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Amplification and Cloning of gene encoding of Cell Surface protein (CSP's) and their Expression in *E.coli* BL21DE3 strain

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Abstract
Escherichia coli also known as *E.coli*, is a Gram-negative, facultative anaerobic, rod-shaped, coliform bacterium of the genus *Escherichia* that is commonly found in the lower intestine of warm-blooded organisms (endotherms). Most *E.coli* strains are harmless, but some serotypes can cause serious food poisoning in their hosts and are occasionally responsible for product recalls due to food contamination. In this study the gene encoding cell surface protein from *E.coli* was cloned, expressed under the control of T7 promoter and the obtained result showed that Pet19b system was very efficient. the results obtained during this investigation promising conclusion was made as;

- The CSP gene isolate from *E.coli* and was incorporated into pET19b vector
- Transformation was done successfully
- The clones were analysed for the presence of insert by PCR and they showed that the insert presence
- The clones were also analysed by SDS-PAGE and one of the clone showed expression of the protein which is 35kdalton.

Keywords: cell surface protein, amplification, cloning and *E.coli*

Introduction
Escherichia coli also known as *E.coli*, is a Gram-negative, facultative anaerobic, rod-shaped, coliform bacterium of the genus *Escherichia* that is commonly found in the lower intestine of warm-blooded organisms (endotherms). Most *E.coli* strains are harmless, but some serotypes can cause serious food poisoning in their hosts and are occasionally responsible for product recalls due to food contamination. The harmless strains are part of the normal microbiota of the gut and can benefit their hosts by producing vitamin K2 and preventing colonization of the intestine with pathogenic bacteria, having a symbiotic relationship. *E.coli* is expelled into the environment within fecal matter. The bacterium grows massively in fresh fecal matter under aerobic conditions for 3 days, but its numbers decline slowly afterwards. (Pernilla *et al*., 2007).
DH5alpha Cells: DH5alpha cells are engineered *E.coli* cells in order to maximize transformation efficiency. They are given their name from Douglas Hanhan an American biologist. The cells are defined by three mutations:

1. recA1
2. endA1 which helps plasmid insertion
3. LacZM15 which enables blue white screening

Cell surface proteins that are embedded in span the layer of cell membrane of more complex organisms. These proteins are integral to the way in which a cell interacts with the environmental around it, including other cells. Some of these proteins, especially one that are exposed to the external side of the membrane, are called glycoproteins because they have carbohydrates attached to the outer surface (Vogt. 1997) [2].
Their main functions are communication-signal transduction, receptors for hormones and neurotransmitters and regulation acting as pores that follow ions, nutrients and waste products to pass into and out of the cell. Proteins can change their confirmation; opening or closing (permeability) allowing for small molecules to pass while blocking others.
The most widely used method for gene cloning in *E coli* uses plasmid as the cloning vector. *E.coli* DH5alpha is a strain widely used for DNA manipulation. It is a rec.A-highly transformable and allows for selection by alpha-complementation (Lin, 2009) [1].

Materials and Methods
Bacterial Strain of *E.coli*
The strains of *E.coli* i.e. DH5alfa and BL21DE3 were obtained from Aristogene Biosciences Pvt. Ltd. Bangalore.

Transformation
Transformation is a very basic technique that is used on a daily basis in a molecular biological laboratory. The purpose of this technique is to introduce foreign plasmid into bacteria to amplify the plasmid in order to make large quantities of it. It is defined as the transfer of genetic from a donor to a recipient using naked DNA. The recipient takes up the DNA from the media and there is no requirement for cell to cell contact (Michelsen, 1995) [4].

Preparation of competent cell
Streak DH5alfa strain provided onto a fresh LB plate.

Incubate the plate overnight at 37˚c. Inoculate 200µl of culture into 5ml LB medium and incubate at 37˚C. Grow until the OD (A600) reaches 0.4 -0.6 (It takes around 2-3hours to reach the required OD). Then quickly chill the culture flask on ice and leave it on ice in refrigerator for 10-20mins. 4^0C temperature should be maintained in all further steps by using ice box. After that transfer the culture aseptically into append off vials tubes and spin down at 6000rpm for 5mins at 4˚c. After spinning discard the supernatant and suspend the pellet add TSSA and TSSB. Centrifuge again at 6000rpm for 5mins at 4˚c (Chudaev, 1997) [3].

Isolation of plasmid DNA

In this method the transformed single colony inoculation has been done. Further Incubated overnight at 37˚C, grown culture was taken and centrifuged at 6K To that, solution was added such that it contains 15% Glucose which provides isosmotic condition to physical shock (Sambrook, Fritch 1989) [10]. The solution pH is raised to basic level with tris that helps to denature DNA. EDTA stabilizes the cell membrane by binding the divalent cations. Then to that solution, solution II is added such that 1%SDS is an ionic detergent which dissolves the phospholipids and protein components of cell membrane.0.2N NaOH in the solution denatures the plasmids and the chromosomal DNA into single strands (Fu, 2009) [1].
Then this solution was mixed with solution III such that, sodium acetate in this solution forms an insoluble precipitates of protein complex and neutralize NaOH. At this neutral pH, the DNA's renature, further3 volume of cold absolute alcohol, the DNA pellet was washed twice with chilled alcohol, air dried and suspended in 50µl of TE buffer.

Amplification of insert DNA by PCR

Polymerase chain reaction is an *in-vitro* technique which allows the amplification of a specific DNA using the *in-vitro* amplification of single copy of gene using klenow fragment of *E.coli* DNA polymerase. The amplification of targeted DNA was carried put in 25µl reaction volume each containing 2mM MgCl2, 25mM Deoxy Nucleoside triphosphate, 400ng primer 1µl of whole cell bacterial lysated and adjusted to 25µl by the addition of HPLC grade water. Amplification was carried out, initial denaturation for 2 min at 94^0C, 30 cycles were completed, each consisting of 1 minute at 94^0C, 1 min at approximate annealing temperature of 50^0C and 2 min at 72^0C as shown in the Table 1. A final extension of 10 min at 72^0C was applied (Saiki *et al.,* 1985) [5]. The reaction is carried out in a programmable thermal cycler capable of changing incubation temperature and incubation times. Typical cycling in a PCR reaction for primers 18-24 nucleotides in length with 50%G+C content and a PCR product length of 0.2-2.0kb would be 30cycles of denaturation, annealing and extension (Matsuda, 2001) [6].

Table 1: Time temperature conditions for PCR

Condition	Temperature	Time	Cycle
Initial denaturation	94˚c	2 min	1 cycle
Denaturation	94˚c	30 sec	30cycles
Annealing	58˚c	30 sec	30cycles
Extension	72˚c	30 sec	30cycles
Final extension	72˚c	2 min	

Restriction Digestion

Restriction digestion is the process of cutting DNA molecules into smaller pieces with special enzymes called Restriction Endonucleases. The restriction digestion of PCR product was digested using the restriction enzymes Nde1 and Xho1. The reaction mixture was composed of 20µg of total DNA as a template. The digestion procedure was executed following instructions from the manufacturers (Sigma Aldrich, Bangalore, India) for each enzyme. The PCR product was digested with 20U of the reaction enzyme in the presence of appropriate 1X restriction enzyme buffer. The total volume of the reaction mixture was made up to 50µl. The Contents of the reaction were mixed thoroughly and incubated at 37^0C. Post incubation, the reaction was terminated by heating the reaction mixture at 65^0C for 10 minutes, the digested DNA was analyzed by agarose gel electrophoresis (John brooks, 1987) [7], as shown in table 2.

Table 2: Composition of restriction digestion

Composition	Vector	Insert
DNA	20µl	20µl
Buffer zero	10µl	10µl
Nde1	3µl	3µl
Xho1	3µl	3µl
Double distilled water	64µ l	64µl
Total Volume	100µl	100µl

Ligation

Ligation is joining of two nucleic acid fragments through the action of an enzyme. It is an essential laboratory procedure in the molecular cloning of DNA where the DNA fragments are joined together to create recombinant DNA molecule, such as when a foreign DNA fragment is inserted into a plasmid. The ends of DNA fragments are joined together by the formation of phosphodiester bond between the 3`hydroxyl of one DNA terminus with the 5`phosphoryl of another. A cofactor generally involved in the reaction and this is usually ATP or NAD+, as shown in the Table 3.

SDS-PAGE for the analysis of protein expression

Electrophoresis is the migration of charged molecules in solution response to an electric field. The rate of migration depends on the strength of the field; on the net charge, size and shape of the molecules and also on the ionic strength, viscosity and temperature of the medium in which the molecule are moving. As an analytical tool, electrophoresis is sample, rapid and highly sensitive. It is used analytically to study the properties of a single charged species, and as a separation technique (Mullis *et al.,* 1985) [8]. SDS-PAGE is to separate proteins based on the size through the use of stacking

gel and resolving gel are cross linked polyacrylamide gels are formed from the polymerization of acrylamide monomer in the presence of smaller N,N,-methylene, bisarylamide which serves as a cross linking agent (Muryoi, 2004) [9].

Table 3: Composition of ligation

Components	Stock	Working	1:2	1:4
A.DH2O				
LIGASE ASSAY BUFFER	10X	1X	2µl	2µl
VECTOR	--	50ng	2µl	2µl
INSERT	--	--	1µl	2µl
T4DNA LIGASE	10units	1unit	1 µl	1µl
Total			20 µl	20 µl

Results

Cloning of CSP Gene Encoding Cell Surface Protein

Transformation

The competent cells of *E.coli* Dh5alfa were prepared by $MgCl_2$ treatment method. The construct was then transformed into the competent cell *E.coli* Dh5alfaz

Plate Screening and Colony PCR

After transformation, the cells were plated on LB agar plates containing ampicillin100ul. These plates were incubated at 37˚C for 24 hrs. Hundreds of colonies were observed on plates which indicate the presence of transformed cells can be seen in fig 1. The several colonies were randomly numbered and patched on fresh LB agar plates containing ampicillin these patched colonies were used to carryout colony PCR. The bands obtained in gel showed the colony PCR product along with 500bp DNA marker ladder. Hence the transformation was successfully done and the desired DNA fragment was successfully cloned into dh5 alpha cells.

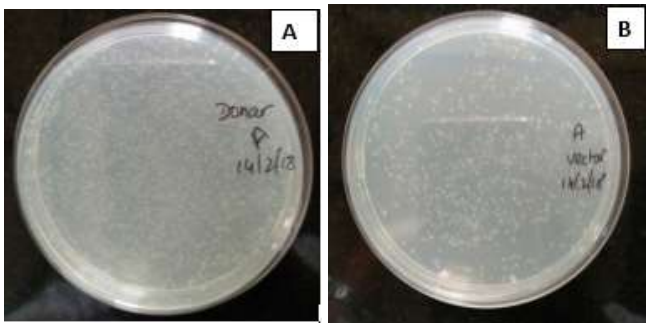

Fig.1: Plate containing Transformed colonies. LB plates (A & B) are containing Insert and Vector DNA into *E.coli* DH5 alfha

Isolation of insert DNA and vector

The plasmid DNA was isolated by alkalyne lysis method and DNA was ananlysed with 0.4% agarose gel and bands was observed under UV transilluminator after staining with Ethidium bromide as shown in the Figure 2.

Amplification of insert gene by PCR

The amplification of insert gene encoding cell surface protein was amplified by using polymerase chain reaction. The PCR amplified product obtained after 30 cycle was run on 0.4% agarose gel and observed under UV transilluminator. The obtained product length was ~600bp as shown in the Figure 3.

Restriction digetion of insert gene and vector

Restriction digestion of insert and vector with Nde1 and Xho1 was done and restricted product was run on agarose gel and clear bands were obtained as shown in the figure 4. Further Silica gel purification has been done for purifying insert and vector DNA as shown in the Figure 5.

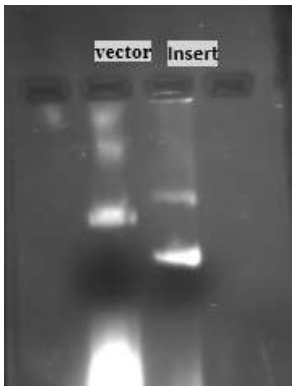

Fig 2: Quantitation of insert DNA and plasmid DNA.

Insert DNA and vector is isolated which is having 100ng of DNA.

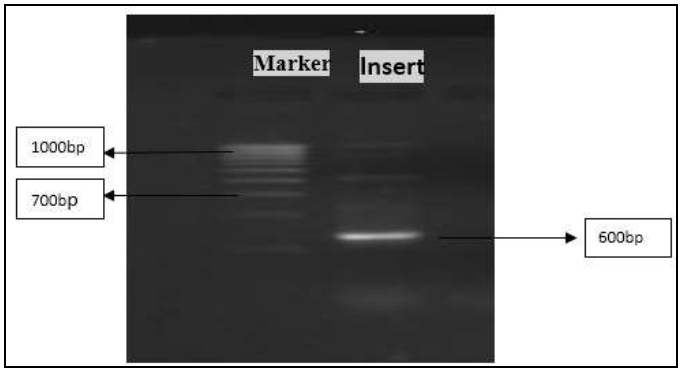

Fig 3: Amplified product of insert gene by PCR. The first lane is of marker 500base pairs and second lane is of amplified insert gene ~600base pairs. Lane M; labeled as Marker of 1kb DNA Ladder; Lane 2: labeled as amplified insert gene ~600bp

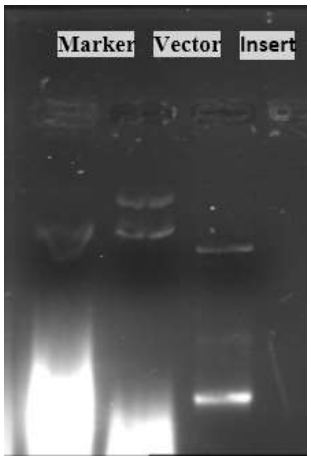

Fig 4: Restriction digestion of Vector and Insert DNA. Lane M: 1kb DNA Ladder; Lane 1-2: Vector and Insert DNA

Ligation

The amplified gene was ligated into pET 19b in two different ratios of 1:2 and 1:4 by using T4 DNA ligase and transformation of ligated sample were done.

Transformation and Screening of positive recombinant Clones.

The competent cells of *E.coli* dh5alfa were prepared by MgCl2 treatment method. The ligated product was then transformed into competent cell *E coli* DH5alfa. After transformation the cells were plated on LB agar plates containing ampicillin 100ul. These plates were incubated at 37˚C for 24 hrs. And a Very few colonies were observed on plates which indicate the presence of recombinant vector as shown in figure 5.

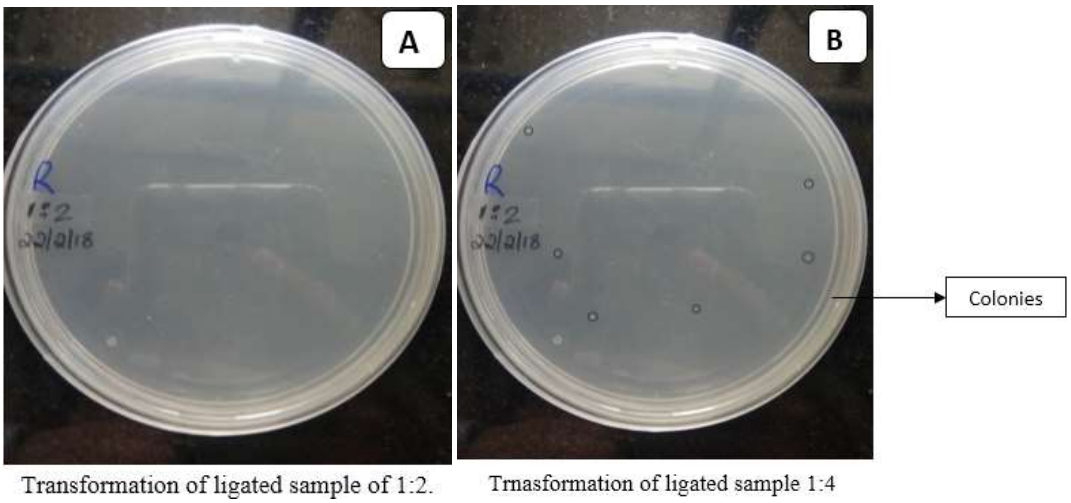

Fig 5: Transformed Colonies of ligation. (Plates A & B are showing transformed colonies containing recombinant Vector)

Isolation of recombinant vector

The recombinant vector was isolated from transformed colonies to confirm the clones as shown in Figure 6.

Transformation of Clones

The competent cells of *E.coli* BL21DE3 were prepared by MgCl2 treatment method. The construct was then transformed to *E.coli* BL21DE3 competent cell.

Plate screening

After transformation the cells were plated on LB agar plates containing ampicillin. Hundreds of colonies were observed which indicates the presence of recombinant vector in BL21DE3 strain of *E.coli*. Transformation of clones into BL21 DE3 can be seen in Figure 7.

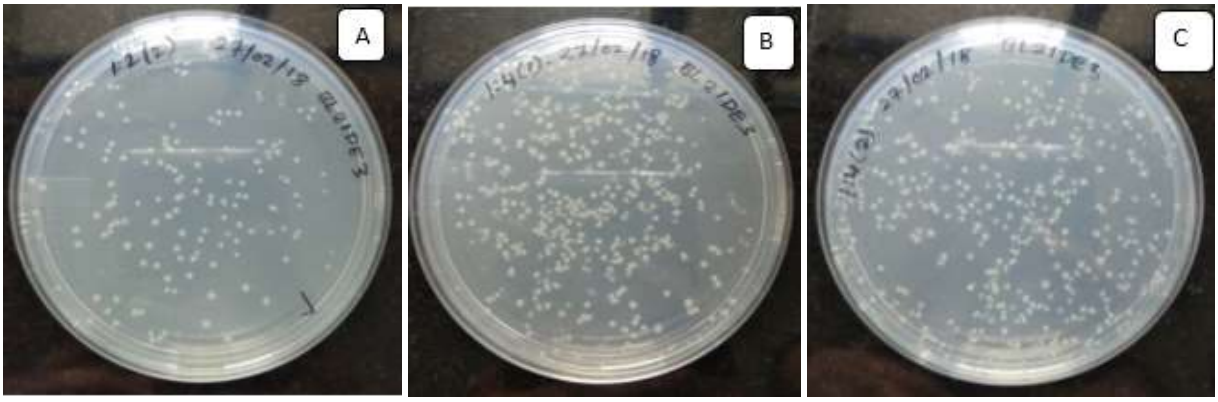

Fig 7: Plate containing transformed clones. (Plates A,B&C are containing transformed colonies)

SDS-Page

The expression of gene encoding cell surface protein was achieved with pET19b the SDS PAGE showed that the *E coli* BL21DE3 upon IPTG induction were shown a thick band of = 35kDa, of cell surface protein can be seen in Figure 8.

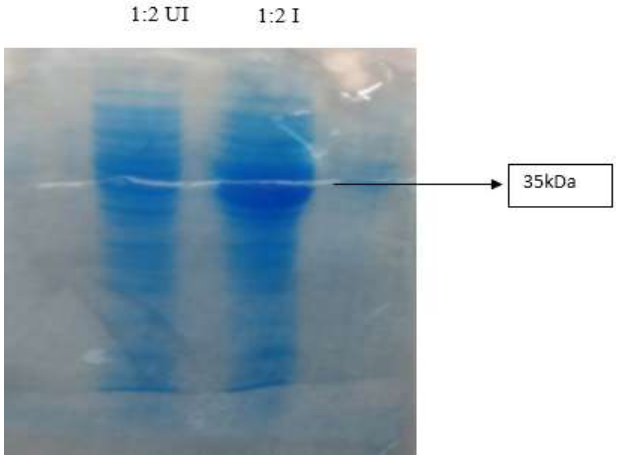

Fig 8: Expression of protein by SDS-PAGE (UI = Uninduced, I = Induced with IPTG) Expression of the cell surface protein can be seen in Induced culture with a size of 35kDa.

Discussion

Vast studies are carried out in research of gene encoding cell surface protein from microbial source especially bacteria due to its advantages. Their main function are communication-signal transduction, receptors for hormones, regulation action as pores that allow ion nutrient and waste product to pass and neurotransmitter. These are the major targets of biomedical research due to their utility as cellular marker and several other applications. (Chen, Cleary infection and immunity, 1989) [10]. In this study the gene encoding cell surface protein from *E.coli* was cloned, expressed under the control of T7 promoter and the obtained result showed that Pet19b system was very efficient.

Abcam's cell surface protein isolation kit provides a simple and efficient method for the isolation of cell surface proteins. In this method, cells are first labeled with sulfo- NHS - SS - Biotin, an amine reactive, thiol- cleavable, biltini raagent. Cells are subsequently lysed and the labeled cell surface proteins are isolated using streptavidin beads. The bound proteins are then released from beads by incubating with DTT solution. The biotinylation reagent is cell membrane-impermeable, with an extended spacer arm to reduce steric hindrances associated with streptavidin binding. This convenient kit provides the entire required component for optimal labelling and isolation of cell surface proteins.

These factors position membranes as prime engineering targets to develop platform technologies ranging from whole cells to synthetic constructs based on concepts derived from cells. Applications for these technologies range from research tools that facilitate drug discovery and help understand complex cell-cell interactions to the creation of non-natural transmembrane protein systems for purposes including energy production, biosensing technology, and mitigation of environmental pollutant (Xia ZG Qian, CS Ki, 2011) [11].

Conclusion

Based on the literature survey and the results obtained during this investigation several promising conclusion remarks can be made.

- The csp gene isolate from *E.coli* and was incorporated into pET19b vector
- Transformation was done successfully
- The clones were analysed for the presence of insert by PCR and they showed that the insert presence
- The clones were also analysed by SDS-PAGE and one of the clone showed expression of the protein which is 35kdalton.

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Conflict of interest statement

We declare that no conflict of interest.

Authors Contribution

All authors are responsible for carrying out the research work, data analysis and optimization of experimental work and Corresponding author is responsible for research planning executing and providing valuable inputs and in writing manuscript.

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