RECOMBINANT EXPRESSION OF GENES *ZLP* and *AMDase* in *E. coli*

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CERTIFICATE

This is to certify that the project report entitled "**Recombinant expression of genes *ZLP* and *AMDase* in *E. coli***", submitted by **Atul Kumar and Vijay Kumar Bhardwaj** is partial fulfillment for the award of degree of Bachelor of Technology in Biotechnology Engineering from Jaypee University of Information Technology, Waknaghat, Solan has been carried out under my supervision.

This work has not been submitted partially or fully to any other university or Institute for the award of this or any other degree or diploma.

Signature of Supervisor   …………………..

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SUMMARY

Zeamatin is a 22 KDa protein produced by plant *Zea mays* in response to fungal infections. It has the ability to be used as an antifungal agent for the treatment of human fungal infections and can also be used in conjunction with other therapeutic proteins. Zeamatin did not stop mammals alpha-amylase and activity of trypsin at high molar ratios. The effect is likely to result in significant clinical toxicity, even at high dose of zeamatin.

AMDase (Arylmalonate decarboxylase) is a rare and underexplored enzyme that explores the potential for effective biocatalytic properties and its applications in the pharmaceutical and bio processing industries. The enzyme helps in the conversion of 2-APA substrates also known as profen and result in the various drug production, such as NSAIDs (non-anti-inflammatory non-steroidal drugs, such as ibuprofen, naproxen, flurbiprofen and many more). They tend to make the decarboxylation of malonic acid disubstituted deravatives for production of active optical products without cofactors. This property could provide a platform to be an attractive candidate for use as biocatalyst in industrial processes.

Recombinant expression of *ZLP* and *AMDase* in the strain of *E. coli* (BL21 (DE3) pLysS) was carried out with the help of vector pET100/D-TOPO that was used for transformation. The transformation was done by using heat shock method. The protein expression was induced with IPTG on different concentration and different temperatures. Later, ethanol was added for solubilization of insoluble proteins. Sonication was performed for the lysis of cells for extraction of proteins. For analysis, SDS-PAGE was performed to visualize the protein bands of desired sizes.

LISTOF SYMBOLS AND ACRONYMS

AMDase - Arylmalonate decarboxylase

Profens – subclass of NSAID's

APA's - Aryl propionic acids

NSAID's - Non-steroidal anti-inflammatory drugs

E. coli -Escherichia coli

NdeI - *Neisseria denitrificans*

XhoI- *Xanthomonas holcicola*

LB- Luria broth

OD- Optical density

IPTG-Isopropyl β-D-1-thiogalactopyranoside

Ni-NTA- Nickel- Nitrilotriacetic acid

SDS-PAGE- Sodium Dodoecyl Sulphate– Poly Acrylamide Gel Electrophoresis.

*BamH*1- *Bacillus amyloliquefaciens*

His-tag- Histidine tag

Amp- Ampicillin

ZLP – Zeamatin Like protein

TLPs- Thaumatin like proteins

NCBI- National Center for Biotechnology Information

SDS – Sodium Dodecyl Sulphate

TEMED – Tetramethylethylenediamine

APS – Ammonium per sulphate

Dil. – Dilution

Conc. – Concentration

NaCl – Sodium chloride

KCl – Pottasium Chloride

PBS- Phosphate Buffered Saline

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1. INTRODUCTION

The genetic manipulation techniques used in the pharmaceutical industry, including the use of genetically modified organisms to produce drugs and for the most part, large-scale production of insulin, human growth hormones, follistism (to treat infertility), human albumin, monoclonal antibodies, antihemophilia factors, vaccines and numerous other medicines. Some pharmaceutical organizations offer products that have been genetically modified and are constantly being made, but the way to make and design a genetically modified pharmaceutical product can take years. Over the past few decades, a significant rise in human fungal infections could be seen in many developed and other developing countries. Improper use of antifungal drugs lead to resistant form of fungi to evolve in a greater pace. Recent healthcare system is facing problems in dealing with increased number of deadly infections due to pathogenic and antifungal resistant fungi. The human fungal pathogens are becoming resistant to the first line drugs in use and due to the ever increasing population the number of individuals at risk is also increasing day by day.

With the evolution of the generations and advancement in the technologies, diseases are also evolving and spreading faster as with the technology and advancements. So, the world really needs fast, cheap and efficient methods for the production of pharmaceuticals to cope up with the upcoming calamities and to satisfy the need of the future.

AMDases can be a new vision of the pharmaceutical and industrial world. *AMDase* has the potential to become a promising candidate to use as a biocatalyst in industrial processes. *AMDase* tends to decarboxylate the α-disubstituted malonic acid derivatives to convert them into individual enantio-products without cofactors. This property of selective and non-cofactor dependent production of an enantio pure product could be a boon to the highly valuable and active fine chemicals that happen to be substrates for pharmaceutical products.

Plants lack the immune system as present in humans and hence they have to device an alternative mechanism to combat the attack of microbes and other pathogens. One such mechanism is production of proteins specific for inhibiting microbial growth. Such proteins are known as plant pathogenesis related proteins (PR proteins). PR proteins are classified mainly on the basis of their structure; however other characteristics like amino acid sequence, serological relationship and biological activity are also taken into account. On basis of shared sequence homology, these proteins are classified into 17 different families.

Zeamatin-like protein (*ZLP*) is an individual from TLPs, which is essentially limited in seeds with higher expression during physiological development and cell differentiation. However a basal amount is found in the leaves of many crop plants. *ZLP* is a potential candidate for treatment of emerging human fungal expressions. *ZLP* could also be used in combination with other antifungal drugs to enhance their activity. Moreover in areas having high rate of fungal crop infections *ZLP* could be transformed and expressed in crop plants and hence yield and productivity could be increased significantly.

This study aims at recombinant expression of two genes named *ZLP* and *AMDase*. The expression and validation of the proteins so produces would also be done by different biochemical assays. Effects of the different variables like temperature, time and agitation speed during the growth of microorganisms would also be studied and optimized. Following are the objectives of the experiment:

1. Selection and retrieval of *AMDase* gene and *ZLP* gene sequence from NCBI database.
2. Analysis of gene sequence and their optimization for expression in *E. coli*.
3. Chemical synthesis of gene and cloning in   pET 100/D TOPO vector (Invitrogen GeneArt).
4. Transformation of protease deficient strain of *E. coli* [BL21 (DE3) pLysS] with expression vector carrying the desired gene.
5. Optimization of protein production process and validation of protein expression.

2. REVIEW OF LITERATURE

2.1. RISE IN FUNGAL INFECTIONS

Paramythiotou et al. (2014) in their study demonstrated that contagious diseases are a developing issue in basically sick patients and are related with expanded mortality. A large part of them is because of *Candida* species, in particular *Candida albicans*. Troublesome candidiasis incorporates *Candidemia candidiasis*, dispersed with the inclusion of deep organs and infinite candidiasis extending. In the last decades, uncommon pathogenic organisms have also been emerged, such as *Aspergillus*, Zygomycetes, *Fusarium* and *Scedosporium* species. In 2007, the results of the epic II study, including 1,265 ICU (intensive care unit) in 75 countries, revealed that 19% of isolated pathogens in ICU patients were fungi (Angus et al. 2007). Current medications are limited to couple of options, and fungicide protection of frontline medications that are used requires the detection and advancement of new antifungal elements with new activity methods.

2.2. ZEAMATIN LIKE PROTEIN (*ZLP*) IN PLANTS

Sajjad et al (2016) showed that with a specific end goal to limit the hypha contagious development, plant cells create antifungal proteins by up or down regulation of anti fungal genes. Pathogenesis-related proteins (PRPs) are grouped into 17 families (PR1 to PR17) in view of serological and amino acid sequence investigations e.g., chitinases, lipid transfer proteins (LTPs), thaumatin like proteins (TLPs), defensins, ribosome inactivating proteins (RIPs) and TIPs and so on. The group incorporates an assorted cluster of proteins with various structures, method of activity and the phase of plant development for their enhanced expression. *ZLP* is a member of TLPs and having antifungal properties against plant and human fungal infections.

2.3. CHARACTERIZATION AND EXPRESSION OF *ZLP* GENE

Malehorn et al (1994) undertook the project for molecular cloning and the heterologous expression of the basic TLP of *Zea mays* seed. The results of over expressing these genes in the cell lines of insects and two species of genetically modified plants were satisfactory, because in both cases, the protein expressed retained antifungal activity. The various antifungal tests are made in fungal species that have been reported to be inhibited by TLP. The antifungal action of the *ZLP* on *Candida albicans*, *Neurospora crassus*, and *Trichoderma reesei* was seen as already represented (Richardson et al. 1991). Ideal results for the inhibition analysis of *C. albicans* were acquired by consolidation of 2 X 10^4 cells/ml and 0.5 mg of nikkomycin per ml in dextrose agar. The heterologous protein expressed in different cell lines of insects and plants were isolated and purified. This purified protein showed inhibition results as seen in earlier reports with slight changes. Although from this experiment, it cannot be concluded that the ambiguity of zeamatin in inhibiting different species of fungi. After boiling of the purified protein, the inhibitory activity was lost in all experimental conditions. Further research is needed to establish the complete role of zeamatin in inhibiting the growth of fungal species.

2.4. MEMBRANE PERMEALIZING ACTIVITY OF ZEAMATIN

Roberts et al (1990) in their experiments determined that the cell wall of fungi appeared an impossible target site for zeamatin as the purified zeamatin protein contained no substantial activity of chitinase, glucanase or protease. In any case, the fungal cell wall remained the possible site of action for zeamatin. Many experimental approaches have been used to examine this possibility. The first experiment measured the zeamatin-induced release of UV absorbent material from *C. albicans* suspensions. Under these experimental conditions less than 1 µg zeamatin per ml created detectable cellular lysis. Other basic proteins that cause cellular lysis are needed in higher concentrations as compared to zeamatin.

A second leakage experiment was done by supplementing the *N. crassa* cells with [1-^{14}C] isobutyric amino acids, a non-metabolizable amino acid. In order to measure the membrane integrity the cells were then treated with zeamatin or antifungal drug with

membrane permeabilizing activity - amphotericin-B. This caused the release of the radioactive amino acid because of cell rupture. The zeamatin induced rupture of the hypha occurred in less than 15s at 23 °C and with as low concentration as 1 pg zeamatin per ml. Most ruptures were observed in the hypha extremities or immediately behind the apical hypha dome. The lysis with zeamatin was much faster when compared to standard antifungal drug amphotericin-B.

Moreover, purified zeamatin did not hydrolyze the mannan or proteins which are the basic components of the fungal cell wall, denying the possibility that the antifungal activity of zeamatin is possibly due to the enzymatic hydrolysis of the cellular wall. This vision was further supported by the fact that the impact of zeamatin on fungi occurred rapidly even at 0 °C. At such low temperature most of the enzymes lose their activity. This experiment proposed a non-enzymatic activity system of zeamatin antifungal activity. These appeared to predict that the zeamatin lyses the fungi by direct insertion of proteins into the cell wall forming the transmembrane pores.

2.5. *AMDases*: CLASS RACEMASES

K. Miyamoto and R. Kourist (2016) studied that *AMDase* belongs to class racemases and promotes conversion of an isomer in an optically pure isomer that is pharmacologically active. Racemisation is a chemical process that promotes the conversion of an enantiomer in its active form that requires tremendous amount of energy to reach the threshold energy level and at very harsh conditions, such as strong acid or basic conditions, for product formation. Because of such disadvantages, it is logical to create the site for need of much simpler and greener processes. A more environmentally friendly chemistry can be effective catalytic methods for rupturing stable C-H bonds that can minimize the use of energy, waste production and the use of harmful reagents. Racemases has become a new intuition to make enzymatic reactions feasible that require special conditions. *AMDase* make the reaction favorable in mild conditions that do not require much energy or the use of harmful chemicals. Baxter and colleagues (2012) with the help of protein engineering, has made the modifications in racemases that have achieved a six-fold increase in N-acyl amino acid racemase activity and increased production of D-amino acids. Gu et al. (2014) obtained an improvement of the activity of six folds, thus showing the vitality of the rational methods for the improvement of the racemases. This underlines the requirement for a good mechanical understanding for the successful engineering of racemases.

The actual biological role of *AMDase* has not been explained, but it is considered to be the robust catalyst decarboxylating the range of various substrates including phenylmalonic acid, naphthylmalonic acid etc. It does not demand any co-factors or biotin to carry out the activity. Furthermore, decarboxylation does not involve the formation of enzyme intermediates which is a prime incentive of several decarboxylase enzymes. Therefore, there is a huge interest in the mechanism of this enzyme. The chiral products of *AMDase* activity was of high potential in pharmaceutical industry. There is a clear need for the development of improved decarboxylase enzymes or their functional mutants derived from such enzymes with modified substrate specificity. They have the potential to come up with immense application in the industrial processes for the production of optically pure active pharmaceuticals.

2.5.1. MODIFIED *AMDase*

Miyamoto K. et al. (2006) investigates the enzyme *AMDase* to improve the activity of the enzyme. They introduced a single change in the amino acid to determine the alteration in performance of the enzyme. Essential amino acid according to reference is revealed to be Cys188 in the active site of *AMDase.* It is supposed to protonate the intermediate enolate form of α-arylpropionates from one side of the enantiomer and promotes the conversion of one enantiomer in its other form that is optically active. The difference between *AMDase* and other enzymes of this class is that others have another Cys located in the opposite side of the enantiomeric face while *AMDase* has no alternative Cys around this region. Therefore, other enzymes follow two-base mechanism for the racemisation of substrates. On the other hand, *AMDase* leads to the production of active decarboxylated products.

Kourist R. et al. (2014) designed a modified G74C (glycine is substituted by cysteine at 74th position) variant of arylmalonate decarboxylase (*AMDase*) obtained by *Bordetella bronchiseptica* presents a unique racemising activity with regard to various substrates (arylaliphatic acids, malonic acid derivatives). Its substrates include several arylpropionate non-steroidal anti-inflammatory drugs (NSAIDs), such as Naproxen, ibuprofen, etc. A technique for racemisation under mild response circumstances would allow the constant reuse of unwanted enantiomer. *AMDase* G74C has the catalytic machinery of the superfamily racemases. It consists of a dioxyanion opening for the coordination of carboxylic acid and two cysteine molecules, one on each side of the substrate that catalyzes the exchange of protons during catalysis.

Maimanakos J. et al. (2016) proposed another option for the synthesis of profen is the enzymatic decarboxylation of malonic corrosive esters that are easily accessible. Arylmalonate decarboxylase (*AMDase*) of *Bordatella bronchiseptica*, proved to be a suitable catalyst for this reaction. The *AMDase* catalyzed asymmetrization of an aryl-α-methylmalonic acids leads to the production of profens in hypothetical performance 100% which is a reasonable favorable performance. The activity and enantioselectivity are excellent in the production of Naproxen, but are much lower with the precursors of ketoprofen and ibuprofen. This enzymatic system can also be used for the production of hydroxycarboxylic acids.
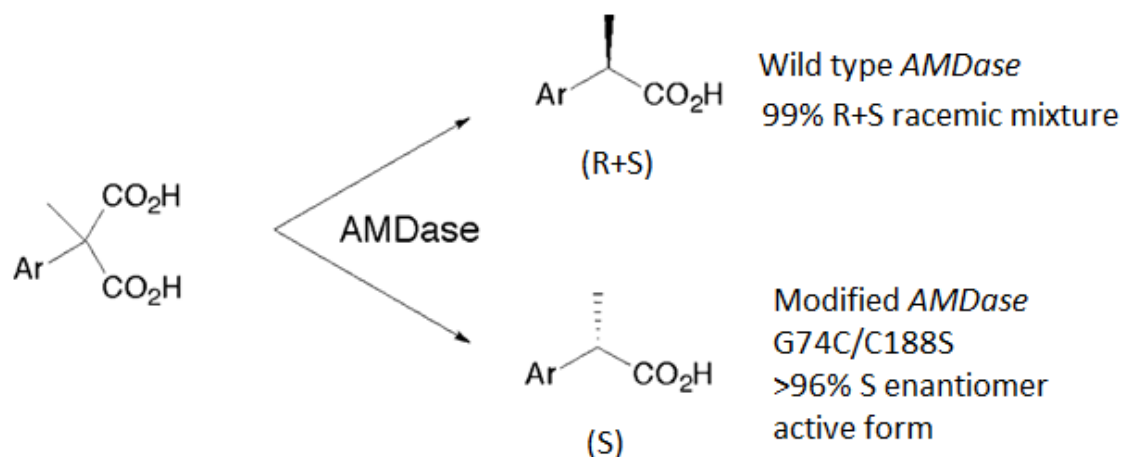
Figure 1: Conversion of substrate by *AMDase* (wild type) and *AMDase* (modified); Kourist R., María P. D. D., Miyamoto K. et al. (2011)

The modification in *AMDase* with respect to its wild type is presented in Figure 1. The modification in the enzyme *AMDase* at specific positions leads to the formation of inverted product from (R)-isomer to (S)-isomer up to 96%. Wild type *AMDase* is modified on the position 74 from where Glycine is being replaced by cysteine and at position 188 where cysteine is replaced by serine and results in the formation of desired pharmacologically pure active chemicals.

2.5.2. RECOMBINANT EXPRESSION OF DIFFERENT VARIANTS OF *AMDase*

Different variants of *AMDase* were developed from different organisms. *AMDa* and *AMDv* were obtained from *Variovax* sp. HH01 and HH 02 respectively. Different constraint sites were determined for cloning and expression of various variants in expression vector pET-21a. The respective sites were identified and were processed with the specific restriction enzymes and were synthesized with the use of codon set to *E. coli*. The confirmation of the constructs was confirmed by sequencing and later by the selection of BL21 *E. coli* transformants. Different constructs were made with the vector pET-21a for the transformation purposes.

3. MATERIAL AND METHODS

3.1. SEQUENCE RETRIEVAL

NCBI is a store of many databases relevant to the field of biotechnology, bio informatics and biomedicine. One of the most used databases is GenBank, which is a DNA sequence database. This database is connected to individual laboratories and other DNA sequence databases.

The sequences for genes *ZLP* and *AMDase* were retrieved from NCBI database with following accession numbers:

4.1.1. Accession: EU725369.1 for *ZLP*.
4.1.2. Accession: S54007.2 for *AMDase*.

On the NCBI portal, the search was filtered using nucleotide option by which only the nucleotide sequences for the related gene was listed. Other articles and papers relating to the genes were filtered out. The sequence was retrieved in FASTA format.

3.2. CODON OPTIMIZATION

The translational efficiency of the target genes could be increased by optimizing the codons according to the expression system and hence the expression of the protein could be increased. Different organisms prefer different synonymous codons for expression of a particular amino acid and the phenomenon is called codon usage bias. By optimizing the codon usage bias, the translational efficiency of any target gene could be increased. There are many bioinformatics tools for optimization of codons according to the expression system.

The retrieved sequences of *AMDase* and *ZLP* were optimized for expression in *E. coli* by the GeneOptimizer software on the ThermoFisher portal.

In the process of optimization the following cis-acting sequence motifs were avoided:

1. Internal TATA boxes.

2. AT or GC rich sequence stretches

3. RNA instability motifs

4. Repeat sequences and RNA secondary structures

5. (Cryptic) Splice donor and acceptor sites in higher eukaryotes.

3.3. GENE SYNTHESIS

DNA printing or the artificial gene synthesis is the process by which the genes are chemically synthesized in the laboratory using synthetic nucleotides. Chemical gene synthesis is based on solid phase DNA synthesis which differs from molecular cloning by having no need of pre existing DNA template for synthesis of DNA. Gene synthesis services are commercially provided by many companies worldwide. The optimized sequences of both the genes ZLP and *AMDase* were analyzed and were chemically synthesized by the Invitrogen GeneArt services. Following is the process of chemical synthesis of the genes.

GeneArt Gene Synthesis process

Bioinformatics

Oligonucleotide synthesis

Gene assembly
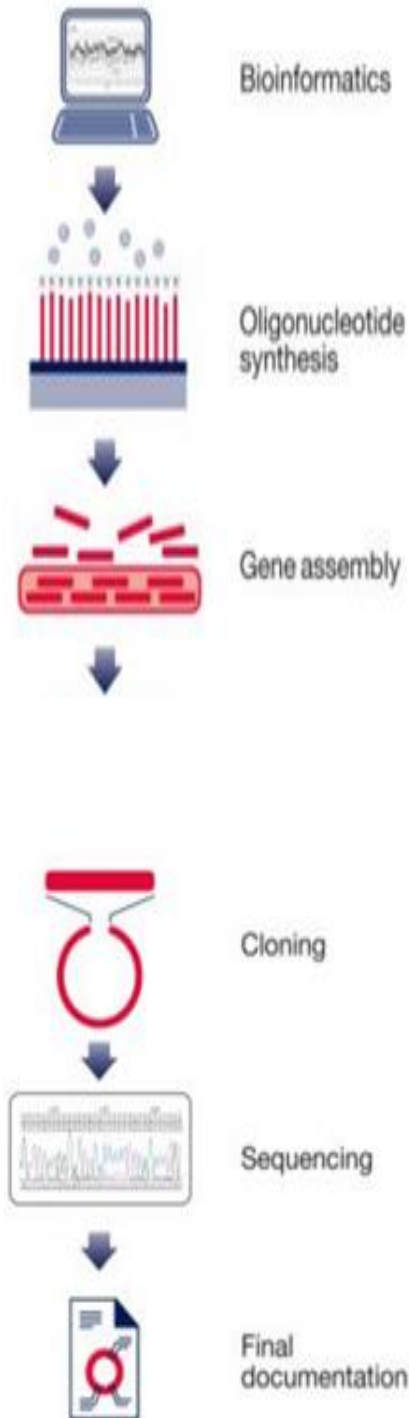
1. Before synthesis the genes were verified and optimized according to the expression system. *ZLP* and *AMDase* sequences were analysed and optimized for expression in E. coli. Different bioinformatics tools were used for this purpose.

2. Solid phase DNA synthesis technology was used for chemical synthesis of *ZLP* and *AMDase*. The synthetic oligonucleotides served as building blocks for the complete synthesis of the cDNA sequences of both the genes. Firstly the genes are synthesised in fragments and then joined together.

3. Small fragments of *AMDase* and *ZLP* were assembled into complete gene using GeneAssembeler. The synthesised genes were now ready for direct cloning or transfection using different genetic engineering tools.

Cloning

Sequencing

Final documentation

4. A standard cloning vector pET 100\D TOPO was used to clone the desired genes. The same vector was further used for the process of transformation.

5. After cloning of both the genes *ZLP* and *AMDase* in separate vectors the sequencing of the recombinant plasmid constructs was done to ensure the genes are placed correctly in with the desired sequence of nucleotides.

6. The quality of each gene synthesis is controlled according to ISO 9001:2015 certified quality management standards. These systems help to ensure the reliability and reproducibility of the synthesis processes.

3.4. VECTORS

In the terms of molecular cloning a vector is a DNA molecule used as an agent for the transfer of a desired DNA sequence into another cell for cloning or expression. Plasmids can be of different types according to their function such as cloning vectors and expression vectors. In this experiment we used an expression vector pET 100\D TOPO for expression of the desired genes. Two different plasmid pET 100\D TOPO vectors containing gene of interests i.e. *ZLP* and *AMDa*ses were used for transformation. These constructs were synthesized from Invitrogen gene art. In each preparation (5 µg of lyophilized DNA) 100µl of T.E buffer was added to make stock concentration of 0.05µg/µl. The final concentration ready to use for transformation of plasmid DNA was made to be 0.02µg/µl.

3.5. STRAINS

BL21(DE3)pLysS chemically competent strains of *E. coli* were used for transformation and expression of recombinant plasmids. These strains were used in combination with plasmids having T7 promoters for expression of the desired product. Moreover these strains were protease deficient, deficient in ion and Omp T proteases for higher protein stability. The strains were ordered from Invitrogen.

3.6. MEDIA ND PLATES:

Three types of media were used for the growth and transformation of bacterial cells. Three types of media used were: Luria broth (LB), Luria broth agar (LBA) for the growth and support of bacterial cells, super optimal broth with catabolite repression (SOC) media was used for the transformation of cells.

3.6.1. LB (Luria broth) media (HIMEDIA) was used for primary and secondary growth of *E. coli.* The composition of LB media is given in Table 1.

Table 1: Composition of LB-Luria Broth Media (components in gram/litre).

S.No.	Ingredients	Gram/Litre
1.	Casein enzymic hydrolysate	10
2.	Yeast extract	5
3.	Sodium Chloride	10
4.	Final pH at 25°C	7.5±0.2

3.6.2. LBA (Luria broth agar) (HIMEDIA) was used for plating (spreading and streaking). The composition of LBA is given in Table 2.

Table 2: Composition of LBA-Luria Broth Agar (components in gram/litre).

S.No.	Ingredients	Gram/Litre
1.	Casein enzymic hydrolysate	10
2.	Yeast extract	5
3.	Sodium Chloride	10
4.	Agar	15
5.	Final pH at 25°C	7.5±0.2

20-25 ml of LBA was poured into plates.

3.6.3. **SOC (Super Optimal Broth with Catabolite Repression) media** was used for transformation of *E. coli.* The composition of SOC media is given in Table 3.

Table 3: Composition for 100 ml SOC media.

S.No.	Components	Concentration
1.	Tryptone	2%
2.	Yeast extract	.5%
3.	NaCl	10mM
4.	KCl	2.5mM
5.	$MgCl_2$	10mM
6.	$MgSO_4$	10mM
7.	Glucose	20mM

3.7. TRANSFORMATION

Chemically competent BL21(DE3)pLysS strains of *E. coli* were used for transformation. The cells were thawed on ice for 30 minutes. Water bath was set at 42°C. Inside laminar air flow cabinet. To 50µl of competent cells, 5 µl of plasmid DNA of concentration 0.02µg/µl was added. The mixture was incubated on ice for 30 minutes. The tubes were then transferred to water bath set at 42°C for exactly 45 seconds. Immediately, the tubes were transferred back to ice for 2 minutes. After 2 minutes, 250µl of pre warmed SOC media was added to tubes. The tubes were incubated overnight at 37°C. The cells were then spreaded into pre warmed LBA containing chloromphenicol (34mg/ml) and ampicillin (50mg/ml). The colonies were observed and transformation frequency was calculated. Procedure was adopted from B. et al. (1994) with slight modifications.

3.8. OPTIMIZATION OF PROTEIN EXPRESSION

For expression of proteins, the expression guidelines by B. et al (1994) were adopted with slight modifications. A single colony of BL21(DE3)pLysS strains of *E. coli* was picked up and grown over night in LB media containing antibiotic chloramphenicol (34mg/ml). 1 ml of overnight culture was taken and added to 200 ml of LB media containing antibiotics chloramphenicol (34mg/ml) and ampicillin (50 mg/ml) and incubated until the OD_{600} reached between 0.4-0.8. At this stage, the media was incubated at 37°C with transformed cells with different genes in different flasks.

- **INDUCTION WITH IPTG:-**

The gene for T7 RNA polymerase is present in pET100/D-TOPO vector under the control of IPTG inducible promoter. The promoter is induced by IPTG at different concentrations. Upon induction, the RNA polymerase binds to T7 promoter and starts transcription of the desired gene. IPTG (different concentrations) was added to standardize the IPTG concentration for optimum induction of protein expression. Different IPTG concentrations used were: - 0.1μM, 0.5μM, 1μM.

- **OPTIMIZATION OF TEMPERATURE:-**

E. coli was grown best at 37°C. Sometimes, the desired heterologous protein/recombinant proteins are produced at different temperatures by the bacteria. Different temperatures were maintained to grow *E. coli* in order to optimize the temperature for maximum yield of desired protein. Different temperature at which the bacteria were grown: 26°C, 30°C, 37°C.

- **EXPRESSION USING ETHANOL:-**

Sambrook et al. (1989) used ethanol 1%, 2%, 3% for the enhanced expression of desired proteins. They found out that some proteins were produced in insoluble form which could not be analyzed. So, solubilization of such proteins can be carried out by using ethanol in culture media. It was recommended from the above study that 3% ethanol gives the best results. So, 3% ethanol was added at the time of induction in the media and the results were analyzed.

3.9. PROTEIN EXTRACTION

Walker JM (2009) protein extraction method was followed with required changes. The cells were centrifuged at 8000 rpm for 10 min. and supernatant was discarded. To the pellet, 5ml of washing buffer (PBS) was added to remove the media. The sample was centrifuged at 8000 rpm for 10 min. The pellet was extracted and processed to cell lysis by two different reagents. Initially, the lysis was carried out by using lysis buffer. The composition of lysis buffer is given in Table 4.

Table 4: Composition of lysis buffer.

S.No.	Ingredients	Concentration
1.	Sodium phosphate	10mM
2.	Glycerol	10% v/v
3.	NaCl	30mM
4.	Lysozyme	1mg/ml
5.	DTT	1mM

After that, the lysis was done by using Tris buffer (pH 7.2±0.2) to carry out cell lysis which gives the best results.

3.9.1. SONICATION

One of the methods to disrupt the cell membrane of bacteria was by using sound energy and the process is called sonication. In this process, the electrical signal was converted into sound waves; thousands of microscopic vacuum bubbles were formed in the solution which collapse into the solution in a process called avitation. This results in formation of waves of vibrations that releases high energy and disrupts different molecular interaction. The heat was generated due to friction caused by sound waves, which was lowered by keeping the sample on ice during the process of sonication. The cycle of sonication was optimized and carried out at 21% amplitude. The sonicator was on on-cycle for 20 sec and on off-cycle for 5 sec. The time duration of whole cycle was 4 min for 15 ml of sample.

3.10. PROTEIN ESTIMATION

Estimation of total protein can be done by various methods. Bradford Assay was performed for the estimation of total protein in the samples. The principle of Bradford Assay is that under acidic conditions upon binding of Coomassie dye to protein molecule, the color changes from brown to blue. Basic amino acids – lysine, histidine and arginine contribute to the formation of protein dye complex which is estimated by calorimetry.

Bradford assay given by M.M Bradford (1976) was followed with slight modifications. The standard curve was prepared using different concentration of BSA (0, 50, 100, 250, 500, 1000, 2000, 3000 µg/ml). 30 µl of different dilutions of protein sample was added to 1500 µl of Bradford reagent. The mixture was incubated for 10 min. at room temperature in dark conditions. O.D. was taken at 595 nm. Results were analyzed and total protein was estimated.

Preparation of Bradford reagent :-

- 100 mg of Coomassie G250 was weighed and dissolved in 50 ml 95% ethanol.
- Add 100 ml 85% phosphoric acid.
- Make up the volume up to 1000 ml.
- Filter with Whatman filter paper to remove precipitates.

3.11. SDS-PAGE

SDS-PAGE (Sodium dodecyl sulphate – polyacrylamide gel electrophoresis) is a technique to separate protein on the basis of their electrophoretic mobility which is function of the size of the protein. SDS is a anionic surfactant which degrades the tertiary structure of the protein and linearizes it into chain form. When electric field is applied the proteins tend to move towards the anode, the speed depends upon the size/mass ratio of the protein. The SDS-PAGE was conducted with an aim to characterize the expression of protein in *E. coli* as per protocol given below.

3.11.1. GEL PREPARATION

Stacking Gel

Two separate gels were prepared for stacking and resolving the proteins. The stacking gel allows all the proteins to start from the same point for resolving. The pH of stacking gel was 6.8 at which the proteins were sandwiched between chlorine and glycine ions. The composition of 5% stacking gel is given in Table 5.

Table 5: The composition of 5 % stacking gel.

S. No.	Components (5% gel conc.)	Volume = 5 ml
1.	Water	3400 µl
2.	Acrylamide/bisacrylamide	830 µl
3.	100mM Tris pH(6.8)	630 µl
4.	10% SDS	50 µl
5.	10% APS	50 µl
6.	TEMED	5 µl

Resolving Gel:

The pH of resolving gel was 8.8 where the glycine ions along with the chloride ion move ahead of the protein molecules and hence allows them to separate on the basis of their size. The composition of 12% resolving gel is given in Table 6.

Table 6: The composition of 12% resolving gel.

S.No.	Components (12% gel conc.)	Volume = 10ml
1.	Water	3300 µl
2.	Acrylamide/bisacrylamide (30%)	4000 µl
3.	1.5M Tris pH 8.8	2500 µl
4.	10% SDS	100 µl
5.	10% APS	100 µl
6.	TEMED	4 µl

After completion of the electrophoresis the protein bands could be seen after staining. TEMED generates free radicals for polymerization and APS acts as a catalyst.

3.11.2. SAMPLE PREPARATION

10 µl of protein sample was added to 10 µl of sample buffer and mixture was heated at 95°C for 10 minutes. This resulted in disruption of tertiary and secondary structure of proteins by disrupting the hydrogen bonds. The composition of sample buffer is given in Table 7.

Table 7: The composition of sample buffer.

S.No.	Components	Concentration
1.	Tris HCl pH 6.8	62.5mM
2.	SDS	2.5%
3.	Bromophenol blue	0.002%
4.	β-mercaptoethanol	0.7135M (5%)
5.	Glycerol	10%

3.11.3. ELECTROPHORESIS

The denatured samples were cooled down to room temperature and loaded onto gel of polyacrylamide for separation of proteins. During the stacking phase, the voltage was set at 80V per cm gel length. Once the proteins entered the resolving gel, the voltage is increased to 100V per cm gel length. The protein molecules moved towards the anode under the influence of electric field. Broad range protein ladder (BIO-RAD) was used as reference. The composition of 10X running buffer is given in Table 8.

Table 8: Composition for 10X running buffer.

S.No.	Components	Amount
1.	Tris base	30gm
2.	Glycine	144gm
3.	SDS	10gm

Make up the volume with water up to 1000 ml.

3.11.4. GEL STAINING

After the completion of electrophoresis, the gel was taken out into staining tank containing staining dye. Coomassie staining was done to visualize the protein bands. It is easy and most commonly used for the staining purposes.

3.11.5. VISUALIZATION

Visualization was done with the help of densitometer. Densitometer is a device that is used to measure the degree of darkness or the optical density of a semitransparent material. It is mainly a light source that emits light and target at photoelectric cell. Density of the sample is determined when it is placed between the light source and the photoelectric cell.

4. RESULTS AND DISCUSSION

4.1.   GENE SEQUENCES RETRIEVED FROM NCBI:

The gene sequences of *ZLP* and *AMDase* were successfully retrieved from the NCBI database. Following are the sequences as provided for synthesis in FASTA format.

***ZLP* Gene Sequence** was obtained by filtering the search to nucleotide option in the NCBI portal where the format of sequence was selected as FASTA.

>EU725359.1 *Zea mays* subsp. parviglumis isolate *ZLP*_z1 zeamatin-like protein (*ZLP*) gene, complete cds

TAAGATGGCAGGTTCCGTGGCAATCGTGGGCATCTTCGTTGCCCTCCTCGCCGTG
GCCGGCGAGGCGGCTGTGTTCACGGTGGTGAACCAGTGCCCGTTCACCGTGTGG
GCCGCGTCCGTGCCGGTGGGCGGCGGGCGGCAGCTGAACCGCGGCGAGAGCTG
GCGGATCACGGCCCCCGCGGGCACGACGGCCGCGCGCATCTGGGCGCGCACGGG
GTGCCAGTTCGACGCGAGCGGGCGCGGGAGCTGCCGCACGGGGGACTGCGGCG
GCGTGCTGCAGTGCACTGGGTACGGGCGCGCGCCCAACACGCTGGCGGAGTACG
CGCTGAAGCAGTTCAACAACCTCGACTTCTTCGACATCTCCCTCATCGACGGCTT
CAACGTGCCCATGAGCTTCCCCCCGACGGCGGGTCCGGGTGCAGCCGCGGCCCG
CGCTGCGCCGTGGACGTGAACGCGCGCTGCCCTGCCGAGCTGCGGCAGGACGGC
GTGTGCAACAACGCGTGCCCCGTGTTCAAGAAGGACGAGTACTGCTGCGTCGGC
TCGGCGGCCAACGACTGCCACCCGACCAACTACTCCAGGTACTTCAAGGGGCAG
TGCCCCGACGCGTACAGCTACCCCAAGGATGACGCCACCAGCACCTTCACCTGC
CCCGCCGGAACCAACTACAAGGTCGTCTTCTGCCCGTGAGGCCGCTGAACTAGC

AMDase **Gene Sequence** was extracted from the NCBI portal by filtering the search to nucleotide option where the format of sequence was selected FASTA.

>S54007.2 *Bordetella bronchiseptica* arylmalonate decarboxylase gene complete cds

CTTTCATCGCGCAGGGTTCGCTGGTGCGCTACCAGCCCGCCGACGCGCTCGCCGC
GTGGCTGCCCGCGCAGGAGCAGCGCTGGGTCGACCTGATCTCGCGCGCCAAGCT
GACCTTCGCCCCTTGAGATTTTCGGTATCCACAGTAGGAGAACTTTTCATGCAGC
AAGCAAGCACTCCCACCATCGGCATGATCGTGCCGCCCGCCGCGGGTCTGGTGC
CGGCGGATGGGGCGCGGCTCTATCCCGATCTGCCCTTCATTGCCAGCGGGCTGG
GGCTGGGCTCCGTCACGCCGGAAGGCTATGACGCCGTGATCGAATCGGTGGTGG
ACCATGCGCGCCGCCTGCAAAAGCAGGGCGCGGCGGTGGTTTCGCTGATGGGCA
CCTCGCTCAGCTTCTACCGGGGCGCGGCCTTCAATGCCGCGTTGACCGTAGCGAT
GCGCGAAGCCACGGGACTGCCATGCACGACCATGAGCACGGCGGTCCTGAACGG
ATTGCGCGCCCTGGGCGTGCGCCGCGTCGCGTTGGCGACGGCCTATATCGACGA
TGTGAACGAGCGCCTGGCGGCATTCCTGGCCGAAGAGAGCCTGGTTCCCACCGG
CTGCCGCAGCCTTGGCATCACGGGCGTGGAGGCCATGGCGCGCGTGGATACGGC
CACGCTGGTCGACCTGTGCGTGCGTGCCTTCGAAGCGGCGCCCGATAGCGACGG
CATCCTGCTGTCTTGCGGCGGCTTGCTGACGCTGGACGCCATACCCGAAGTCGAG
CGCCGCCTGGGCGTGCCGGTGGTGTCGAGTTCGCCGGCGGGGTTCTGGGACGCC
GTGCGGCTTGCGGGGGGGAGGGGCCAAGGCAAGGCCCGGATACGGCCGGCTGTTC
GACGAGTCCTGACGATAGCGGCTCGCCGGCGGCAAGGCCGCAATCGCGCGCTAG
CCGCGCACCGCAGTGACATGAAACCCGCGCAAGCGGGTTTCTTACTTTGTCCTTA
ATGCAAAACTTATTTTCATGTCAAGAAAATCGATCTGATATGGTCTGTTGAGCGT
ATCAGATGGCTTAAAAAGCGCATAATGTGCGCACCCTTTCCCAAGCTGCATGTCA
TGGGGATCGGGTCGATTTTCTGTAAGCGAAGAGCGTGTTGTCACTAGCTCGGCGG
TTCGTGTTCCCAGGTTCATGTTCGGTAGCTAGGACAACTTGGAGTATTCACGGAG
ATGTCTATGACAGAAGACGCAAAGCCGCGCAGGCGGTTCCTGCAG

4.2.   PLASMID CONSTRUCTS

The gene *ZLP* and the *AMDase* were synthesized with synthetic oligonucleotides. The constructed genes were incorporated into the Vector pET100/D-TOPO expression system. For cross-checking, plasmid DNA was extracted from the transformed bacteria and OD was calculated using ultraviolet spectroscopy to determine the concentration of the desired product. The genetic maps of the genes *ZLP* and *AMDase* in vector pET100/D-TOPO is shown in Figure 2 and Figure 3 respectively.
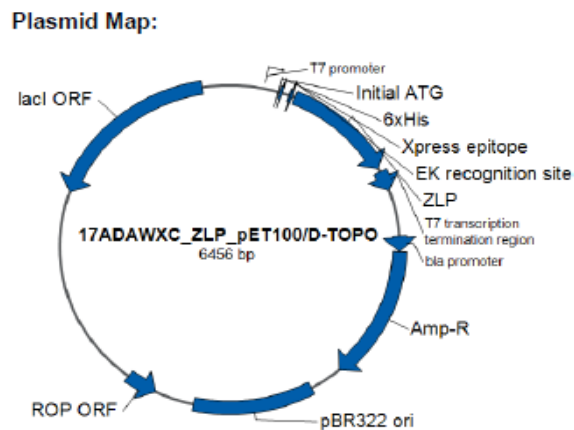
Plasmid Map:

Figure 2. Plasmid map of vector pET100/D- TOPO with gene *ZLP* ready for transcription.
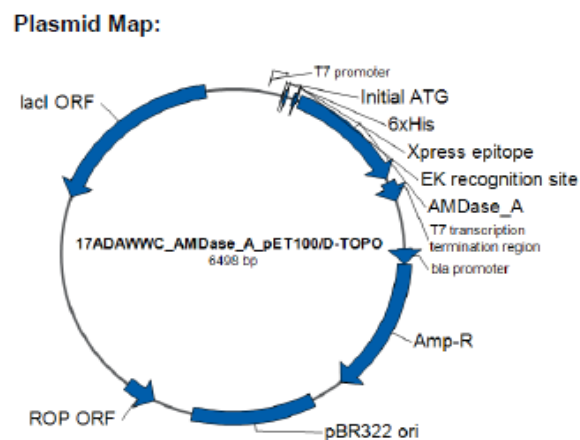
Plasmid Map:

Figure 3. Plasmid map of vector pET100/D- TOPO with gene *AMDase* ready for transcription.

4.3.   TRANSFORMATION

Transformation of cells was done successfully on plates containing selection antibiotics –

Chloramphenicol (34 mg/ml) and Ampicillin (50mg/ml)

The transformation efficiency of both the samples was calculated using the following formula:-

Colonies on plate/ng of DNA plated X 1000 ng/μg

1. **Gene *ZLP***

The transformed cells containing foreign gene *ZLP* are shown in Figure 4.

DNA concentration (in μg/μl) for transformation : 0.020 μg/μl

Volume (in μl) of DNA used in transformation mix: 5 μl

Volume (in μl) plated fortransformed colonies (for a single plate): 100 μl

Number of colonies observed: 207

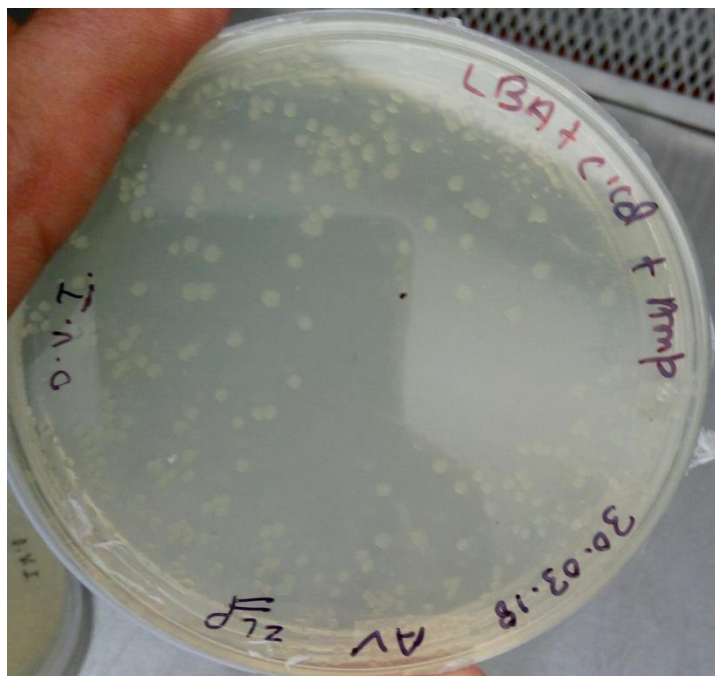Transformation Efficiency: $6.3135*10^3$ Transformants / μg DNA

Figure 4: Colonies obtained after transformation with gene *ZLP* with selectable markers, ampicillin and chloramphenicol

2. **Gene *AMDase***

The transformed cells containing foreign gene *AMDase* are shown in figure 5.

DNA concentration (in µg/µl) for transformation: .020 µg/µl

Volume (in µl) of DNA used in transformation mix: 5 µl

Volume (in µl) plated fortransformed colonies (for a single plate): 100 µl

Number of colonies observed: 263

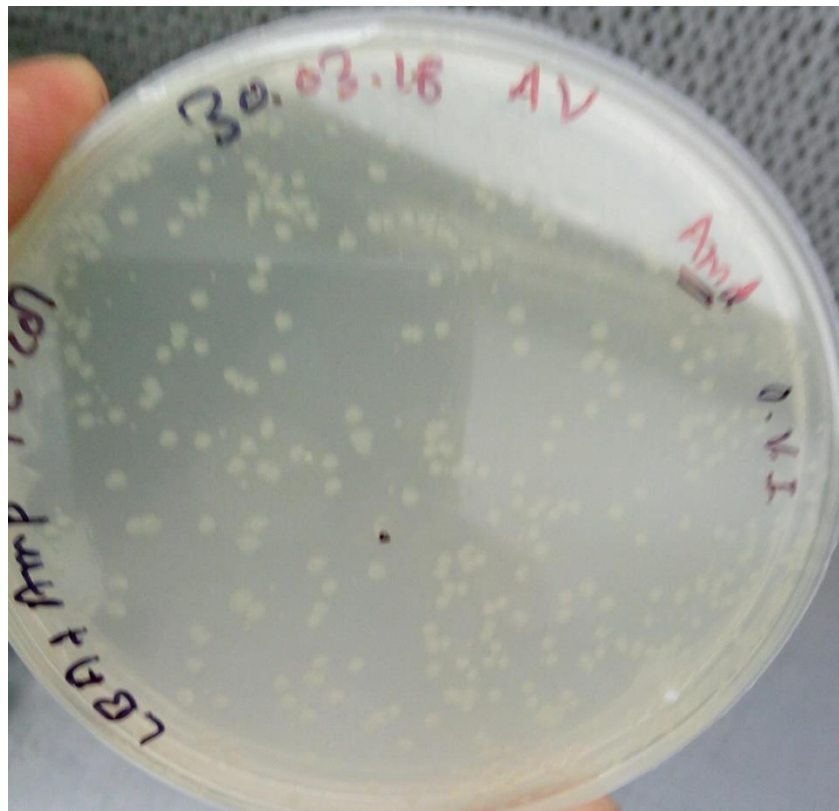Transformation Efficiency: $8.0215*10^3$ Transformants / µg DNA

Figure 5: Colonies obtained after transformation with gene *AMD* with selectable markers, ampicillin and chloramphenicol

4.4.   PROTEIN ESTIMATION BY BRADFORD

The total protein was estimated by Bradford assay. The readings obtained by using UV spectrophotometer and its corresponding concentration calculations are mentioned in Table 9 below.

Table 9: O.D. obtained with the help of spectrophotometer and concentration is calculated.

S.No.	OD 1	0.465	0.465	0.497	0.49	0.513	0.597	0.681	0.796
1.	OD 2	0.463	0.47	0.503	0.504	0.532	0.635	0.71	0.843
2.	OD 3	0.487	0.504	0.498	0.51	0.572	0.624	0.733	0.861
3.	AV	0.47166	0.47966	0.49933	0.50133	0.539	0.61866	0.708	0.8333
4.	CONC.(μg/μl)	0	50	100	250	500	1000	2000	3000
5.	OD-BLANK	0	0.008	0.0276	0.0296	0.0673	0.147	0.2363	0.3616

The graph representing the standard curve that was obtained using different concentrations of BSA is provided in the Figure 6.
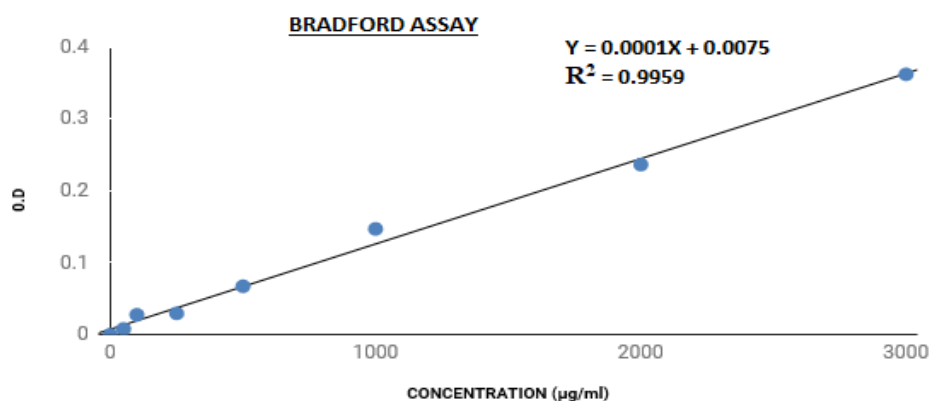

Figure 6: Bradford assay; Standard graph using different concentrations of BSA

- **O.D. OF SAMPLES :-**

The formula for calculating the concentration is :

Concentration (µg/ml)= (O.D. – 0.0075)/0.0001

The calculated concentrations that were determined using different dilutions are presented in the Table 10.

Table 10: Concentration of samples of different dilutions in µg/ml.

S.No.	Sample	ZLP no dil.	AMD no dil.	ZLP dil 1/15	ZLP dil 1/6	AMDdil 1/15	AMD dil 1/6
1.	Conc. (µg/ml)	7295	6068.33	811.66	1805	951.66	2281.66
2.	OD-Blank	0.737	0.6143	0.08866	0.188	0.102	0.235

- **CALCULATIONS :-**

The formula to calculate final concentration is:

Final concentration = Concentration (µg/ml) * Dilution factor

The final readings for the concentration of different samples of various dilutions are given in Table 11.

Table 11: Final concentration of samples of different dilutions.

S.No.	Sample	ZLP dil 1/15	ZLP dil 1/6	AMD dil 1/15	AMD dil 1/6
1.	Final conc.	12175	10830	14275	13690

4.5.   SDS-PAGE

Sodium dodecyl sulfate (SDS) is a strongly denaturing anionic detergent with unique characteristics. It unrolls completely and denatures all the proteins, essentially ignoring specific secondary structures or hydrophobic domains and generating SDS protein complexes that are mainly characterized by a uniform mass-charge ratio. This makes SDS-PAGE a generally very simple and reliable technique for protein separation and (with exceptions) for mass determination.

Different expression parameters were applied for expressing the desired protein in *E. coli*. Although expression of proteins were very low. To counter this, different troubleshooting methods were incorporated which include changing concentration of IPTG, incubation temperature, addition of 3% ethanol for solubilization of proteins and use of different buffers for lysis of cells.

Confirmation of transformation by plasmid isolation or gene sequencing is an important step before heading to expression. The plasmid was isolated from the transformed cells but unclear bands were obtained because of its smaller and circular size. Analysis of such bands was difficult.

Since the expression of protein was very low so it was difficult to analyze the protein using SDS-PAGE. Other detection methods like 2 –D electrophoresis, affinity chromatography could be used for detection of proteins. The size of desired proteins *ZLP* and *AMDase* are 22kDa and 23 KDa respectively. Ladder used as reference is of size 1 KDa (Broad Range).

4.5.1.   SDS-PAGE RESULT 1

IPTG concentration = 1mM

Temperature = 37°C   Incubation period = 24 hrs

In first attempt, the desired parameters were set up for the induction of protein expression. 1mM IPTG was added to culture media for optimum protein expression and the culture is incubated at 37°C for 24 hrs. Various protein bands were obtained on gel while performing SDS-PAGE.

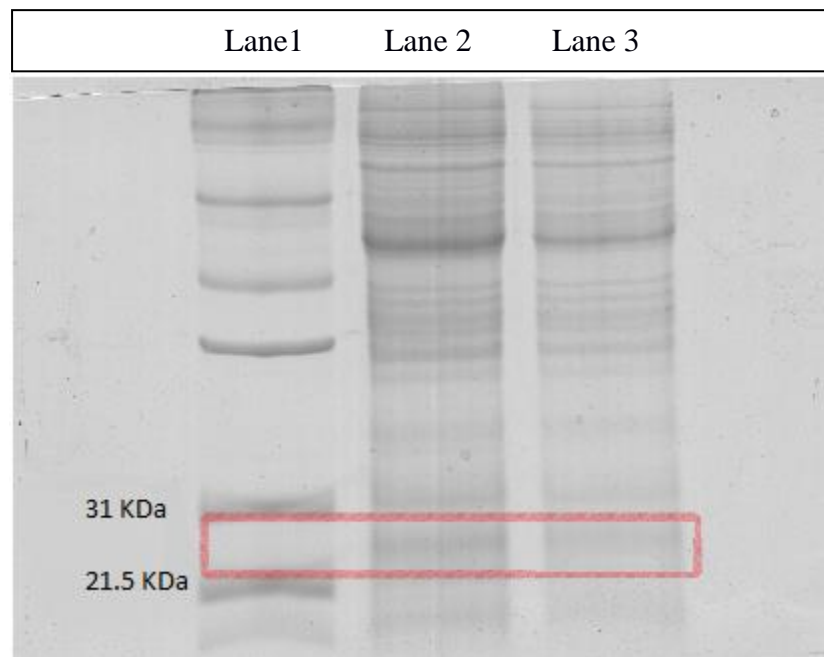The bands that were obtained using the above mentioned parameters are validated in Figure 7.

Figure 7: Protein bands of different sizes obtained after SDS-PAGE. Loaded samples were ladder and induced samples with IPTG (1mM).

Lane 1: Ladder

Lane 2: *ZLP* induced with IPTG and incubated for 24 hours

Lane 3: *AMD* induced with IPTG and incubated for 24 hours

The expression of proteins was very low in this case. The desired proteins were of sizes 22KDa and 23 KDa respectively.

4.5.2.   SDS-PAGE RESULT 2

IPTG concentration = 1mM

Temperature = 37°C   Incubation period = 16 hrs

In second attempt, the parameters were kept same for the induction but the incubation time was 16 hrs. In addition to this, uninduced samples were also loaded to compare the expression of proteins with the expression of proteins in case of induced ones. Bands that were obtained when above mentioned parameters were used are presented in Figure 8.
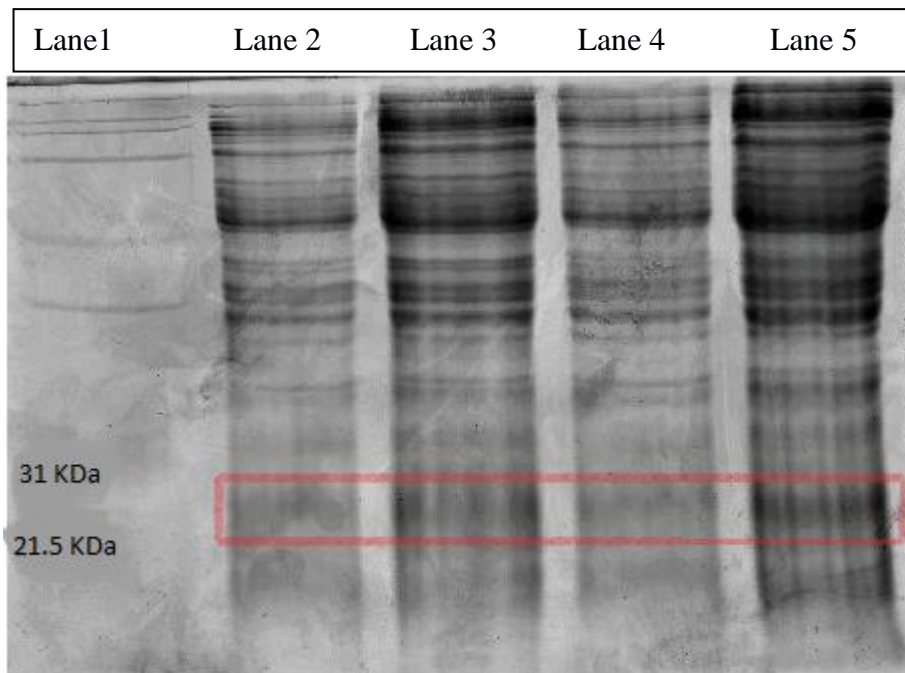

Figure 8: Bands of proteins of different sizes obtained after running SDS-PAGE. Loaded samples were ladder, uninduced and induced genes by IPTG (1mM)

Lane 1 : Ladder (Broad range)

Lane 2 : *ZLP* uninduced

Lane 3 : *ZLP* induced with IPTG and cultured for 16 hours

Lane 4 : *AMD* uninduced

Lane 5 : *AMD* induced with IPTG and cultured for 16 hours

*Ladder is not properly visible.

The results obtained were not optimum as the expression is very low.

4.5.3. SDS-PAGE RESULT 3

IPTG concentration = 0.5mM

Temperature = 37°C   **Incubation period = 8 hrs and 16 hrs**

In the third attempt, the concentration of IPTG was decreased to 0.5mM and temperature kept 37°C. The culture was incubated for16 hrs.

Above explained parameters were used to validate the protein bands on electrophoresis gel. The protein bands are presented below in Figure 9.
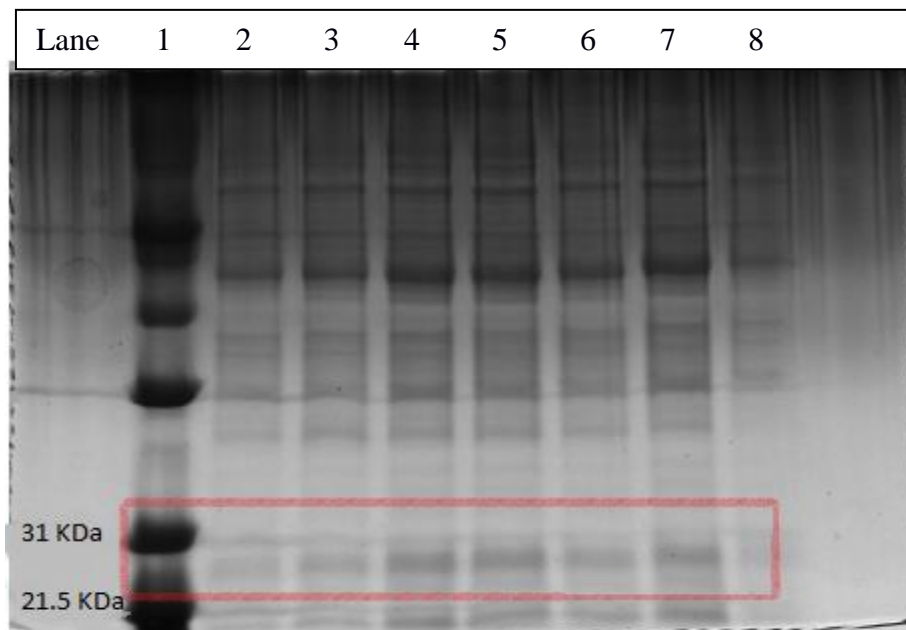

Figure 9: Protein bands obtained after SDS-PAGE. Loaded samples were ladder, uninduced and induced samples; IPTG used is 0.5mM.

Lane 1 : Ladder

Lane 2 : No plasmid

Lane 3 : *ZLP* uninduced

Lane 4 : *ZLP* induced (8 hrs incubation)

Lane 5 : *ZLP* induced (16 hrs incubation)

Lane 6 : *AMD* uninduced

Lane 7 : *AMD* induced (8 hrs incubation)

Lane 8 : *AMD* induced (16 hrs incubation)

4.5.4. SDS-PAGE RESULT 4

IPTG concentration = 0.1mM

Temperature = 26°C   Incubation period = 8 hrs and 16 hrs

In the last attempt, the concentration of IPTG was maintained to 0.1mM and the temperature was reduced to 26°C whereas the incubation period was kept same as earlier. Above parameters were maintained to validate the expression of proteins. The protein bands obtained are provided in Figure 10.
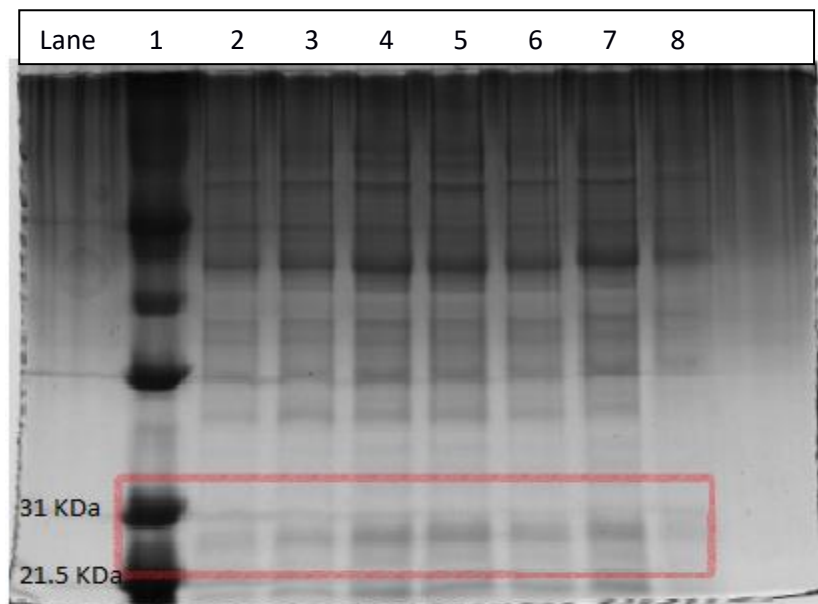

Figure 10: Protein bands obtained after changing the concentration of IPTG to 0.1mM and introduction of 3 % ethanol for the induction purposes and cultured at 26 °C

Lane 1 : Ladder

Lane 2 : No plasmid

Lane 3 : *ZLP* uninduced

Lane 4 : *ZLP* induced (8 hrs incubation)

Lane 5 : *ZLP* induced (16 hrs incubation)

Lane 6 : *AMD* uninduced

Lane 7 : *AMD* induced (8 hrs incubation)

Lane 8 : *AMD* induced (16 hrs incubation)

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