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Cloning Expression of Arsenic Binding Protein with GFP Tag in Bl21de3 Strain and its Chracterization

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Abstract: ***This review explores the cloning, expression, and of arsenic binding protein bind with green fluorescent protein (GFP) tags in Escherichia coli BL21DE3 strain. Arsenic contamination poses significant environmental and health risks, necessitating the study of proteins involved in arsenic detoxification and sequestration. GFP tagging facilitates visualization and tracking of these proteins, aiding in their characterization and potential applications. The cloning process involves selecting suitable expression vectors and host strains, followed by DNA manipulation and vector construction. Expression optimization parameters, including inducer con - centration, temperature, and culture media, play crucial roles in achieving high protein yields. Characterization techniques such chromatography and SDS - PAGE, and fluorescence microscopy allow for the assessment of protein folding, stability, and arsenic binding affinity. Studies have implications for biosensing, bioremediation, and biomedical research. Future directions include structural elucidation and protein engineering to enhance the efficiency and specificity of arsenic binding proteins.***

Keywords: Arsenic binding protein (ABP), GFP tag, BL21 (DE3), Arsenic biosensor, Protein expression and purification

1. Introduction

Arsenic is a naturally occurring element present in the earth crust and can be present in various modes in the environment. While it is naturally present in soil, water, and air, human activities such as mining, smelting, and industrially processes can also lead to increased levels of arsenic contamination in the environment. Arsenic is highly toxic to humans and other organisms, and exposure to high levels of arsenic can lead to severe health issues, including cancer, skin lesions, cardiovascular diseases, and neurological effect. to mitigate the health - related problems when exposure to arsenic, researchers (Chen C J; Chuang Y C; You S L; Lin T M; Wu H Y 1986) have been exploring various methods for arsenic removal and detoxification. One such approach's to use of arsenic binding proteins. These proteins have to ability to specifically bind to arsenic molecules, thereby reducing their toxicity and facilitating their removal from the body. Arsenic –binding proteins found in a variety of organisms, including bacteria, algae, and even some higher organisms like humans.

The mechanism by which arsenic - binding proteins bind to arsenic molecules can vary depending on the specific protein and organism involved. (Saravanamuthu Thiyagarajan 2008) Some protein may bind arsenic through specific binding sites, whereas other protein may experience conformational changes as a arsenic binding, leading to sequestration or detoxification of the arsenic molecule. **To develop methods for arsenic detoxification and remediation, it is crucial to com - prehend the structure and activities of arsenic - binding protein**. Researchers are actively studying these proteins to elucidate their mechanisms of action and explore their potential ap - plications in environmental cleanup and medical treatment for arsenic poisoning. Arsenic bind to a protein can changes its conformation and inhibit its function. Arsenic present in 3 allosteric forms like grey yellow & black. The researchers found that the arsenic binding protein adverse effect on to the proteome. A group of researchers found that the arsenic binding protein they used arsenic affinity chromatography to find the arsenic binding proteins.

1.1. Vector

Using vector builder software, a vector for the bacterial ex - pression system's GFP protein expression was created. The ampicillin resistance gene and the majority of the tags chosen for the purification system's optimization, elements necessary, in addition to the T7 promoter sequence selected for the promoter region at position one. Six histidine tags have been chosen for the purification process. This tagging technique is particularly helpful since it makes it simple to purify and identify the recombinant protein. When introducing a novel polyhistidine tag to a protein, a six - histidine tag is generally a suitable option for the initial experiment. Recombinant proteins often include polyhistidine affinity tags attached to either the N or C terminus.

Maltose - binding protein (MBP), chosen as a solubilization tag, is a secretion - enhancing tag for systems that express proteins in mammals. MBP is a dependable solubility tag for the creation of recombinant soluble proteins. Although solubility is a crucial component of a protein production campaign, solubility should not always be the ultimate aim. TEV (tobacco etch virus) was chosen as a sequence that may be broken. Among these enzymes, TEV's nuclear inclusion protease is arguably the most well - studied. TEV protease cleaves between Q and G after recognizing the amino acid sequence ENLYFQ/G with great efficiency.

1.2. Green Fluoresce Protein

When blue to ultraviolet light is present, proteins such as green fluorescent protein (GFP) and others flash bright green. The protein that was extracted from Aequorea Victoria jelly - fish is known by the name GFP, while it is also occasion - ally called avGFP. Numerous creatures, including copepods,

lancelets, zoanthids, corals, and sea anemones, have been found to contain GFPs. **Two excitation peaks can be seen in Victo - ria's GFP: a larger peak at 395 nm and a smaller peak at 475 nm.** Its emission peak is at 509 nm, in the lower green area of the visible spectrum. The fluorescence quantum yield (QY) of GFP is 0.79.

The structure of GFP is a beta barrel made up of eleven β - strands arranged in a pleated sheet pattern. The covalently bonded chromophore 4 - (p - hydroxy benzylidene) imidazolidin - 5 - one (HBI) is located in the center of an alpha helix. Matura - tion is the term used to describe this post - translational modification process.

The colors, intensity, and photo stability of GFP and its many variants are influenced by the hydrogen - bonding network and electron - stacking interactions with these sidechains. Be - cause of the barrel's dense packing, solvent molecules are kept out, preventing water from inhibiting the chromophore fluorescence.

1.3. Cloning

Cloning is the molecular biology techniques used to copies the desired DNA copies. the Cloning is the process was first discovered by (Dr. LanWilmut) a Scottish scientist he said that world to be created by using cloning. There are three types of cloning DNA copies created by gene cloning. Whole copies of genes created by animals by reproductive cloning. Embryonic stem cells copies created by therapeutic cloning.

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1.3.1. Gene of interest and vector ligation

After the restriction the cutter DNA fragments will be joined by T4 DNA ligase. T4 DNA ligase act as a joining of DNA fragment by phosphor diester bonds.

1.4 Transformation

After the ligation process the r (DNA) will be introduced into bacterial host. In this the r (DNA) and the plasmid mixed with bacterial host then the replication will be started. Selection and culturing of transformed cells. The transformed cells onto a nutrient agar medium that contain specific antibiotic then after recombinant DNA isolation.

1.4. GFP Protein Importance

GFP green fluorescent protein is a protein it exhibits green light it exposed to light and UV range. This protein was first isolated from the jelly fish Agoura vectoria (Prendergast and Mann, 1978) that's why it is also known as the avGFP protein have a invaluable tool in biochemistry and molecular biology.

The protein consists of 238 amino acids and its molecular mass 27kDa. The discovery of GFP the scientist Roger Y. Tsien, Osamu Shimomura and Martin and Calfie were awarded Nobel prize in biochemistry (2008) for their work related to protein.

1.4.1. *Reporter gene application*

The primary application of GFP in protein expression studies and it is use as a reporter gene. By fusing the GFP gene to the gene of interest can track the expression, the protein's lo - cation, dynamics, and state within living things. Many biological processes, including the control of gene expression, protein transport, and cell communication pathways, have been made clearer by this technique.

1.4.2. *Protein localization studies*

GFP has been extensively employed to study protein localization within cells. By tagging proteins with GFP and visualizing their fluorescence using microscopy techniques, re - searchers can determine the subcellular localization of proteins in real time. This gives new information into the spatial organization of cellular components and dynamic changes in protein distribution during various cellular processes.

1.4.3. *Fusion protein constructs*

GFP is frequently used to generate fusion protein constructs, wherein it is genetically linked to the protein of interest. This to study the fusion, interaction, and behaviour of the target protein in living cells. Moreover, GFP can be fused to either the N - or C - terminus of the protein significantly altering its structure or function, making it a versatile tool for protein expression studies.

1.4.4. *Expression of protein systems compatibility*

GFP works with many different expression protein systems, include bacterial (e. g., Escherichia coli), yeast (e. g., Saccharomyces cerevisiae), insect (e. g., Drosophila melanogaster), and mammalian (e. g., human cell lines) systems. Its small size (27kDa) and lack of cofactors simplify its expression and localization in diverse host organisms, facilitating the study of protein function across different biological contexts.

1.4.5. *Engineering GFP variants*

Variants of GFP with unique spectral characteristics, including blue, cyan, yellow, and red fluorescence, have been discovered over time. These variants have expanded the toolkit for multiplex imaging and Fluorescence Resonance Energy Trans - fer (FRET) experiments, enabling simultaneous visualization and analysis of multiple proteins or cellular processes within the same sample.

1.4.6. *Quantitative analysis techniques*

GFP - based assays allow for quantitative analysis of gene ex - pression, protein - protein interactions, and protein localization using fluorescence intensity measurements and imaging analysis software. This quantitative data facilitates the characterization and comparison of protein expression levels, localization patterns, and dynamic changes under different experimental conditions.

1.4.7. *Visualization of cellular structure and processes*

GFP binds to the protein it visualization of various cellular process like movement structure trace the specific gene.

1.4.8. *Expression of gene studies*
GFP act as a reporter to monitor the gene expression patterns and activity.

1.4.9. *Protein - protein interaction studies*
GFP binds to the protein observe the florescence resonance energy transfer between GFP and another fluorophore can observe the protein - protein interaction in real time. In vivo imaging GFP non - toxic and small suitable in vivo imaging studies it also helps in the disease progression, drug efficiency.

1.4.10. *Biomedical and biotechnological application*
GFP has variable applications in biotechnology and biomedical like gene therapy, biosensor, environmental monitoring, its stability.

1.5. *BL21DE3 Ecoli Strain Importance*
The BL21 (DE3) E. coli strain, often referred to as the BL21 strain, is a widely used bacterial strain in biotechnology and molecular biology research. Here's a detailed explanation of its importance:

1.5.1. *Expression System*
BL21 (DE3) is commonly used as a host strain for protein expression Under the regulation of the lacUV5 promoter, it harbors the T7 RNA polymerase gene, which facilitates the effective expression of target genes cloned into T7 expression vectors 13. Tight control over the quantities of protein expression is facilitated by this technology, which is essential for a number of applications, including the synthesis of recombinant proteins for structural research, industrial enzymes, and therapeutic proteins.

1.5.2. *High Protein Yield*
The BL21 (DE3) strain offers high protein yields due to its efficient expression machinery. The T7 RNA polymerase stimulates the T7 promoter to produce enormous amounts of tran - scription, which results in the target protein being produced in large quantities. Applications requiring huge amounts of pro - tein can benefit from this high expression level.

1.5.3. *Enhanced Protein Folding*
The strain variant of BL21 (DE3) is engineered to improve the folding of recombinant proteins. It contains mutations in the lon and ompT protease genes, which reduce the degradation of target proteins and enhance their solubility. This feature is particularly beneficial for the expression of proteins prone to misfolding or aggregation, improving the quality and yield of functional recombinant proteins.

1.5.4. *Versatility*
BL21 (DE3) strains are compatible with a variety of expression vectors and cloning techniques, making them flexible instrumentation for molecular biology research. Researchers can easily manipulate and express different target proteins using this system, enabling the study of protein structure, function, and interactions

1.5.5. *Commercial Availability*
The well - characterized expression system of BL21 (DE3) is readily accessible to researchers due to its commercial availability from many sources. The genetically modified variants like the BL21DE3 strain further expands the capabilities and applications of the system.

1.5.6. *Track Record of Success*
BL21 (DE3) has been extensively used in numerous re - search studies and industrial applications, accumulating a track record of success. Its reliability, efficiency, and compatibility with standard laboratory techniques have made it a trusted workhorse in the field of molecular biology and biotechnology. Cloning and expression of arsenic binding protein with GFP tag in BL21DE3 strain and its characterization and the objectives are

- To design vector in vector builder to design the specific PET vector with linker sequences
- To prepare the ligated sample inserted into the GFP and arsenic binding protein sequence
- To study the transformation of a specific E. Coli strain (BL21DE3) and with GFP tag expressions
- To express the arsenic binding protein by using the purification techniques
- To study the expression of protein by using different techniques

2. Review of Literature

Arsenic contamination in water poses a significant threat to human health (Cullen. W. R 2008). Developing efficient methods for arsenic detection and removal is crucial. Recombinant proteins with arsenic binding capabilities offer a promising avenue for bioremediation strategies (Biswas A., Das S., Seth S., Maulik S. K., Bhargava B., Rao V. R. Dual 2016). This focuses on the research surrounding the expression, and cloning characterization of arsenic binding proteins (ABPs) fused with a Green Fluorescent Protein (GFP) tag in the BL21 (DE3) strain of Escherichia coli (E. coli). The GFP tag allows for non - invasive monitoring of protein expression and localization within the bacterial cells.

2.1 *Advantages of EGFP - tagged ABPs in BL21 (DE3)*

2.1.1 Non –invasive monitoring
EGFP fluorescence enables real time visualization of ABP expression in living E. coli cells. This removes the requirement for time - consuming purification and protein extraction processes for analysis. (Chalfie M, Tu Y, Euskirchen G, 1994)

2.1.2 Localization
EGFP allows researchers to define the cellular location of the ABP (e. g., cytoplasm, periplasm). This information is crucial to understand the protein's mechanism of action and potential optimization strategies (Yang F, Moss LG, Phillips GN, 1996).

2.2 *BL21 (DE3) expression system*

For the expression of heterologous proteins in E. coli, BL21 (DE3) is a reliable and well - established system. It offers high protein yield and efficient control over expression using the T7 lac promoter system (Studier et al., 1990).

2.3 *Cloning and Expression of Arsenic Binding Protein with GFP Tag*

Providing an overview of the cloning process, including the selection of the arsenic binding protein and EGFP tag. Describe the design of the expression vector and strategy for inserting the gene of interest.

2.3.1 Key Features of BL21DE3 for Protein Expression

2.3.1.1 T7 RNA polymerase regulated by the lacUV5 promoter

The gene for T7 RNA polymerase is located on BL21DE3. An enzyme very efficient at transcribing DNA into RNA. This gene is under presence of a strong, inducible lacUV5 promoter. You can "switch on" T7 RNA polymerase with a molecule called IPTG, allowing for controlled protein production (studier, et al 2019)

2.3.1.2 Reduced proteolytic activity

Mutations in the strain cause some proteases—enzymes that break down proteins—to become inactive. This aids in stopping the target protein from being created and breaking down.

2.4 *Benefits of using BL21DE3*

2.4.1 High protein yield

The efficient T7 RNA polymerase and reduced proteolysis often lead to high amounts of the desired protein being produced. (Singh et al., 2015).

2.4.2 Inducible expression

The lacUV promoter allows you to control when protein pro - duction starts, which can be helpful to the proteins that might be harms to the bacteria if produced constantly (Rosano and Ceccarelli, 2014; Studier, 2014).

2.4.3 Well - characterized system

BL21DE3 and the plasmids used with it are very well understood by scientists, making it a reliable and easy - to - use system, (Shiloach and Fass, 2005)

2.4.4 Not ideal for all proteins

BL21DE3 is mainly used for cytoplasmic protein expression. Some proteins, such as membrane proteins, may require differ - ent expression systems (Shiloach and Fass, 2005).

2.4.5 Can be leaky

The lacUV5 promoter can have some background activity, even without IPTG induction. This might be toxic to the bacte - ria.

2.4.6 Characterization of the expressed protein

Describe the methods used to conclude the expression of the arsenic binding protein with GFP tag. (Lallemand - Breitenbach V.; Jeanne M. Science 2010) Discuss purification and separation techniques to isolate the recombinant protein from bacterial lysates. This data on protein yield, purity, and stability. Analyze the functionality of the expressed protein in arsenic binding assay (Gopal and Kumar, 2013; Rosano and Ceccarelli, 2014).

2.5 Literature Review

This research focused on an ABP from other organism like mouse, fish. They cloned the ABP gene with a C - terminal GFP tag into a pET expression vector and transformed it into BL21 (DE3) (T. T. Yip, Y. Nakagawa, and J. Porath, Anal. Biochem., 1989). Following IPTG induction, they **observed strong GFP fluorescence, indicating successful ABP expression**. (Zhuang, J. Environ. Sci., 2010). Western blotting was used to demonstrate that the full - length ABP - GFP fusion protein was present.

Research by investigated the effect of different linker sequences between the ABP and GFP on arsenic binding affinity. They expressed several constructs with varying linker lengths and compositions in BL21 (DE3). Their findings demonstrated that a short and flexible linker preserved the ABP's binding capacity while facilitating proper GFP folding. Explored the use of the GFP tag for in vivo monitoring of ABP activity. They engineered an ABP - GFP construct where arsenic binding resulted in a conformational change affecting GFP fluorescence intensity. This approach allowed for real - time detection of arsenic uptake by the E. coli cells expressing the ABP - GFP fusion.

2.6 *Challenges and Considerations*

2.6.1.1 Fusion protein functionality

The addition of a GFP tag might affect the ABP's folding, stability, or arsenic binding affinity. Careful selection of linker sequences and optimization of expression conditions can mitigate these issues.

2.6.1.2 Background fluorescence

BL21 (DE3) can exhibit basal levels of autofluorescence. Choosing appropriate controls and employing fluorescence microscopy techniques with high signal - to - noise ratios are essential for accurate analysis.

2.6.1.3 Cytotoxicity

In some cases, overexpression of ABP - GFP fusions might be detrimental to E. coli cell viability. Optimizing expression levels and growth conditions is crucial for successful character –

2.7 *Future Directions*

2.7.1. Development of genetically encoded biosensors

The ABP - GFP system can be further developed into a biosensor for sensitive and specific arsenic detection in environ - mental samples (S. Daunert, G. Barrett, 2000) By incorporating additional reporter elements, researchers can engineer a system that translates arsenic binding events into easily detectable signals (J. Stocker, D. Balluch, M. Gsell).

2.7.2. Exploring alternative tags

While GFP offers valuable advantages, alternative tags with different properties (e. g., smaller size, different excitation/ emission wavelengths) could be explored to address potential limitations associated with GFP.

2.7.3. In vivo applications

The ABP - GFP system can be tested in more reliable

biological systems, such as engineered bacteria for bioremediation applications. Monitoring ABP expression and activity in vivo pro - vide valuable insights for optimizing arsenic removal strategies.

3. Materials and Methods

3.1 *Vector designing*

Procedure: Firstly —Vector builder‖ page was opened in google (Fig 1). Next setting option (3 - line bar option) was selected then next a vector system i. e. –Recombinant protein expression vector was selected. A bacteria option appeared, from where PET option was selected then next the tag (i. e. N - terminal) - 6xHis+MBP+TEV selected. Now for (petase en - zyme) petase ORF region was added then selected the EGFP protein selected then after another of sequence gene of interest sequence copied and paste into vector builders and named as ABP and then the linker sequence 3XGGGGS sequence also added then go for the design. Arsenic binding protein (ABP) sequence also added.

Materials: PCR tube, micropipette, oral ligation kit (Fig - 2), mini - thermal cycler, refrigerator.
Procedure: Firstly, a clean PCR tube was taken. Next a ligation kit was taken. Now the ligation sample was added based on the measurements with the micropipette. Ligation sample preparation following measurements 2µl - Vector DNA 4µl – gene of interest 2µl – 10x Ligase buffer + ATP 2µl - T4 DNA Ligase Now the following measurements add to the PCR tube with help of micropipette. Now leave the Ligated sample tubes in refrigerator for 1 hour. After 1 hour the ligated sample tubes were taken and kept in the mini - thermo cycler for 5 minutes at 65°C and ran the above setting. After the 5 minutes the ligated sample taken out from the mini thermo cycler. Then kept into fringe at 4°c.

Figure 1: Vector Builder

Figure 2: Ligation Kit

3.2. *Ligation*

Materials: PCR tube, micropipette, oral ligation kit (Fig-2), mini-thermal cycler, refrigerator.

Procedure: Firstly, a clean PCR tube was taken. Next a ligation kit was taken. Now the ligation sample was added based on the measurements with the micropipette. Ligation sample preparation following measurements 2µl - Vector DNA 4µl –gene of interest 2µl – 10x Ligase buffer + ATP 2µl - T4 DNA Ligase Now the following measurements add to the PCR tube with help of micropipette. Now leave the Ligated sample tubes in refrigerator for 1 hour. After 1 hour the ligated sample tubes were taken and kept in the mini-thermo cycler for 5 minutes at 65°C and ran the above setting. After the 5 minutes the ligated sample taken out from the mini thermo cycler. Then kept into fringe at 4°c.

3.3. *Competent cell preparation*

Materials: Conical flask, magnetic loop, centrifuge, hot plate, refrigerator, E. coli sample (Fig - 3), Luria Bertani broth

Procedure: Firstly, prepare the LB broth for 20 ml. LB broth prepared then take conical flask and add lb 0.5gm dissolved in 20 ml of distilled water. Then after cover the top of the conical flask with cotton plug or paper around to rubber band then put it on the hot plate. When the bubbles came out remove the conical flask on to the plate. Then after cool it for few minutes. Then add the BL21DC E. coli stain to the Lb broth put it on to the orbital zshaker 24 hours.

Then the growth will be observed put into the trans il - luminator observe the green florescence on UV light. Then take the new eppendrof tubes and add 1.5 ml of E. coli into 2 to the eppendrof tubes with the help of micropipette Centrifuge at 4000rpm for 10 minutes then remove the supernatant and re - suspend the pellet with 400µl of ice - cold solution (mgcl2) then incubate the cell in ice for 15 minutes. Then again centrifuge at 4000rpm for 10 minutes at 4°C. Remove the supernatant by aspiration with pipette and resuspend the pellet with 800µl of ice - cold solution added with the help of micropipette and incubate the cells in refrigerator for 30 minutes then centrifuge at 4000 rpm for 10 minutes at 4°C.

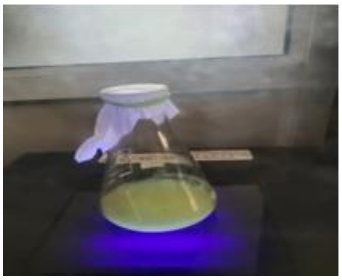

Figure 3: E. coli growth observed in transilluminator

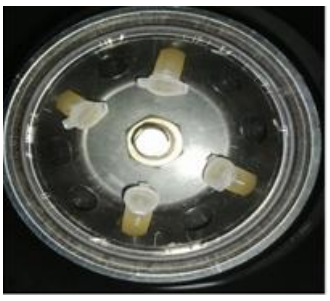

Figure 4: Centrifugation of competent cells

3.4. *Transformation*

Material: Petri plates (Fig - 5), L shaped rod, microfuge tubes, micropipette, cotton swab, IPTG (isopropyl - B - D -

thiogalactopyranoside), Ampicillin, L. B broth, plate count agar, distilled water, ligated sample, competent cell, incubator shaker refrigerator micropipette, laminar air flow.

Procedure: Add 10µl of ligated sample into the 200µl of competent cell mix gently then after gently tap the microfuge tube and incubate on ice for 5 minutes. Then give the heat shock the cells by placing the microfuge in 42°C water bath for 2 minutes then keeps the microfuge tube on ice and chill for 5 minutes. Pipette out 05µl of L. B broth aseptically to the microfuge tubes and incubates at 37°C with shaking for an hour this is to allow bacteria to remove from heat shock and express the protein. Prepare the lb agar media then cool it add the IPTG and ampicillin to the lb agar medium then pour into the Petri plates. After solidifying the media Then the mixed competent cells and ligated sample 200µl added to the Petri plate with the help of micropipette spread the sample with the help of spreader under the laminar air flow. Then another plate added 200µl of competent cells to the petri plate spread through the spreader. Then after incubate the plates at 37°C for overnight and observe the growth on the media.

3.5. *Antibiotic screening of the transformed cells*

Procedure: Prepare the Lb agar plates supplemented with appropriate ampicillin antibiotic. Inoculated transformed cell onto the Lb agar plate using sterile loop. Spread the cells evenly across the agar plate Incubate the plate appropriate temperature for bacterial growth Observe the plates on presence or absence of bacterial colonies. Identified colonies that grow on antibiotic contains plates as potential antibiotic resistant trans - formants Selected antibiotic for further analysis and experimentation. Documented the result and procedure for further use.

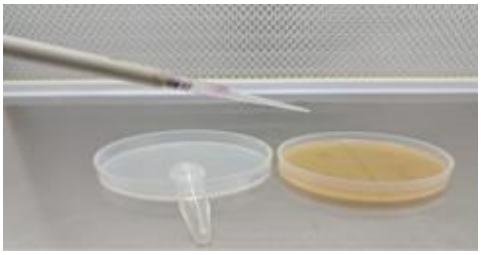

Figure 5: Petri plate with antibiotic

Figure 6: Ni - MTA affinity column

3.6. *Purification (affinity chromatography Ni - NTA column)*

Principle: Affinity chromatography used to separate purify the specific biomolecule such as proteins enzymes antibodies and nucleic acids based on between the ligand and target molecule interaction. The immobilized ligand is attached to a solid matrix in chromatography column.

Materials: Affinity column (Fig - 6), buffers, pH Equilibration buffer Sodium phosphate mono basic, Sodium phosphate diabasic, Nacl, pH.

Wash buffer: Same to equilibration buffer Elusion buffer: PBS buffer + imidazole, NaOH (0.5M) – 100 ml, EDTA (100mM) - 10 ml

Procedure: Plug the column to the strand and pass the 10 ml of NaOH solution through column this is used to remove the unbounded protein impurities from column. Next passes the 10ml EDTA solution through column then after NaOH pass 10 ml distilled water to remove completely remove nickel from the column.

Equilibration: The column is initially equilibrated with a buffer that provide suitable condition for the specific binding. Sample application: The sample that contain the target molecule is applied to the column the target molecule selectively binds to the immobilized ligand. Prepare the LB for culturing the transforming cells and inoculate 100µl of trans - formed cells after 3 hours add IPTG and incubate for overnight at shaker. Pass the NiSO4 through the column to add nickel until column become green. Pass the equilibration buffer to the column until the pH matches the column Then take the suspension culture to pass through the column then the flow through collected.

Washing: Non - specifically bounded molecule is washed out of the column leaving the target molecule bound to the ligand. Then pass the 10 ml wash buffer in the column and collect the flow through.

Elution: the target molecule is eluted or released from the column this is achieved by changing pH or using competitive ligand the replace the target molecule then pass the elution buffer to the column and collect the flow through protein sample.

Collection: The eluted purified target molecule is gathered for further examination. Run the purified sample on the SSDs page.

3.7. *SDS Page*

Principle: SDS - PAGE (sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis) this technique is used to separate and analyze protein based on their molecular weight. SDS de - natures proteins, unfolding them into linear chains, and binds to them imparting a negative charge proportional to their mass. As a result, protein migrate through the gel primarily based on size, allowing for their separation.

Materials: Acrylamide/Bis - acrylamide stock solution, Tris - HCL buffer, SDS (Sodium Dodecyl Sulphate), TEMED (Tetraacetylethylenediamine) (Fig - 7), Ammonium persulfate (APS), Protein sample, Protein ladder, running buffer (Tris Glycine - SDS buffer), Loading buffer, distilled water, Gel casting apparatus, Electrophoresis tank, Power supply, Coomassie Brilliant blue staining solution.

Procedure:

- **Preparing the Gel**

Acrylamide and bisacrylamide stock solution were mixed in the proper volumes to create the resolving gel, tris - hcl buffer, SDS, TEMED, and APS in a flask. The gel casting apparatus's glass cascade are filled with the resolving gel solution and a comb was inserted to create wells for sample loading. The gel was allowed to polymerize. The stacking gel was prepared by using a similar procedure but with a lower acrylamide concentration. The comb was inserted and allowed the stacking gel to polymerize.

- **Preparation of sample**

Take PCR eppendrof tube add 40µl of eluted PB into one tube and add 10µl oof sample buffer eluted. Take another PCR tube and add 40µl of sample buffer add 10µl of wash buffer to an - other tube. Then add another tube 40µl of elution buffer and 10µl of sample buffer to the PCR tube. Then put insert into the PCR for 95°C for 10 minutes.

- **Preparing Samples**

The loading buffer containing SDS and a reducing agent (such as β - mercaptothions) was combined with the protein sample. To denature the proteins (of both enzymes), the sample was heated to 95°C for five minutes.

- **Loading Samples**

The comb was gently removed from the gel. The well was rinsed with running buffer. The protein samples (of both the enzymes) and protein ladder loaded into the wells.

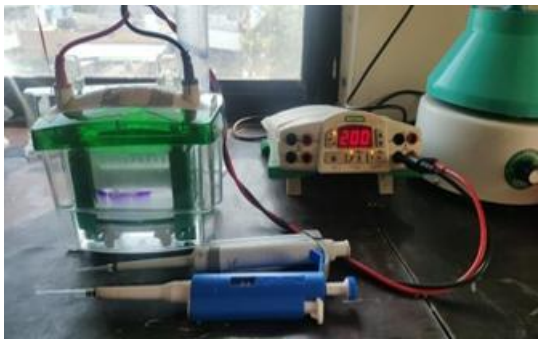

Figure 7: SDS Page

- **Electrophoresis**

The gel cascade was placed in the electrophoresis tank and filled it with running buffer until the gel is fully immersed. The electrodes were connected to the power supply. The gel was run at a constant voltage until the dye front to reaches the lowest portion of the gel.

- **Staining and visualization**

The gel was disassembled from the glass plates. Coomassie Brilliant Blue distaining solution for protein visualization. The gel was distained with distaining solution to remove back - ground staining. The separated protein bands (of both the samples) were visualised.

- **Analysis**

The molecular weight of protein bands was measured by using a protein ladder as a reference. The result was analysed to determine the size and relative abundance of proteins in the sample.

3.8. *BCA Assay*

Principle: Bicinchonic acid assay is technique used to detect the concentration of the unknown protein present in sample.

Working principle of BCA protein assay: Bi cinchonic acid react with OH charged ions of the copper sulphate and it will visualize the green colour change. The BCA assay relies too on the reaction that the peptide bonds present in the proteins reduce CU2+ ions to CU+. The amount of the CU2+ reduced is proportional to the amount of protein concentration present in the sample. BCA chelate with each CU2+ ion, forms the purple colour that absorbs light at a wave length of 562nm. The
deeper the colour the higher the protein concentration.

Procedure: Firstly, prepare a working reagent is prepared. Reagent A - Bi cinchonic acid. Reagent B – copper sulphate working reagent was taken in the 50: 1 ratio. Sample and working reagent were taken in (1: 8 ratio). i. e. (sample: working reagent = 1: 8 ratio (10micro litre: 80micro litre). Firstly, added about 10 micro litre of the sample in the wells. Next added stan - dard sample in the other wells. Again added 80 micro litre of the working reagent in all the wells. After incubated the plate in incubator for 30 minutes. There will be a change in colour. Finally checked the O. D values.

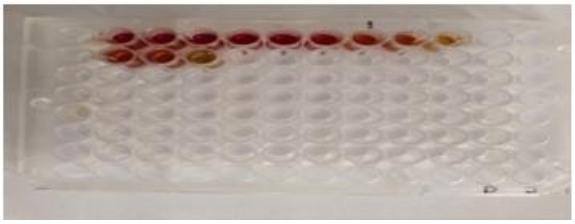

Figure 8: BCA wells coated with standers and unknowns

3.9. *ELISA (Enzyme Linked Immune Sorbent Assay)*

Principle: The indirect ELISA technique to detect the presence of an antibody in a sample. It involves immobilizing the antigen of interest onto a solid surface, such as a microplate well. Then, the sample containing the antibody is added. If the antibody is present, it will bind to the immobilized antigen. After washed away unbound components, a secondary antibody conjugated with is an enzyme, is added. This secondary anti - body binds to the primary antibody. Finally, a substrate solution is added, and the enzyme catalyzes a reaction that produces a measurable signal, indicating the presence of the antibody.

Materials: Microplate wells, Antigen solution, blocking buffer (e. g., BSA or milk), Sample containing the antibody, Primary antibody detection solution, Secondary antibody con - jugated with an enzyme (e. g., HRP), Substrate solution (e. g., TMB or ABTS), Wash buffer (e. g., PBS with Tween)

Procedure: Prepared the antigen solution at the desired con - centration and add it to the microplate wells and the blocking the remaining surface area of the wells with the blocking buffer to prevent nonspecific binding. Added the sample containing the antibody to appropriate wells and incubated to al - low antibody - antigen binding and incubated to allow anybody antigen binding then washed the wells to PB buffer to remove unbounded sample components with PBS buffer.

Added the primary antibody detection solution to each well and to detection solution each well and incubated to allow binding of the primary antibody to antigen - antibody complex. Then washed the wells again to remove unbound primary antibody. Added the secondary antibody conjugated with an enzyme to each well and after incubated to allow binding of the secondary antibody to the primary antibody. Washed the wells to remove unbound secondary antibody. Added the substrate solution to each well and then incubated to allow the enzyme substrate re - action occur. Stopped the reaction by adding a stop solution (if necessary), and measured the absorbance at the appropriate wavelength using a microplate reader. Analyzed the data to determine the presence and concentration of the antibody in the complex.

4. Result and Discussion

4.1 *Vector Designing*

Recombinant protein expression of E. coli is achieved using potent and extensively utilized PET vector system (Fig - 10). Under the guidance of the robust transcription and translation regulatory mechanism of bacteriophage T7, the target gene is cloned into the pET vector. Introducing T7 RNA polymerase into the cell causes the expression to become activated. The expression of the target gene takes up almost all of the cell's resources when the system is completely activated. The desired protein may make up almost half of the total protein in the cell after only a few hours of induction.

Figure 9: ELISA plate coated with samples

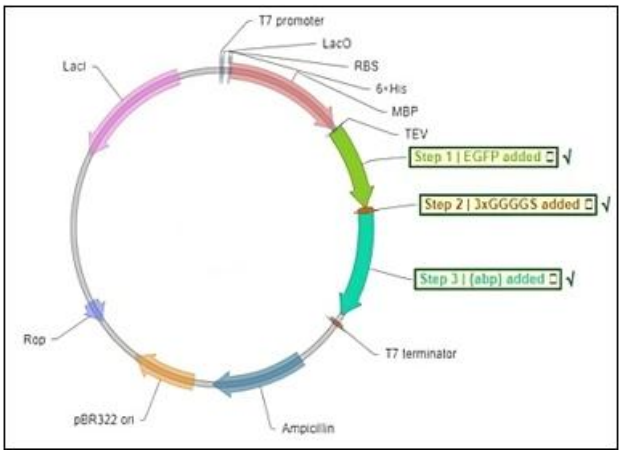

Figure 10: Vector Designing PET vector with EGFP protein with arsenic binding protein

4.2 *Ligation*

The formation of recombinant DNA by the joining of the vector DNA and the gene of interest with the help T4 DNA ligase enzyme. This ligase enzyme catalyses the formation of covalent phosphodiester linkages, which joins the both the vector DNA and the gene of interest permanently (Fig - 11 (a)).

4.3 *Competent cells*

This method involves treating cells with a solution containing ice cold solution (MgCl2). This treatment weakens the cell wall and membrane, making it easier for our gene of interest enters into the cell.

4.4 *Transformation*

Recombinant transformed plate) typically refers to a plate used to observe the successful transformation of a recombinant

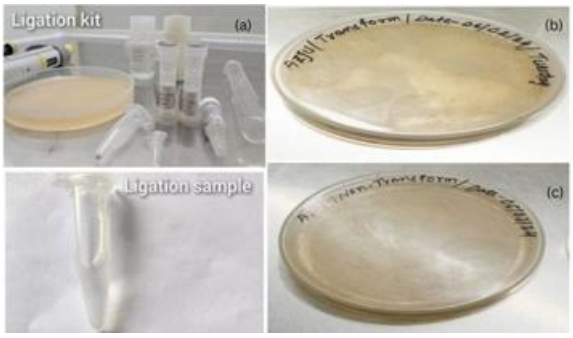

Figure 11: (a) 2 Ligation kit and the Ligation sample, (b) 1 Transformed plate, (c) Non Transformed plate

vector containing arsenic binding protein to GFP into host cells. The result of control plate of Fig - 11 (b) shows the successful transformation. Colonies on the plate exhibit resistance to certain antibiotics and may show the desired phenotype associated with arsenic gene expression.

4.5 *Affinity (Ni - NTA) column chromatography*

The affinity chromatography is purified techniques eluted sample contain the more unbound proteins are comes out and washing buffer contain the gene of interest and the elution buffer contain the purified protein.

4.6 *SDS Page*

Arsenic binding protein band at 33 kDa: The presence of a protein band corresponding at 33 kDa shows that the recombinant arsenic binding protein has been successfully expressed and purified. The molecular weight of 33 kDa is consistent with the expected size of arsenic binding protein, indicating that properly folded and processed.

Significance of the 33 kDa band

- **Identify conformation:** The appearance of a band at 33 kDa confirms the presence of arsenic protein in sample. This is crucial for verifying the success of protein expression and purification.
- **Purity assessment:** The intensity and sharpness of the 33 kDa band can provide insights into the purity of the arsenic binding protein preparation. A strong, well - defined band indicates a high level of purity, whereas additional band or smearing may suggest contamination or degradation products.

4.7 *BCA ASSAY*

Fig - 12 (b) shows that the gradual decrease in the concentration of arsenic (with PB, Sample, PBS, PBS+NaCl) standards absorbance (990, 750, 650, 399, 200, 108, 0) on x - axis than concentrations (2500, 1500, 750, 500, 250, 125, 0) on y - axis with the help of graph will be plotted.

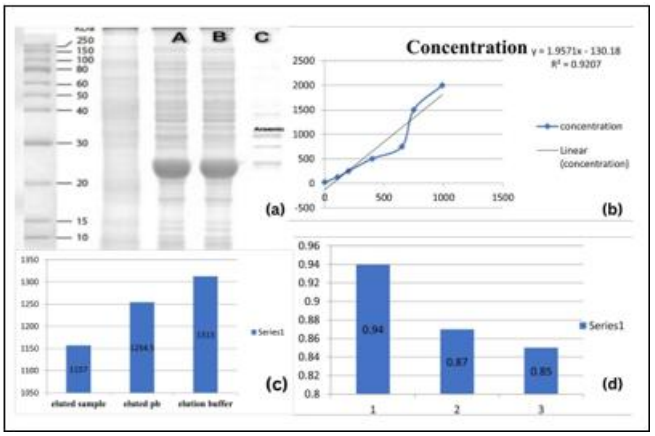

Figure 12: (a) Protein band on arsenic binding protein, (b) Concentration of standard BCA Assay, (c) Concentration of samples on BCA Assay, (d) Con - centration of standards on samples on ELISA

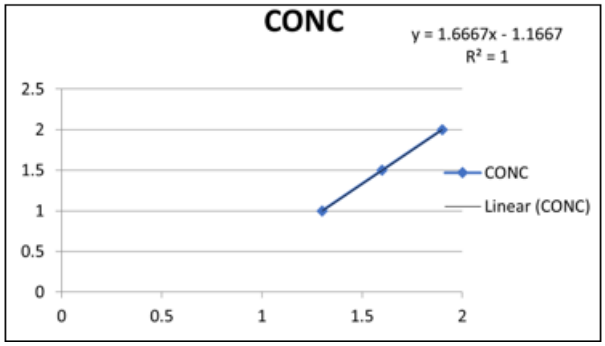

Figure 13: Concentration of standard on ELISA

Then Fig - 12 (c) represents the Gradual increase in the con - centration of arsenic (eluted sample, wash buffered, elu - tion buffer) absorbance (660, 710, 740) on y - axis concen - tration (1157, 1254.5, 1313) on x - axis by using the formula (y=1.9571*absorbance - 130.18) and plot a graph the eluted buffer more conc it will be purified form.

4.8 *ELISA*

Fig - 13 represents gradually decrease in concentration of absorbance (1.9, 1.6, 1.3) and concentration (2, 1.5, 1) then plot the graph.

The Fig - 12 (d) the Gradual decrease in the concentration of arsenic binding protein Samples (eluted Sample, PBS, PBS + NaCl): absorbance (0.94, 0.87, 0.85) and concentration (0.344, 0.234, 0.2) and by using the formula of standards (y=1.6667*absorbance - 1.1667) gives R2 value by this formula we known the concentration of the sample and graph will be plotted.

5. Conclusion

The cloning and expression of arsenic binding proteins with GFP tags in BL21DE3 strain bacteria have emerged as valu - able techniques in **the study of arsenic detoxification mechanisms and environmental monitoring**. Through the synthesis of literature, it is in optimizing cloning strategies, expression conditions, and characterization techniques. First, the choice of expression vector and host organism is critical for achieving high - level expression of the target protein. Studies have **demonstrated the superiority of BL21DE3 strain bacteria for efficient protein expression**, particularly coupled with ap - propriate inducer concentrations and culture conditions.

Furthermore, characterization of the expressed arsenic binding proteins has been providing its structural and functional properties. Techniques such as affinity chromatography and SDS - PAGE, and fluorescence microscopy have enabled re - searchers to assess protein folding, stability, and arsenic binding affinity. **These results add to our knowledge of the mechanisms by which arsenic detoxifies and have potential uses in biosensing and bioremediation, among other fields**.

Despite the advancements made in cloning, expression, and characterization of arsenic binding proteins, several challenges and opportunities remain. One notable challenge is the het - erogeneity of arsenic binding protein, in their arsenic binding affinities, specificities, and subcellular localization. Future re - search focus on elucidating the structural determinants of arsenic binding and the molecular mechanisms underlying protein–arsenic interactions.

Moreover, while GFP tagging provides a convenient technique for monitoring protein expression and localization, it is essential to consider potential drawbacks such as steric hindrance and alteration of protein function. Alternative tagging strategies or label - free detection techniques may offer complementary approaches for studying arsenic binding proteins in di - verse biological contexts.

In conclusion, the cloning, expression, and characterization of arsenic binding proteins with GFP tags in BL21DE3 strain bacteria represent a significant advancement in the field of arsenic research. By combining molecular biology techniques with protein biochemistry and environmental science, researchers can gain deeper knowledge into **arsenic detoxification mechanisms and develop innovative solutions for arsenic contamination mitigation and detection**. Continued interdisciplinary efforts are essential to address remaining challenges and harness the full potential of arsenic binding proteins for environmental and biomedical applications.

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