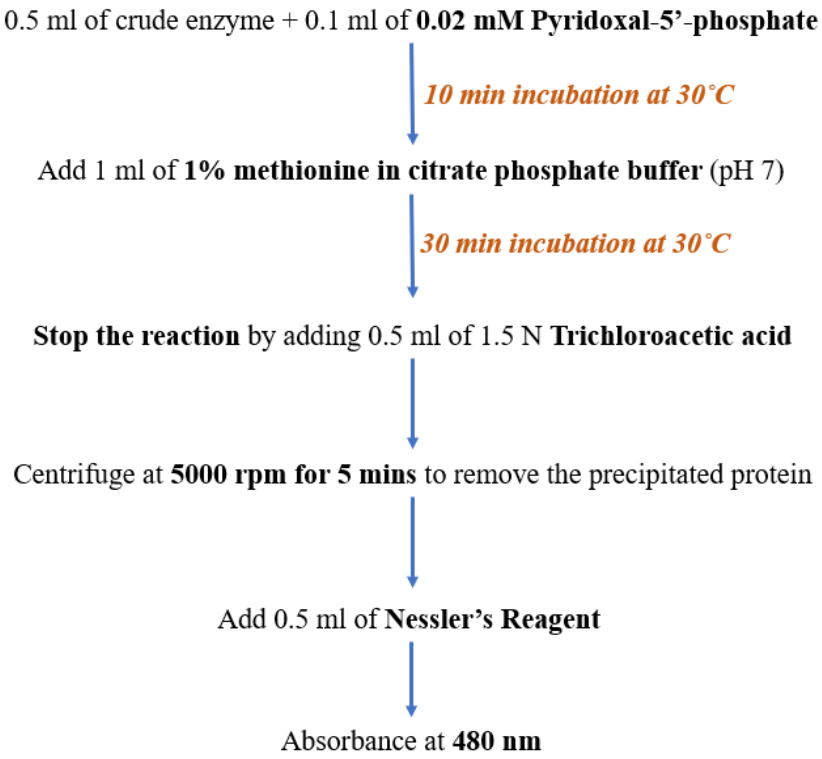


Fig 8: Flow chart representing nesslerization method for enzyme activity determination.

The ODs are extrapolated on the graph by the command '=TREND' in excel sheet or by using the formula- $\frac{1}{slope\ of\ the\ graph}\ x\ OD\ obtained$

The value obtained gives us the concentration of ammonia in the sample. This value is utilized in a formula, to determine the total activity of the enzyme. The formula of total activity of the enzyme is-

$$\frac{Value\ from\ graph\ x\ total\ reaction\ volume}{volume\ of\ enzyme\ x\ incubation\ time}$$

Enzyme activity is expressed in U (units). One unit is defined as the volume of enzyme that releases 1 mmole of ammonia per min per ml of the reaction under optimum conditions.

Once the three parameters were established, specific activity of each enzyme was calculated by determining protein concentration with the help of the Folin-Lowry method. The steps of Folin-Lowry have been described in Fig 9.

Preparation of solutions-

Solution A: 2% Sodium carbonate in 0.1 N NaOH

Solution B: 0.5% copper sulphate in 1% potassium-sodium tartrate

Solution C: 50 ml solution A + 1 ml solution B

Folin Ciocalteu Reagent: 5 ml 2N F_cR + 5 ml distilled water

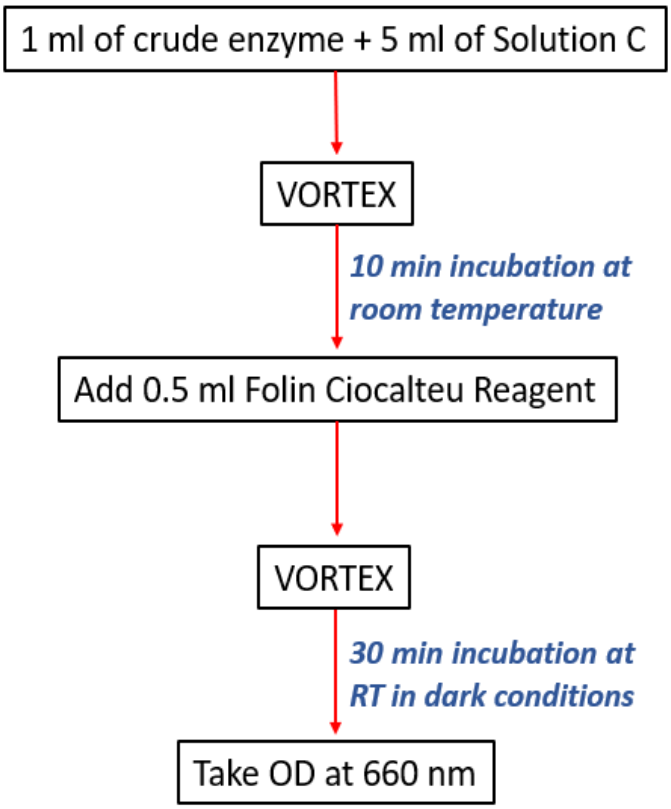


Fig 9: Representation of Folin-Lowry method for protein estimation.

3.2.8 Characterization of enzymes: The enzymes were characterized for few properties- their optimum temperature and pH for enzyme activity, their thermal stability and their affinity towards the substrate of interest i.e., Methionine.

A. Optimum Temperature: This was determined by changing the temperature of the enzyme assay at 30 mins incubation of enzyme reaction. The temperatures tested were- 30°C, 40°C,

50°C, 60°C and 70ºC. These temperatures were maintained with the help of incubators and water baths.

B. Optimum pH: The optimum pH was determined by changing the pH of the substrate buffer. The buffer utilized for this purpose was- citrate-phosphate buffer and pH range was form 4-8. Rest of the reaction was kept the same as the original.

C. Thermal Stability: Thermal stability of the enzyme is determined by keeping the enzyme at different temperatures for 30 mins, prior to the enzymatic assay. The reaction was carried out at 30ºC, same as in the original assay.

3.2.9 Determination of K_m value of the enzymes: The K_m value of each enzyme towards the substrate can be determined by plotting a Lineweaver burk plot between reciprocal values of substrate concentration used and respective rate of reaction. For different substrate concentrations, a stock solution of 100mM of methionine in citrate phosphate buffer was prepared and the rest of the concentrations were satisfied by diluting the stock solution according to the desired concentration. This was done using the formula-

$$C_1V_1 = C_2V_2$$

Table 5: Different concentrations of substrate utilized for Lineweaver burk plot

Substrate concentration	*Volume of Stock solution (in ml)*	*Volume of distilled water (in ml)*	*Total volume (in ml)*
10 mM	1	9	10
20 mM	2	8	10
40 mM	4	6	10
60 mM	6	4	10
80 mM	8	2	10
100 mM	10	0	10

The reactions were carried out according to the same methodology used before and the enzyme activity that we got was plotted on to the graph to obtain a straight line. The equation of the graph is written as-

$$Y = mx + c$$

Where,

Y corresponds to: $\frac{1}{V_{\circ}}$

M (Slope of the graph) corresponds to: $\frac{Km}{Vmax}$

C corresponds to: $\frac{1}{Vmax}$

CHAPTER 4: RESULTS AND DISCUSSIONS

4.1 In-silico search for bacterial species:

As per the detailed procedure outlined in material and methods for the selection of bacterial species for production and optimization of MGL, three bacterial species depicted in Table 1, were selected to be investigated in this study.

Table 6: Bacterial Species selected on the basis of results from in-silico search and literature review.

S. NO.	ORGANISM	FAMILY	GROWTH TEMPERATURE
1	*Pseudomonas fragi*	Pseudomonadaceae	10-30ºC
2	*Pseudomonas lurida*	Pseudomonadaceae	10-30ºC
3	*Citrobacter braakii*	Enterobacteriaceae	30-37ºC

The selected bacterial species have not yet been tested for the secretion of this enzyme and the enzymes produced by these organisms can be characterized with the help of a few parameters. So, these obtained species were procured from MTCC, Chandigarh and were revived, to utilize them for the same.

4.2 Culture revival and validation by Gram's staining:

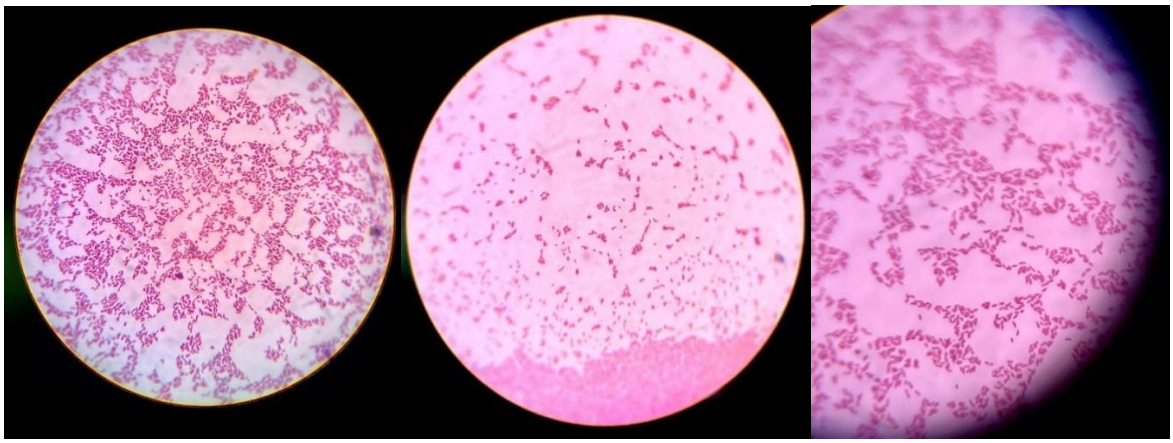
Culture revival: The cultures of selected bacterial species obtained from MTCC were revived using recommended media. Once the growth was observed by cloudy appearance of the media in the flask (after 24 to 48 hrs), the grown bacteria were inoculated onto the agar plates via quadrant streaking, to obtain pure colonies. The pure colonies were observed after 24 hrs incubation (Fig 10)

(A) **(B)** **(C)**

Fig 10: Bacterial growth on agar plates after 24 hrs. (A) *Pseudomonas lurida*, (B) *Citrobacter braakii*, (C) *Pseudomonas fragi*

Gram's Staining: The selected 3 bacterial species were tested with respect to Gram's staining**.** *Pseudomonas lurida* appeared rod-shaped and pink in colour when observed under 100x (Fig 11A). This means that it was gram's negative. Literature also suggests that the given species is gram's negative. *Citrobacter braakii* appeared pink in colour with small rod-shaped entities indicating it to be gram negative (Fig 11B). The gram staining outcome of *Pseudomonas fragi* culture is shown in fig 11C. This bacterial species was observed to give pink colour after gram's staining and the observed shape was rod, under the microscope. In this case also the observations were as per the existing literature.

The observed morphological characters of three bacterial species matched with the expected information available in the literature. So, it could be believed that the cultures we received were pure and of correct species, and further work could be carried out.



A **B** **C**

Fig 11: Microscopic images of bacterial smears after gram's staining. A. *Pseudomonas lurida*, B. *Citrobacter braakii*, C. *Pseudomonas fragi*

4.3 Standard curves for enzyme activity and concentration assay

Nesslerization: The standard graph of OD vs different concentrations (0.1 mM to 1 mM) of ammonium sulphate was made using stock solutions of 1 mM, 1.2 mM and 1.4 mM. The results obtained are presented in Table 7 and the graph formed is presented in Fig 12.

Table 7: Representation of different concentrations of ammonium sulphate solutions and their corresponding ODs.

Concentration of Ammonium sulphate (in mM)	Volume of Stock solution used (in ml)	Volume of citrate phosphate buffer (in ml)	Volume of Nessler's reagent (in ml)	Mean OD obtained at 480 nm
0	0	2	0.5	0
0.2	0.4	1.6	0.5	0.006
0.4	0.8	1.2	0.5	0.09
0.6	1.2	0.8	0.5	0.213
0.8	1.6	0.4	0.5	0.402
1	2	0	0.5	0.551
1.2	2 (of 1.2 mM stock)	0	0.5	0.64
1.4	2 (of 1.4 mM stock)	0	0.5	0.759

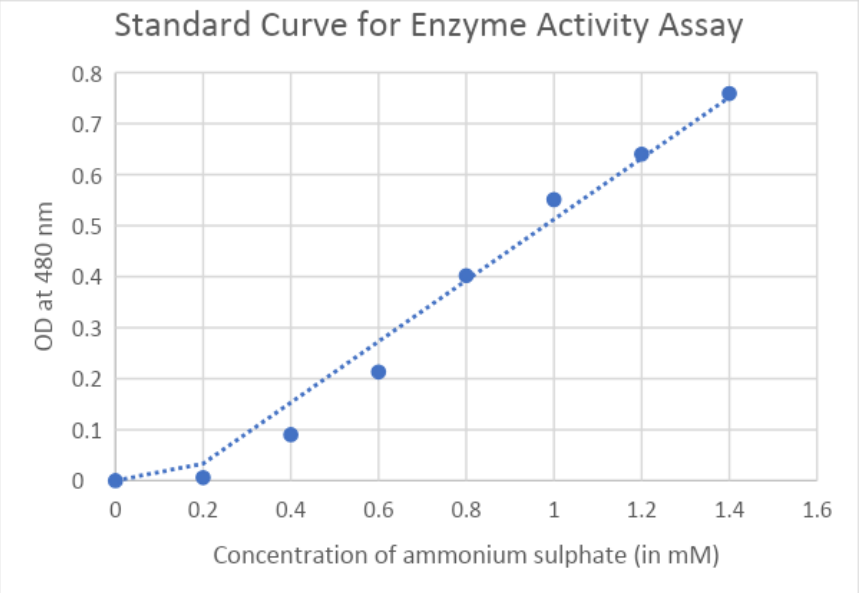


Fig 12: Standard graph for enzyme activity assay.

Folin Lowry Assay: The standard graph was made with Bovine Serum Albumin. The stock solution was of 1 mg/ml concentration and different concentrations were achieved by diluting the stock solution. The ODs obtained are presented in Table 8.

Table 8: Representation of different BSA concentration and corresponding ODs.

Concentration of BSA	Volume of stock (in ml)	Volume of Distilled water (in ml)	Mean OD obtained at 660 nm
0	0	1	0
0.2	0.2	0.8	0.2655
0.4	0.4	0.6	0.421
0.6	0.6	0.4	0.6175
0.8	0.8	0.2	0.7775
1	1	0	0.8785

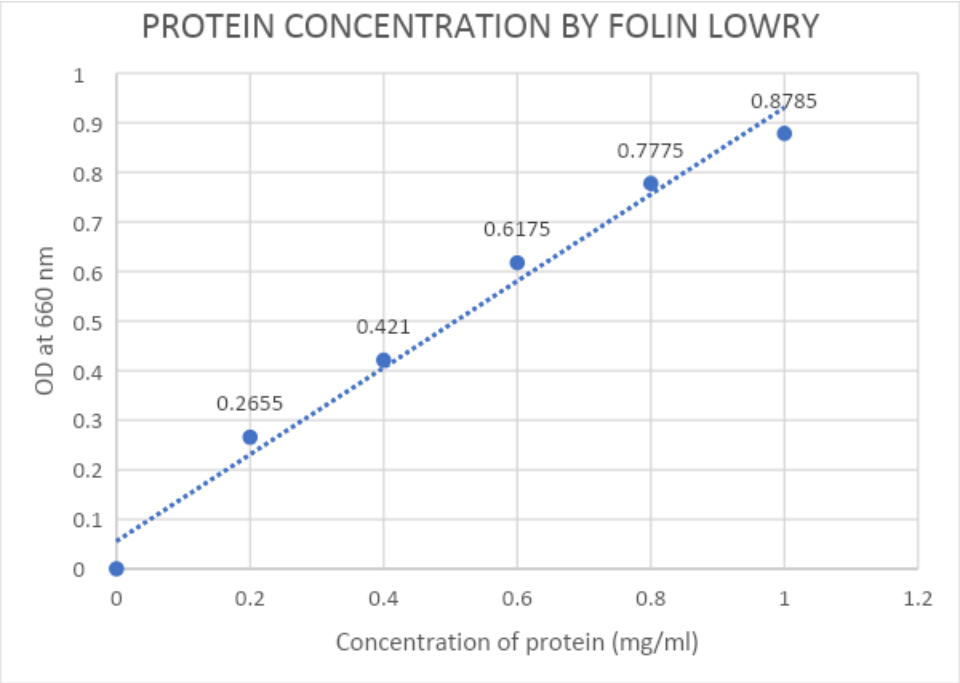


Fig 13: Standard graph for protein estimation

4.4 Optimization of enzyme assay:

White precipitates were observed during the initial enzyme assay, which were interfering with the OD at 480 nm. So different conditions were changed one by one to address this problem. As these white precipitates were forming much more in case of intracellular enzymes, the components of the lysis buffer were first changed one-by-one.

Components of lysis buffer- Tris HCl, NaCl, Triton X100 and EDTA

Test 1: Removing EDTA.

The removal of EDTA had no significant effect on the formation of white precipitates. The OD obtained was in the same range as before, which was from 2.0 to 2.5.

Test 2: Using Rochelle Salt.

Few drops of Na-K tartrate (Rochelle salt) were added to the samples, 2 mins prior to their treatment with Nessler's reagent. This too showed no significant difference in the formation of white precipitates and ODs were obtained in the same range.

Test 3: Replacing Tris HCl with HEPES buffer.

The components of the lysis buffer were changed to less interacting ones. The new components were- HEPES buffer, Triton X 100 and NaCl. This step observed no visible

difference in the formation of white precipitates, but the OD range changed from 2.0-2.5 to 1.5-1.9.

Test 4: Reducing the incubation time.

The assay time was changed from one hour to 30 mins. Reducing the assay time showed much difference in the formation of the white precipitates in case of *Pseudomonas lurida* and *Citrobacter braakii*. The ODs obtained were also in the range of 0.5- 1.2. But large amounts of white precipitates were still forming in the case of *Pseudomonas fragi*.

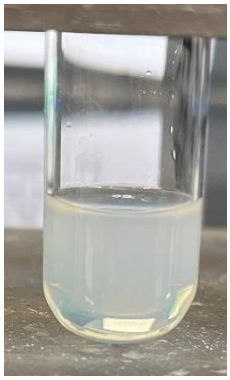
Test 5: Reducing time and enzyme volume.

This test provided the least amount of white precipitates in case of all the three bacteria. But it also came with a problem that the yellow precipitates, that were supposed to form due to interaction of ammonia with nessler's reagent, were also very less and the OD was coming in the range of 0.09-0.3. This meant that the amount of product formed was very low.

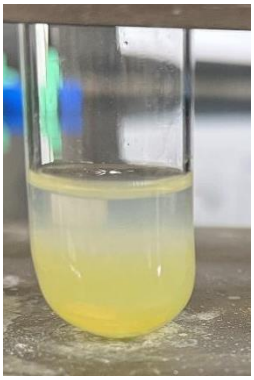
Test 6: Pre-incubation with co-factor.

Before adding the substrate, the enzyme was incubated with PLP for 10 mins. This provided an extra time for the enzyme to interact with the cofactor for its proper activation. As a result, the formation of yellow precipitates was visible and the nesslerization results were finally in an acceptable range of OD, i.e., 0.4-0.8.

(A)



(B)



(C)

Fig 14: Comparative results obtained from different tests for optimization of enzyme assay: (A) Test 1- Removal of EDTA, (B) Test 5- reduction of time and enzyme volume and (C) Test 6- Pre-incubation with the cofactor.

These tests provided us with a more accurate and specific approach to calculate the enzyme activity of the enzyme without any interference of other molecules. Fig 14 shows the results obtained after test-1, 5 and 6.

4.5 Optimization of protein secretion conditions:

A. Methionine Concentration: The first parameter that was optimized for each enzyme was the concentration of methionine needed in the media for maximum MGL production. Four flasks with 0, 1, 2 and 3 g/L of methionine were prepared and inoculated with the three bacterial species. The following results were obtained after enzyme assay of the enzymes produced in each flask.

A1.1. *Pseudomonas lurida* (Intracellular enzyme)

Table 9: Results obtained from nesslerization of intracellular enzyme of *P. lurida.*

Concentration of methionine (g/l)	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)
0	0.2435	0.55529	0.078
1	0.2595	0.58127	0.081
2	0.2575	0.57802	0.081
3	0.219	0.51551	0.072

A1.2. *Pseudomonas lurida* (Extracellular enzyme)

Table 10: Results obtained from nesslerization of extracellular enzyme of *P. lurida.*

Concentration of methionine (g/l)	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)
0	-0.095	-nil-	-
1	-0.068	-nil-	-
2	-0.087	-nil-	-
3	-0.05	-nil-	-

The results obtained revealed that MGL was produced intracellularly in case of *Pseudomonas lurida*. No enzyme activity was seen in case of extracellular crude. Intracellularly, MGL showed highest activity at 1 g/l concentration of methionine meaning that the highest amount of MGL was produced in this condition. Intracellular enzyme also showed activity in the absence of methionine, indicating that it was produced independent of the presence of methionine.

A2.1 *Pseudomonas fragi* (Intracellular enzyme)

Table 11: Results obtained from nesslerization of intracellular enzyme of *P. fragi.*

Concentration of methionine (g/l)	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)
0	0.235	0.54149	0.076
1	0.255	0.57396	0.080
2	0.263	0.58695	0.082
3	0.3485	0.72578	0.102

A2.2 *Pseudomonas fragi* (Extracellular enzyme)

Table 12: Results obtained from nesslerization of extracellular enzyme of *P. fragi.*

Concentration of methionine (g/l)	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)
0	-0.152	0	0
1	0.307	0.65839	0.092
2	0.307	0.65839	0.092
3	0.513	0.99287	0.139

In case of *pseudomonas fragi*, enzyme was produced both extracellularly and intracellularly. This condition was different from the one observed in *Pseudomonas lurida.* Intracellular

MGL showed highest activity when the media had 3 g/l of methionine and the same held true in case of extracellular enzyme where highest activity was seen at 3 g/l concentration.

The extracellular enzyme only showed activity in the presence of methionine, which means that enzyme secretion in the outside environment was dependent on the availability of the methionine. The intracellular enzyme showed activity independent of methionine's presence.

Both *P. lurida* and *P. fragi* were selected due to a previously studied organism of the same genus, i.e., *Pseudomonas putida.* It is interesting to note that the enzyme was only found intracellularly in the case of *Pseudomonas putida*. Although the same was observed for *P. lurida*, the enzyme was also produced extracellularly in case of *P. fragi.* This shows the diversity in properties of the enzyme obtained from different species of the same genus.

A3.1 *Citrobacter braakii* (Extracellular enzyme)

Table 13: Results obtained from nesslerization of extracellular enzyme of *C. braakii.*

Concentration of methionine (g/l)	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)
0	-0.095	0	0
1	0.378	0.7737	0.108
2	0.482	0.9425	0.155
3	0.341	0.7136	0.100

A3.2 *Citrobacter braakii* (Intracellular enzyme)

Table 14: Results obtained from nesslerization of intracellular enzyme of *C. braakii.*

Concentration of methionine (g/l)	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)
0	0.2235	0.52282	0.073
1	0.2415	0.55204	0.077
2	0.2755	0.60725	0.085

3	0.1695	0.43514	0.061

The results obtained suggested that the enzyme was both intracellular and extracellular in nature in case of *Citrobacter braakii*. The enzyme showed activity in the extracellular crude sample, indicating that it had similar properties to the previously studied species- *C. freundii*. The enzyme showed highest activity when the media was supplemented with methionine at a concentration of 2 g/l. The activity of the intracellular crude was much less than extracellular enzyme, this could be due to either partial lysis of the cell or the difference in properties of intracellular and extracellular enzyme because of different environment.

B. Growth Temperature: Once the optimum methionine concentration was determined, the flasks were kept at different temperatures for 24 hrs incubation to note the effect of temperature on the production of enzyme. The concentration of methionine in the media was set according to the results obtained from the previous parameter.

B1.1 *Pseudomonas lurida* (Intracellular enzyme)

Table 15: Results obtained from nesslerization of intracellular enzyme of *P. lurida* for different growth temperatures.

Growth Temperature	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)
25	0.5355	0.604	0.085
30	0.559	0.64216	0.090
35	0.53475	0.60278	0.084
40	0.51325	0.56787	0.080

The literature suggests that the optimum temperature of growth for *P. lurida* is 30°C. And the highest activity was also observed at the same temperature. This shows that the amount of enzyme increases with the increase in bacterial growth.

B2.1 *Pseudomonas fragi* (Extracellular enzyme)

Table 16: Results obtained from nesslerization of extracellular enzyme of *P. fragi* for different growth temperatures.

Growth Temperature	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)
25	0.451	0.8922	0.125
30	0.5085	0.98556	0.138
35	0.592	1.12114	0.157
40	0.52	1.00424	0.141

B2.2 *Pseudomonas fragi* (Intracellular enzyme)

Table 17: Results obtained from nesslerization of intracellular enzyme of *P. fragi* for different growth temperatures.

Growth Temperature	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)
25	0.306	0.65677	0.092
30	0.31875	0.67747	0.095
35	0.351	0.72983	0.102
40	0.3235	0.68478	0.096

Both extracellular and intracellular enzymes were tested after incubating the culture at different temperatures. The optimum growth temperature for *Pseudomonas fragi* can be from 25-30ºC. But still the highest activity was observed when the cultures were kept at 35ºC, indicating that the enzyme produced much more in temperatures higher than the optimum. This stood true for both extracellular and intracellular enzymes. The enzyme activity saw a dip at 40°C which could be due to less growth.

B3.1 *Citrobacter braakii* (Extracellular enzyme)

Table 18: Results obtained from nesslerization of extracellular enzyme of *C. braakii* for different growth temperatures.

Growth Temperature	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)
25	0.212	0.50414	0.071
30	0.27	0.59832	0.084
35	0.315	0.67138	0.094
40	0.305	0.65515	0.091

B3.2 *Citrobacter braakii* (Intracellular enzyme)

Table 19: Results obtained from nesslerization of intracellular enzyme of *C. braakii* for different growth temperatures.

Growth Temperature	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)
25	0.2055	0.49359	0.069
30	0.2934	0.63631	0.089
35	0.299	0.6454	0.090
40	0.28225	0.61821	0.087

The optimum growth temperature for *Citrobacter braakii* is 37°C. As that temperature was not part of the parameter, the organism showed highest production of the enzyme at 35°C. This was the case for both extracellular and intracellular enzymes. The previously reported species, *C. freundii*, of this genus produced the highest amount at a range of 40-50°C.

C. Growth pH: After determining the optimum growth temperature for each culture, the next parameter that was tested was optimum pH for enzyme production.

B1.1 *Pseudomonas lurida* (Intracellular enzyme)

Table 20: Results obtained from nesslerization of intracellular enzyme of *P. lurida* for different growth pH.

Growth pH	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)
5	0.241167	0.551502	0.077
6	0.285833	0.624026	0.087
7	0.2805	0.615366	0.086
8	0.252167	0.569362	0.079

The pH of the growth medium was kept at 7 for the previous parameters. To achieve different pH of the growth media NaOH was added to increase the pH and HCl was added to decrease the pH. The activity of *Pseudomonas lurida* was highest in case of pH 6. Which means that *P. lurida* produced more MGL in slightly acidic conditions.

B2.1 *Pseudomonas fragi* (Extracellular enzyme)

Table 21: Results obtained from nesslerization of extracellular enzyme of *P. fragi* for different growth pH.

Growth pH	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)
5	0.209333	0.499815	0.070
6	0.376	0.770427	0.108
7	0.574667	1.092996	0.153
8	0.239667	0.549066	0.077

B2.2 *Pseudomonas fragi* (Intracellular enzyme)

Table 22: Results obtained from nesslerization of intracellular enzyme of *P. fragi* for different growth pH.

Growth pH	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)
5	0.176	0.445693	0.062
6	0.276	0.60806	0.085
7	0.328	0.692491	0.097
8	0.2263	0.527417	0.074

As *Pseudomonas fragi* was kept at different pH for 24 hrs, it showed that the highest amount of enzyme was produced only when the pH was kept at neutral, i.e., 7. This stood true for both extracellular and intracellular enzyme. Few strains of *P. putida* showed similar results in the previous studies.

B3.1 *Citrobacter braakii* (Extracellular enzyme)

Table 23: Results obtained from nesslerization of extracellular enzyme of *C. braakii* for different growth pH.

Growth pH	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)
5	-	0	0
6	0.305667	0.656229	0.092
7	0.317333	0.675171	0.095
8	0.295333	0.639451	0.090

B3.2 *Citrobacter braakii* (Intracellular enzyme)

Table 24: Results obtained from nesslerization of intracellular enzyme of *C. braakii* for different growth pH.

Growth pH	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)
5	-0.0645	0	0
6	0.205167	0.49305	0.069
7	0.256833	0.576939	0.081
8	0.228167	0.530394	0.074

The results showed that *C. braakii* produced the highest enzyme when the media pH was kept at 7. This means that it requires neutral conditions to produce the highest amount of MGL.

It was also interesting to note that the culture was not able to grow when media pH was at 5 as no enzyme activity was detected in this case.

D. Specific Activity of each enzyme: The cultures were grown in conditions optimum for enzyme secretion and the specific activity of MGL from each source was calculated. This was done by performing enzyme activity assay as well as Folin-lowry assay on each enzyme after 24 hrs of culture growth. The results have been presented in Table 20.

Table 25: Total protein, Total enzyme activity and Specific activity of enzyme from each source.

ORGANISM	TOTAL PROTEIN (mg/ml)	TOTAL ACTIVITY (U/ml)	SPECIFIC ACTIVITY (U/mg)
Pseudomonas lurida	0.413	0.088	0.214
Pseudomonas fragi (intracellular)	0.472	0.103	0.218

Pseudomonas fragi (extracellular)	0.773	0.157	0.203
Citrobacter braakii (intracellular)	0.349	0.085	0.242
Citrobacter braakii (extracellular)	0.433	0.096	0.223

Specific activity tells us about the amount of enzyme present in the sample, as compared to the total amount. Highest specific activity was observed in *C. braakii* and the lowest was observed in *P. fragi*. Even with such low values of *C. braakii's* total activity, the specific activity came out to be highest, because the total amount of enzyme produced by it was also very low, which means it gave a sufficient amount of activity even at low concentration of MGL.

4.6 Characterization of enzyme: Once the optimum conditions for each bacterial source were achieved, the enzyme from each source was characterized on the basis of few parameters.

A. Optimum Temperature

A.1. *Pseudomonas lurida* (Intracellular enzyme)

Table 26: Enzyme activity variation observed for enzyme obtained from *P. lurida* at different temperatures.

Temperature	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)
30	0.251	0.567468	0.079
40	0.289	0.629167	0.088
50	0.266	0.591823	0.083
60	0.1655	0.428644	0.060
70	0.1275	0.366945	0.051

A.2. *Pseudomonas fragi* (Extracellular enzyme)

Table 27: Enzyme activity variation observed for enzyme obtained from *P. fragi* (extracellular) at different temperatures.

Temperature	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)
30	0.4795	0.938477	0.131
40	0.5625	1.073241	0.150
50	0.5575	1.065123	0.149
60	0.4255	0.850798	0.119
70	0.2965	0.641345	0.089

A.3. *Pseudomonas fragi* (Intracellular enzyme)

Table 28: Enzyme activity variation observed for enzyme obtained from *P. fragi* (intracellular) at different temperatures.

Temperature	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)
30	0.3395	0.711163	0.099
40	0.376	0.770427	0.107
50	0.281	0.616178	0.086
60	0.204	0.491155	0.069
70	0.092	0.309304	0.043

A.4. *Citrobacter braakii* (Extracellular enzyme)

Table 29: Enzyme activity variation observed for enzyme obtained from *C. braakii* (extracellular) at different temperatures.

Temperature	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)
30	0.2805	0.615366	0.086
40	0.319	0.677877	0.095
50	0.243	0.554479	0.078
60	0.195	0.476542	0.067
70	0.0645	0.264653	0.037

A.5. *Citrobacter braakii* (Intracellular enzyme)

Table 30: Enzyme activity variation observed for enzyme obtained from *C. braakii* (intracellular) at different temperatures.

Temperature	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)
30	0.3125	0.667324	0.093
40	0.3345	0.703044	0.098
50	0.3005	0.64784	0.091
60	0.199	0.483037	0.068
70	0.1735	0.441633	0.062

For all the three sources of enzyme, the highest activity was observed at 40°C. From the previously known species, the highest activity was observed at 45-55°C for *P. putida*. MGL from *Pseudomonas fragi* (extracellular enzyme) showed almost the same activity at 50°C. This could mean that it works almost equally efficiently at 50°C as it works at 40°C. The mere difference in the readings could also be accounted for by any pipetting error. The intracellular enzyme from *P. fragi* showed much difference in the enzyme activity at temperature 40 and 50°C.

B. Thermal Stability

B.1. *Pseudomonas lurida* (Intracellular enzyme)

Table 31: Enzyme activity of the enzyme obtained from *P. lurida* after treatment with different temperatures.

Temperature	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)	Relative activity (%)
30	0.2875	0.626732	0.088	100
40	0.2855	0.623485	0.087	98.86
50	0.281	0.616178	0.086	97.73
60	0.038	0.221626	0.031	35.23
70	0.0045	0.167233	0.023	26.14

The enzymes were treated at different temperatures for 30 mins and then were utilized in the enzyme assay in the same manner as in the previous processes. It was observed that the enzyme was able to retain 26% of its original activity at 70°C. This shows that the enzyme can be considered as thermostable. Fig 15 represents the enzyme activity of MGL from *P. lurida* after heat inactivation.

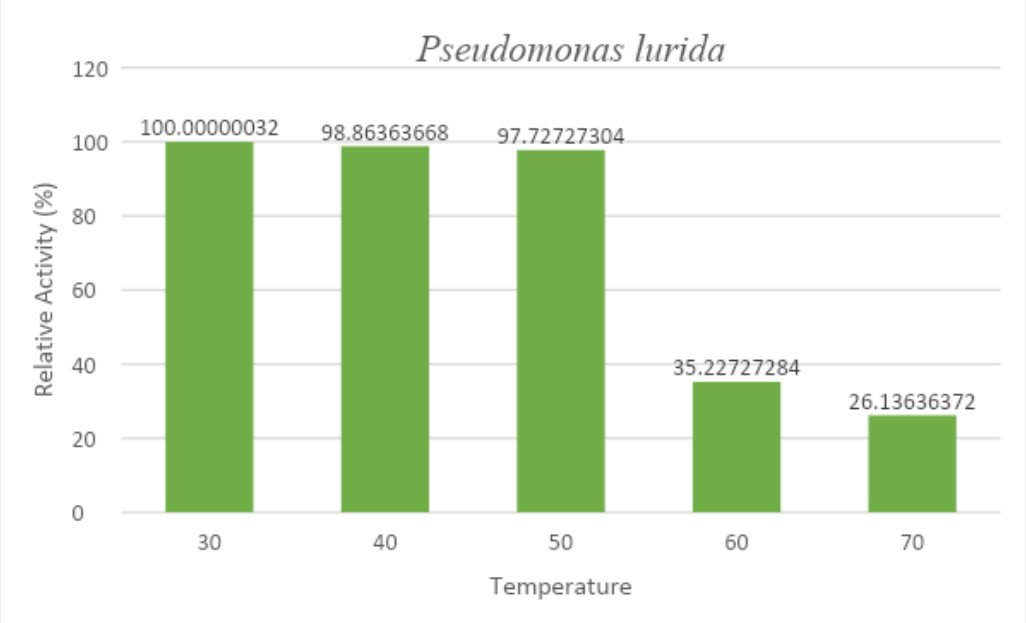


Fig 15: Heat inactivation of MGL from *P. lurida.*

B.2. *Pseudomonas fragi* (Extracellular enzyme)

Table 32: Enzyme activity of the enzyme obtained from *P. fragi* after treatment with different temperatures.

Temperature	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)	Relative activity (%)
30	0.584	1.10815	0.155	100.00
40	0.563	1.074053	0.150	96.77
50	0.356	0.737953	0.103	66.45
60	0.106	0.332036	0.046	29.68
70	-0.011	0.0142	0	0.00

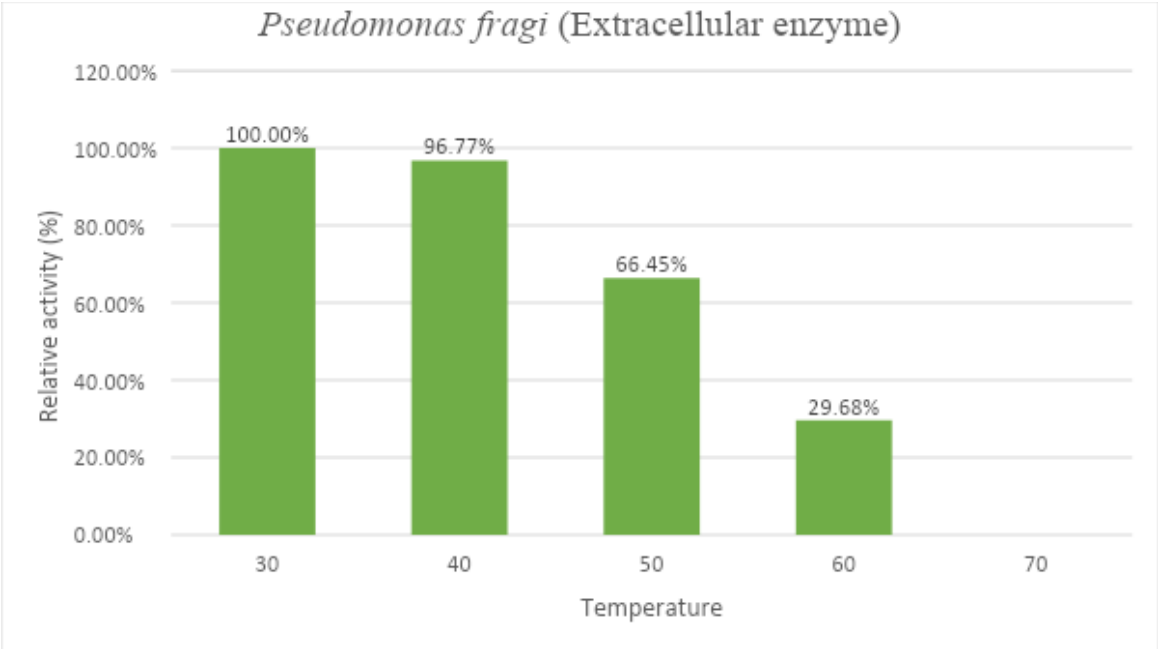


Fig 16: Heat inactivation of extracellular MGL from *P. fragi*.

B.3. *Pseudomonas fragi* (Intracellular enzyme)

Table 33: Enzyme activity of the enzyme obtained from *P. fragi* after treatment with different temperatures.

Temperature	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)	Relative activity (%)
30	0.3575	0.740389	0.104	100
40	0.327	0.690867	0.097	93.26923
50	0.2705	0.599129	0.084	80.76923
60	0.075	0.281702	0.039	37.5
70	-0.117	-0.03004	0	0

The extracellular enzyme showed 0 activity when treated at 70°C. Same was the case for intracellular enzyme. But extracellular enzyme was able to retain 29% of its activity at 60°C. And intracellular enzyme was able to retain 37% of its activity after the 60°C treatment. This indicates that intracellular enzyme is much more thermostable than extracellular. Fig 16 and 17 represents the enzyme activity of MGL extracted from *P. fragi* (extracellular and intracellular, respectively) after heat inactivation.

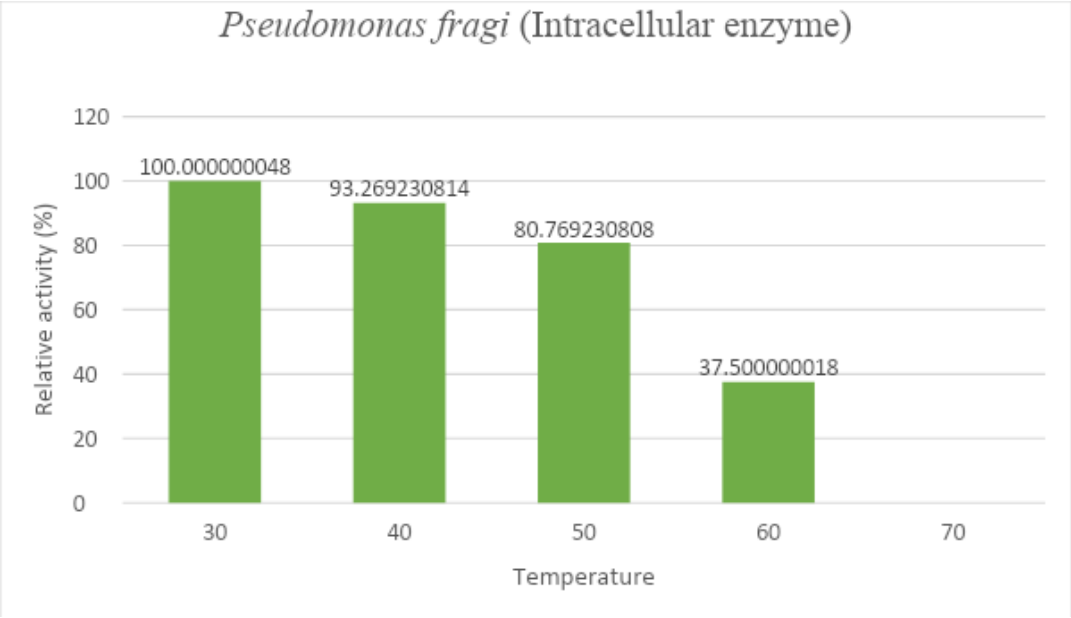


Fig 17: Heat inactivation of intracellular MGL from *P. fragi*.

B.4. *Citrobacter braakii* (Extracellular enzyme)

Table 34: Enzyme activity of the enzyme obtained from *C. braakii* after treatment with different temperatures.

Temperature	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)	Relative activity (%)
30	0.323	0.684372	0.096	100
40	0.257	0.57721	0.081	84.375
50	0.134	0.377498	0.053	55.20833
60	0.008	0.172916	0.024	25
70	-0.0785	0	0	0

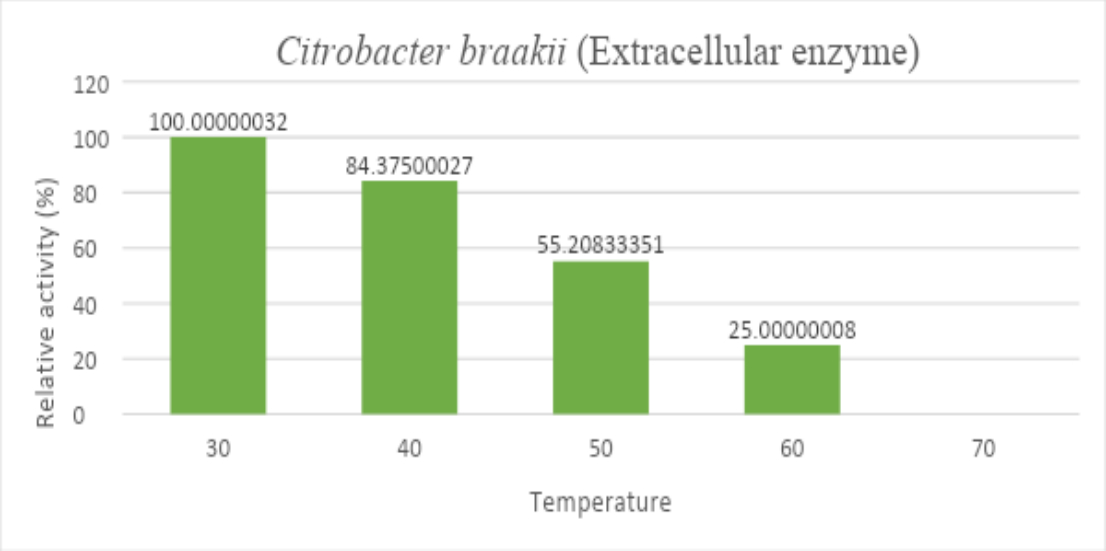


Fig 18: Heat inactivation of extracellular MGL from *C. braakii*.

A.5. *Citrobacter braakii* (Intracellular enzyme)

Table 35: Enzyme activity of the enzyme obtained from *P. lurida* after treatment with different temperatures.

Temperature	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)	Relative activity (%)
30	0.2755	0.607248	0.085015	100.000
40	0.2265	0.527688	0.073876	86.8977
50	0.188	0.465177	0.065125	76.6042
60	0.0645	0.264653	0.037051	43.5817
70	-0.0705	0.045458	0.006364	7.4857

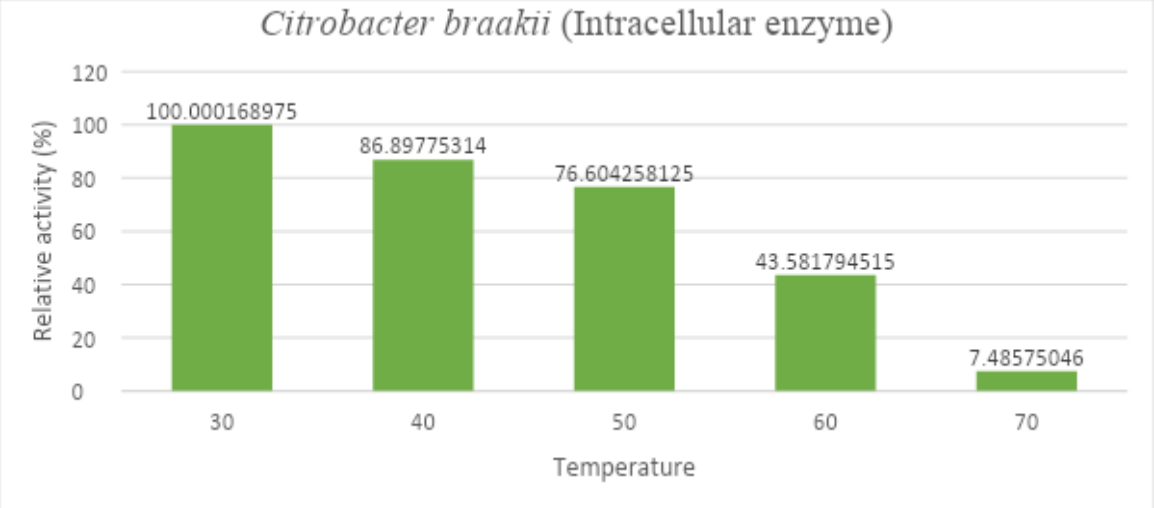


Fig 19: Heat inactivation of intracellular MGL from *C. braakii*.

MGL of *C. braakii* was able to retain 25% and 43% of its activity after 60°C treatment in case of extracellular and intracellular enzyme, respectively. The results pointed to the fact that intracellular enzyme was more thermostable than extracellular enzyme. Fig 18 and 19 represents the enzyme activity of MGL extracted from *C. braakii* (extracellular and intracellular, respectively) after heat inactivation.

C. Optimum pH

C.1. *Pseudomonas lurida* (Intracellular enzyme)

Table 36: Enzyme activity of enzyme obtained from *P. lurida* at different pH.

pH	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)
4	0.172	0.439198	0.061
5	0.24	0.549608	0.077
6	0.29	0.630791	0.088
7	0.2825	0.618614	0.086
8	0.2415	0.552043	0.077

MGL extracted from *Pseudomonas lurida* showed highest activity at pH 6. If the pH was increased or decreased by a factor of one, the activity started decreasing. There was no literature on optimum pH of MGL from *P. putida* was available to compare these results with.

C.2. *Pseudomonas fragi* (Extracellular enzyme)

Table 37: Enzyme activity of enzyme obtained from *P. fragi* at different pH.

pH	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)
4	-0.0255	0.118523	0.016
5	0.0705	0.274395	0.038
6	0.2125	0.504957	0.071
7	0.596	1.127634	0.158
8	0.219	0.51551	0.072

C.3. *Pseudomonas fragi* (Intracellular enzyme)

Table 38: Enzyme activity of enzyme obtained from *P. fragi* at different pH.

pH	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)
4	0.1175	0.350708	0.049
5	0.019	0.190776	0.027
6	0.251	0.567468	0.079
7	0.3545	0.735518	0.103
8	0.2195	0.516322	0.072

Both intracellular and extracellular enzymes showed highest activity at pH 7, which was the initial pH of the assay. These results matched with the previous one's where optimum growth pH for maximum protein production was tested for this species.

C.4. *Citrobacter braakii* (Extracellular enzyme)

Table 39: Enzyme activity of enzyme obtained from *C. braakii* at different pH.

pH	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)
4	-0.003	0.155056	0.022
5	0.201	0.486284	0.068
6	0.302	0.650275	0.091
7	0.3195	0.678689	0.095
8	0.2205	0.517946	0.072

C.5. *Citrobacter braakii* (Intracellular enzyme)

Table 40: Enzyme activity of enzyme obtained from *C. braakii* at different pH.

pH	Mean OD obtained at 480 nm	Amount of ammonia produced (value from std. graph) [mM]	Total activity (U/ml)
4	0.096	0.315799	0.044
5	0.1905	0.469236	0.066
6	0.233	0.538242	0.075
7	0.279	0.612931	0.086
8	0.2215	0.51957	0.073

MGL extracted from *C. braakii* showed optimum activity at pH 7. This was observed in both extracellular and intracellular enzyme. Even a slight increase or decrease in the pH resulted in loss of some activity.

4.7 Determination of K_m value:

The substrate range chosen for this purpose lies between 10-100 mM. The results obtained for all three organisms showed that the velocity of each reaction was almost the same, signifying that the range of substrate concentrations chosen was higher than was needed to obtain different velocities for different substrate concentrations. The readings were considered and utilized for calculating the K_m value of the enzyme for our substrate-Methionine.

1. *Pseudomonas lurida* (Intracellular enzyme)

Table 41: Representation of enzyme activity obtained for different concentrations of substrate [*P. lurida*].

Concentration of substrate [S]	OD obtained	Enzyme activity (mmole/min) [V]	1/[S]	1/[V]
10	0.215	0.07126	0.1	14.0327

20	0.262	0.08195	0.05	12.20316
40	0.266	0.08286	0.025	12.06925
60	0.273	0.08445	0.016667	11.84183
80	0.274	0.08467	0.0125	11.81004
100	0.274	0.08467	0.01	11.81004

The Lineweaver graph plotted with the above readings has been presented in Fig 20.

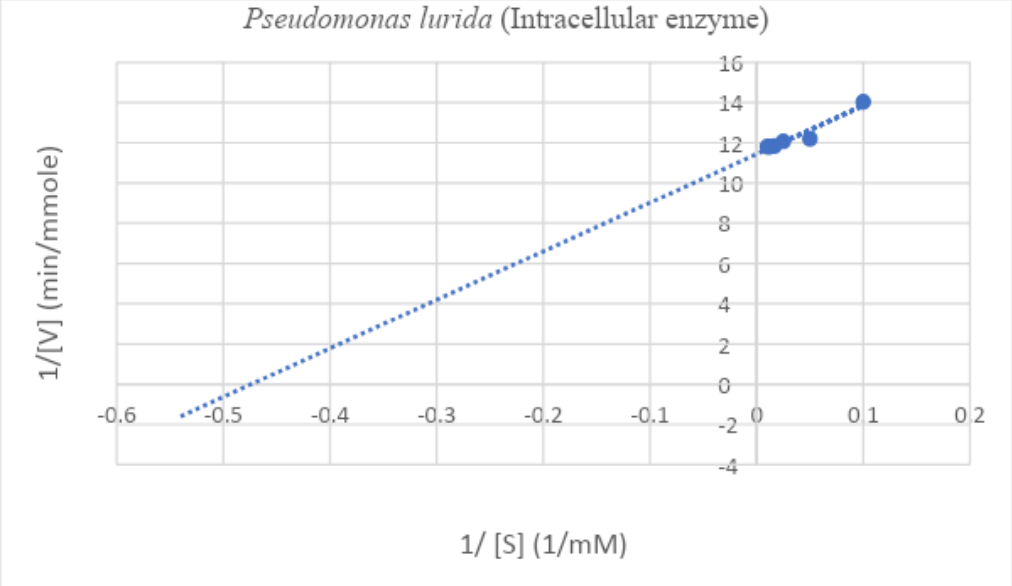


Fig 20: Lineweaver plot of MGL extracted from *P. lurida.*

The equation of the graph obtained is:

Y = 24.138 + 11.433

As per literature, x- intercept = $-\frac{1}{Km}$ As, x-intercept = -0.473

So, **Km = 2.11 mM**

This shows that MGL from *P. lurida* has an almost high affinity for methionine, which could make it a pretty good candidate for utilization in cancer therapy. But the Km value of existing sources was much lower, indicating that *P. lurida* lacks the ability to replace the existing sources for production of MGL for cancer therapy.

2. *Pseudomonas fragi* (Extracellular enzyme)

Table 42: Representation of enzyme activity obtained for different concentrations of substrate [*P. fragi]*.

Concentration of substrate [S] (mM)	OD obtained	Enzyme activity (mmole/min) [V]	1/[S]	1/[V]
10	0.543	0.145821	0.1	6.857715
20	0.547	0.14673	0.05	6.81522
40	0.559	0.149458	0.025	6.690835
60	0.578	0.153777	0.016667	6.502917
80	0.61	0.161051	0.0125	6.209206
100	0.62	0.163324	0.01	6.122787

The Lineweaver graph plotted with the above readings has been presented in Fig 21.

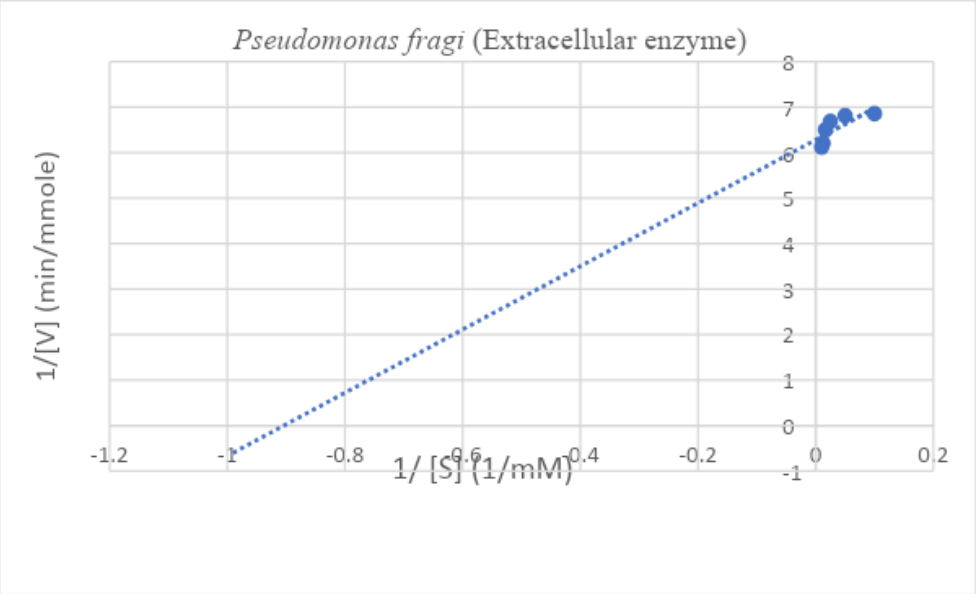


Fig 21: Lineweaver plot of extracellular MGL extracted from *P. fragi.*

The equation of the graph obtained is:

Y = 6.9588x + 6.2847

As per literature, x- intercept = $-\frac{1}{Km}$

x-intercept = -0.903

So, **Km = 1.11 mM**

3. *Pseudomonas fragi* (Intracellular enzyme)

Table 43: Representation of enzyme activity obtained for different concentrations of substrate [*P. fragi*].

Concentration of substrate [S]	OD obtained	Enzyme activity (mmole/min) [V]	1/[S]	1/[V]
10	0.339	0.099449	0.1	10.05539
20	0.351	0.102177	0.05	9.786949
40	0.367	0.105814	0.025	9.450553
60	0.377	0.108087	0.016667	9.251802
80	0.379	0.108542	0.0125	9.213051
100	0.38	0.108769	0.01	9.193796

The lineweaver graph plotted with the above readings has been presented in Fig 22.

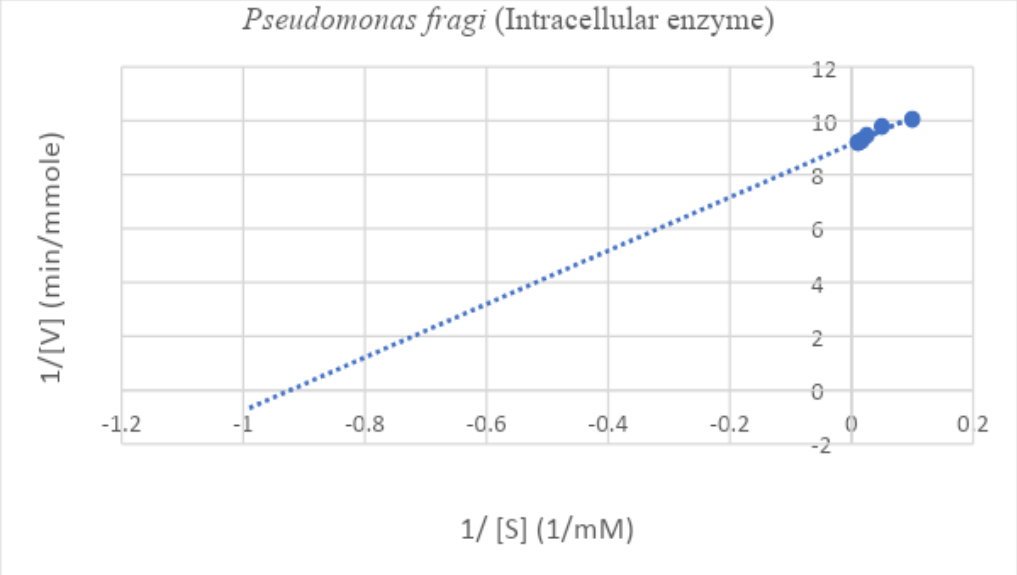


Fig 22: Lineweaver plot of intracellular MGL extracted from *P. fragi*.

The equation of the graph obtained is:

Y = 9.9085x + 9.1382

As per literature, x- intercept = $-\frac{1}{Km}$

x-intercept = -0.922

So, **Km = 1.08 mM**

The results showed that MGL from *P. fragi* has higher affinity towards the substrate, making it a more suitable candidate for utilization in cancer therapy. The previously reported species, *P. putida* has a Km value of 1 mM.

4. *Citrobacter braakii* (Extracellular enzyme)

Table 44: Representation of enzyme activity obtained for different concentrations of substrate [*C. braakii*].

Concentration of substrate [S]	OD obtained	Enzyme activity (mmole/min) [V]	1/[S]	1/[V]
10	0.407	0.11491	0.1	8.70273
20	0.432	0.12059	0.05	8.29261
40	0.429	0.11991	0.025	8.33977
60	0.442	0.12286	0.016667	8.13918
80	0.447	0.124	0.0125	8.06458
100	0.459	0.12673	0.01	7.89099

The Lineweaver graph plotted with the above readings has been presented in Fig 23.

The equation of the graph obtained is:

Y = 7.3816x + 7.9748

As per literature, x- intercept = $-\frac{1}{Km}$

x-intercept = -1.080

So, **Km = 0.926 mM**

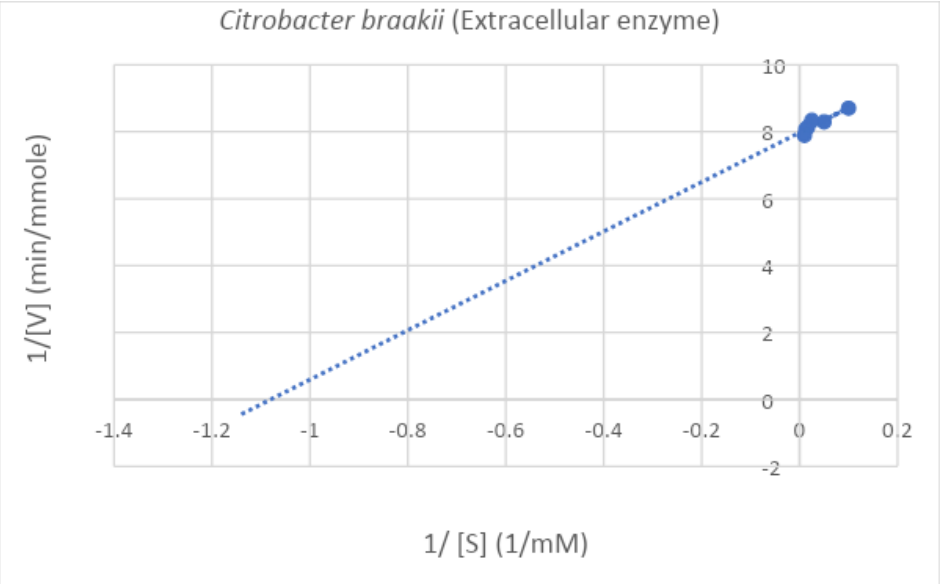


Fig 23: Lineweaver plot of extracellular MGL extracted from *C. braakii*.

5. *Citrobacter braakii* (Intracellular enzyme)

Table 45: Representation of enzyme activity obtained for different concentrations of substrate [*C. braakii*].

Concentration of substrate [S]	OD obtained	Enzyme activity (mmole/min) [V]	1/[S]	1/[V]
10	0.433	0.120817	0.1	8.277006
20	0.439	0.122181	0.05	8.184611
40	0.441	0.122635	0.025	8.15427
60	0.468	0.128773	0.016667	7.765626
80	0.473	0.129909	0.0125	7.697685
100	0.482	0.131955	0.01	7.578341

The Lineweaver graph plotted with the above readings has been presented in Fig 24.

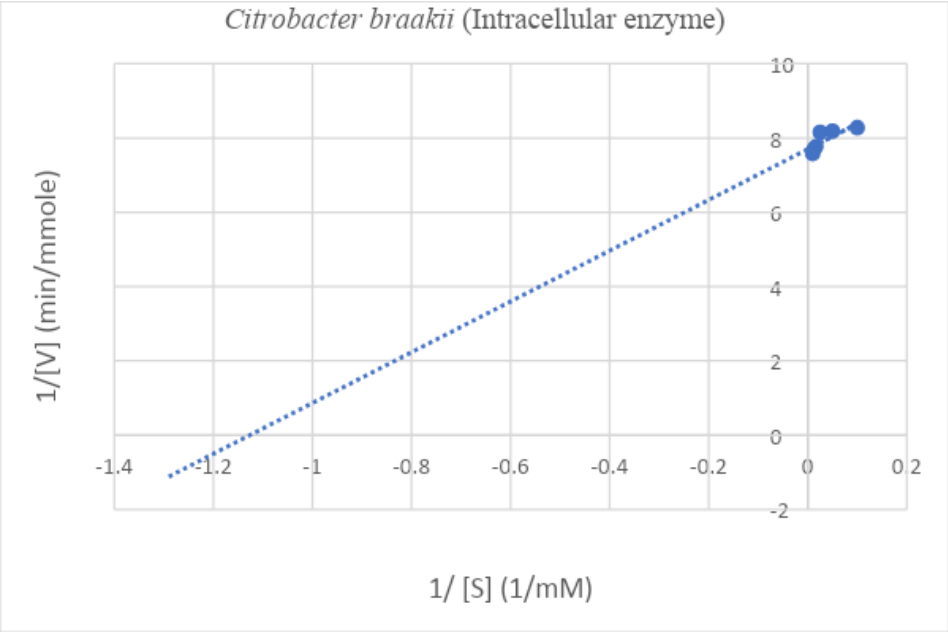


Fig 24: Lineweaver plot of intracellular MGL extracted from *C. braakii*.

The equation of the graph obtained is:

Y = 6.8352x + 7.6989

As per literature, x- intercept = $-\frac{1}{Km}$

x-intercept = -1.126

So, **Km = 0.888 mM**

The results obtained suggest that amongst the three bacterial species, *C. braakii* has the highest affinity towards our substrate. The minor difference between the Km value of intracellular and extracellular enzyme could be due to the fact that the enzymes were analysed in their crude form and were not purified, which means that these could contain different substances that can interfere with enzyme activity.

Lowest Km was shown by the enzyme of *C. braakii*, which means that this enzyme has higher affinity for the substrate and can be utilized as a source for production of MGL.

4.8 Comparison of enzymes obtained from all three organisms:

A comparative analysis was done on the enzymes produced from the three bacterial sources and the results were tabulated in Table 46.

Table 46: Comparison table of enzyme's specific activity and Km value for methionine.

ORGANISM	SPECIFIC ACTIVITY (U/mg)	Km VALUE (mM)
Pseudomonas lurida	0.214	2.11
P. fragi (extracellular)	0.203	1.11
P. fragi (intracellular)	0.218	1.08
Citrobacter braakii (extracellular)	0.242	0.926
C. braakii (intracellular)	0.223	0.888

As per the results, the highest specific activity is shown by MGL from *C. braakii* (Extracellular enzyme) and the highest affinity towards methionine is also shown by the same (Intracellular enzyme). This makes *C. braakii* the most efficient source out of the three selected organisms. As these results were obtained from crude enzymes, the possibility of presence of any interfering agent cannot be ignored and purified enzyme could show slightly different outcomes.

CONCLUSION

The rapidly increasing cases of cancer in the world need to be addressed by either preventive measures introduced in the lifestyle or by introducing new cancer therapies. Introducing a new and effective therapy for curing any type of cancer becomes a necessity when existing drugs in the market are not able to work alone effectively.

One such therapy being tested is the administration of MGL protein into the body of a cancer patient via different means of administration. As research regarding its effects on different types of cancer increases so does the demand to discover novel resources for its production. Three such bacterial sources were analysed for the production of MGL in this study. Two of these resources showcased formation of both intracellular and extracellular MGL- *P. fragi* and *C. braakii.*

The enzymes from these resources were analysed for various properties. The characterization was preceded by optimization of culture conditions. In case of thermal stability, MGL from *P. lurida* excelled in maintaining its enzyme activity even after 30 mins of treatment at 70°C. and only *P. lurida* showed highest activity at slightly acidic conditions (pH 6), the other two showed highest value at neutral pH and also lost their activity at 70°C. The most important parameter was the enzyme affinity towards methionine (Km value). *Citrobacter braakii* turned out to be the best organism in this regard as it showed the lowest Km value, indicating that it has high affinity towards methionine. As the enzyme kinetics has been carried out on crude enzymes, the results could vary in case of a purified enzyme, as it may show high or low affinity values due to absence of any interfering element in the enzymatic reaction.

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APPENDIX

Composition of reagents, buffers and media used:

Reagents	Components	Concentration
M9 media	di-Sodium hydrogen phosphate dihydrate	6 g/L
	Potassium dihydrogen phosphate	3 g/L
	Sodium Chloride	0.5 g/L
	Magnesium sulphate heptahydrate	0.25 g/L
	Calcium chloride	0.014 g/L
	Glucose	2 g/L
Tris HCl Lysis Buffer	Triton X-100	1%
	Sodium Chloride	150 mM
	Tris HCl	50 mM
	EDTA	2 mM
HEPES Lysis Buffer	HEPES Buffer	50 mM
	Sodium Chloride	150 mM
	Pyridoxal 5’ Phosphate	20 mM
	Triton X-100	1%
Potassium phosphate buffer	Potassium phosphate dibasic	8.141 g/L
	Potassium phosphate monobasic	443.9 mg/L
Citrate Buffer	Sodium Citrate dihydrate	0.0437 M
	Citric Acid	0.006302 M