Gene Cloning, Protein Expression and FPLC Purification of Lec A from Pseudomonas Aeruginosa in E.Coli

M.Suganya
Department of Biotechnology
Vivekanandha College of Engineeering For Women, Tiruchengode, Tamilnadu, India

K.I.J .Melani Jerine
Department of Biotechnology
Vivekanandha College of Engineeering For Women, Tiruchengode, Tamilnadu, India

E.Swarnambigai
Department of Biotechnology
Vivekanandha College of Engineeering For Women, Tiruchengode, Tamilnadu, India

R Karpagam
Department of Biotechnology
Vivekanandha College of Engineeering For Women, Tiruchengode, Tamilnadu, India

Abstract - Pseudomonas aeruginosa DNA fragment contains the structural gene coding for the galactophilic PA-I lectin (PA-1L, 369 bp) that can be used as a subunit vaccine. The aim of the current project entitled "Gene cloning,protein expression and FPLC purification of Lec A from Pseudomonas aeruginosa in E.coli" is to isolate the gene using primers specific for gene amplification, clone the gene into a prokaryotic expression vector – pET-32a, transform into prokaryotic expression host E.coli –BL-21, over expression of the protein, purification using chromatography and detection of the protein on SDS-PAGE.The primers for specific gene amplification are designed to have restriction endonuclease site for BamHI for forward primer and EcoRI site for reverse primer. The amplified product and the vector can be digested with these two enzymes and then ligated to obtain a recombinant molecule. The recombinant molecule with the gene of interest can transformed in to an expression host. The cells carrying the recombinant plasmid can be induced by various inducers to obtain the protein in bulk. The protein thus obtained can be purified using chromatography technique and detected by running the protein in SDS PAGE gel with a protein marker. Since, the cloned gene in pET-32a will be expressed along with a GST tag with it, affinity chromatography using the anti-GST column can be used to purify the protein.

Keywords: Lec A gene , lectin protein , pET-32a

1. INTRODUCTION

Pseudomonas aeruginosa is a member of gamma proteobacter class of bacteria under family *Pseudomonadaceae*. It is gram-negative, aerobic rod measuring 0.5 to 0.8 μm by 1.5 to 3.0 μm commonly found in soil and water. Its metabolism is respiratory and never fermentative, but it will grow in the absence of O_2 if NO_3 is available as a respiratory electron acceptor. The typical bacterium in nature might been found in biofilm, attached to surface or planktonic by means of its flagellum. It act as a dreadful pathogen by having optimum temperature ranges from 37˚C to 42˚C as well as resistant to high concentrations of salts and dyes, weak antiseptics, and many commonly used antibiotics including fluoroquinolones, gentamicin and imipenem. Moreover, *Pseudomonas* maintains antibiotic resistance plasmids, both R-factors and RTFs, and it is able to transfer these genes by means of the bacterial mechanisms of horizontal gene transfer (HGT), mainly transduction and conjugation. One type of colonization has a fried-egg appearance and an elevated appearance. Another type, frequently obtained from

respiratory and urinary tract secretions, has a mucoid appearance, which is attributed to the production of alginate slime. Multiple and diverse determinants of virulence leads to septicemia, urinary tract infections, pneumonia, chronic lung infections, endocarditis, dermatitis, and osteochondritis. The pili of bacterium adhere to specific galactose or mannose or sialic acid receptors on epithelial cells on the upper respiratory tract and injured tissues (in case of influenza virus) and in *Pseudomonas* keratitis and urinary tract infections. The receptor on tracheal epithelial cells for *Pseudomonas* pili is probably sialic acid (N-acetylneuraminic acid). Mucoid strains, which produce an exopolysaccharide (alginate) forms the matrix of biofilm, have an additional or alternative adhesin which attaches to the tracheobronchial mucin (N-acetylglucosamine). . Biofilm mucoid strains often seen in lung tissue of cystic fibrosis individual are also less susceptible to antibiotics than their planktonic counterparts. It is isolated on blood agar or eosin-methylthionine blue agar and identified on the basis of its inability to ferment lactose, a positive oxidase identification of *P. Aeruginosa* colonies and by fluroscence. Pseudomonas aeruginosa genome contains PA-IL gene(369 bp) codes for galactophilic lectin PA-IL protein found in cellmembrane of bacterium. The mature proteins lack the initiator methionine and display acidic characters. Lectin have protective role for the *P. aeruginosa* cells by inducing agglutination of other bacteria or uni- and oligocellular eukaryotic organisms. This lectin were demonstrated to act directly in vitro and in vivo as cytotoxic compounds. LecA (PA-IL) is a cytotoxic lectin and adhesin binds hydrophobic galactosides with high specificity and affinity forming biofilm. Passive immunization studies were carried out with *P. aeruginosa* strain K pilus-specific (PK3B, PK99H) and cross-reactive (PAK-13) monoclonal antibodies (MAbs). When A.BY/SnJ mice were passively immunized with a pilus-specific MAb (PK99H), which inhibited pilus-mediated adherence to respiratory epithelial cells, mice challenged with 5 x LD 50 of P. aeruginosa were completely protected while mice were not protected when animals were passively immunized with a pilus specific MAb (PK3B), which did not inhibit pilus adherence to epithilial cells. MAb PAK-13 was found to cross-react with the C-terminal portion of pili of different strains of P. aeruginosa. When mice were passively immunized with MAb PAK-13, subsequent challenge with KB7 (3 x LD50), PAO (8 x LD50) and PAK (3 x LD50) strains of *P. aeruginosa* resulted in a 70%, 60% and 90% protection of the mice, respectively. Similarly the lectin protein found on the cell membrane of *Pseudomonas aeruginosa* can be used to produce subunit vaccine. The adhesion and biofilm formation properties of lectin can be utilised to produce vaccine.

II. MATERIALS AND METHOD

A. Genomic DNA Isolation (Potassium Acetate Method):

Harvest the overnight grown bacterial culture (Pseudomonas aeruginosa), till good pellet is formed by centrifuge at 5000rpm for 5minutes in a 1.5ml microfuge tube. Resuspend the pellet in 575µl of (1X) TE buffer. Add 150µl of 10% SDS, mix well and incubate for one hour at 37°C. Add 150µl of 5M potassium acetate, mix well by inverting. Incubate on ice for 15 minutes. Centrifuge at 10000rpm for 10 minutes and transfer the supernatant to a fresh vial. Add 1µl of RNase A and incubate for 30 minutes at 37°C. Add 0.6 volume of isopropanol, mix gently and precipitate at -20°Cfor 15-30 minutes. Centrifuge at 12000rpm for 8 minutes at -4°C, discard the supernatant. Add 400µl of 70% ethanol, centrifuge at 12000rpm for 5 minutes at -4°C. Discard the supernatant, dry the pellet by inverting the vial on tissue paper towel. Dissolve the pellet in 40µl of TE buffer(0.1X).Store at 4°C. Observe DNA by electrophoresing on 0.8% agarose gel.

B. Agarose Gel Electrophoresis:

Prepare te boat for casting the agarose gel by making it grease free using methanol. Prepare 0.8% agarose in 1X TAE buffer (50ml),Bol to dissolve. Add 5µl of ethidium bromide (stock concentration 5mg/ml) when it reaches about 50-60°C and swrl taking care that there is no air ubble formation. Place the comb in the boat and pour the prepared gel without formation of air bubbles into it and allow for polymerization. The sample to be loaded is mixed with 5µl of gel loading dye. The comb is removed carefully to avoid breaking of wells. The boat is now placed in the tank and 1X TAE is poured into it. The prepared samples are then loaded into the wells and the gel is electrophoresed at 50V. Visualise the gel under the UV transilluminator when the dye reaches 3/4th the length of the gel.

C. Plasmid DNA Isolation:

1.5ml of an overnight culture is transferred in a vial and is centrifuged at 12000rpm for 5 minutes. Repeat till a good pellet is obtained. Discard the medium and suspend the pellet in 100µl of ice-cold Solution I. Mix by vortexing and keep the vials on ice for 5 minutes. To the above vial, add 200µl of freshly prepared Solution II and mix by inverting gently4 times and incubate the vial at room temperature for 3-5 minutes. Add 150µl of ice cold Solution III. Invert the tube four to five times to mix the contents. Centrifuge at 10000rpm for 10 minutes. Collect the supernatant in a fresh vial, add 1µl of RNase and incubate for 30 minutes at 37°C. Add double volume of chilled ethanol, precipitate at -20°Cfor 30 minutes. Centrifuge at 12000rpm for 5 minutes at 4°C. Discard the supernatant and rinse the pellet in 70% ethanol by centrifuging at 5000rpm for 2 minutes at 4°C. Discard the supernatant and air dry the pellet. Dissolve the pellet in 40µl of TE buffer. Observe DNA by electrophorescing on 1% agarose gel.

D. Gene Isolation (Using Pcr)

To a sterile PCR vial ,add the following reagents in the same order

Water	-17.5 µl
PCR Buffer	-2.5 µl
dNTPs	-1.5 µl
Forward primer	-1.0 µl
Reverse primer	-1.0 µl
DNA	-1.0 µl
Taq polymerase	-0.5 µl____
	25.0 µl

Set the mixture in the thermal cycler with following conditions.
Step1: 94°C for 2minutes
Step2: 93°C for 1minute
45°C for 1minute
72 °C for 1minute
Repeat 3 cycles
Step3: 93°C for 1minute
65°C for 1minute
72°C for 1minute
Repeat 20 cycles
Step4: 72°C for 3minutes
4°C forever
End
After thermal cycling ,add 5µl of gel loading dye to the samples.
Electrophorese the samples on 1.2% agarose gel and visualise under UV transilluminator.

E. Concentration Of Pcr Product

Pool all the PCR product in 1.5ml PCR vial. To 120 µl of PCR mixture add double the volume of absolute alcohol and keep at 20 °C for 1hour. Centrifuge at 12000rpm for 10 minutes at -4°C.
Discard the supernatant and add 100 µl of 70% ethanol. Centrifuge at 12000rpm for 5 minutes at 4°C. Decant the supernatant and air dry the pellet. Resuspend the pellet in 60 µl 0.1X TE buffer.

F. Restriction Digestion

To a fresh vial add the following components in the same order

Sterile distilled water	-34.4 µl
NEB buffer H	-5.0 µl
Substrate(PCR product/plasmid)	-10.0 µl
Restriction enzyme (EcoRI)	-0.4 µl
Hind III	-0.2 µl
	50.0 µl

The vial is then incubated for I hour at 37 °C. After incubation ,the reaction was arrested by adding 5µl of gel loading dye. he samples were electrophoresed on a 1% agarose gel and visualised under UV transilluminator.

G. Ligation

To afresh vial add the following components in the same order

	L_1	L_2
Sterile distilled water	-5.5 µl	-4.5 µl
T_4 ligase buffer	-2.5 µl	-2.5 µl
Substrate(PCR RD product)	-12.0 µl	-12.0 µl
Substrate(plasmid RD product)	-3.0 µl	-4.0 µl
T_4 ligase enzyme	-2.0 µl	-2.0µl
	25.0 µl	25.0 µl

The vials are then incubated at 16°C for 2 hours or overnight.

Observe the ligation results by electrophorescing on 1.0% agarose gel.

H. Transformation In Cloning Host

1) Competent cell preparation

Pick an isolated colony of competent cell strain of E.coli DH-5α from a master plate and inoculate into 5ml LB broth. Incubate in a shaker incubator at 200 rpm at 37°C overnight. Inoculate the 3ml of overnight culture to 100ml LB broth and incubate in ashaker incubator. When the optical density at 600 nm reaches 0.3,arrest the growth by chilling on ice for 20 minutes. Transfer the culture into sterile centrifuge tubes and centrifuge at 5000rpm for 10 minutes at 4°C. Discard the culture supernatant ,keep the tubes on ice and resuspend the bacterial pellet in 20ml of ice cold 0.1M $CaCl_2$ solution. Incubate on ice for 20 minutes and centrifuge at 5000rpm for 10 minutes at 4°C. Discard the supernatant and resuspend the pellet in 1ml of ice cold $CaCl_2$ solution. Make 5 aliquots of 125 µl in chilled 1.5ml vials.

2) Transformation

To one 125 µl of the competent cell aliquot ,add 10 µl of isolated plasmid sample,tap and mix the contents, label as TC.Take another 125 µl of competent cell aliquot and label as CC. Incubate the vials (both TC and CC) on ice for 20 minutes. Tap gently and keep the tubes in water bath adjusted to 42°C for 2 minutes,immediately chill on ice for 10 minutes. Add 0.5ml of LB aseptically to both TC and CC and incubate for 60 minutes at 37°C in shaker incubator. Pour plate 100 µl and 150 µl of the transformation culture (TC) to LB agar with ampicillin. Similarly pour plate 100 µl of untransformed competent cells(CC) into LB agar with ampicillin and also LB agar without ampicillin. Incubate the plates overnight at 37°C and observe the results.

I. Screening Of Positive Clones

The transformed cells can selected by isolating the plasmid present in the E.coli host, which is then used to run PCR so as to amplify the PA-IL gene if present in the transformed cells.Further the presence of transformed clones can be confirmed by electrophorescing on agarose gel .The procedure for plasmid isolation and PCR is as given above.

J. Transformation In Expression Host

1) Competent cell preparation

Pick an isolated colony of competent cell strain of E.coli BL-21 from a master plate and inoculate into 5ml LB broth. Incubate in a shaker incubator at 200 rpm at 37°C overnight. Inoculate the 3ml of overnight culture to 100ml LB broth and incubate in ashaker incubator. When the optical density at 600 nm reaches 0.3,arrest the growth by chilling on ice for 20 minutes. Transfer the culture into sterile centrifuge tubes and centrifuge at 5000rpm for 10 minutes at 4°C. Discard the culture supernatant ,keep the tubes on ice and resuspend the bacterial pellet in 20ml of ice cold 0.1M $CaCl_2$ solution. Incubate on ice for 20 minutes and centrifuge at 5000rpm for 10 minutes at 4°C. Discard the supernatant and resuspend the pellet in 1ml of ice cold $CaCl_2$ solution. Make 5 aliquots of 125 µl in chilled 1.5ml vials.

2) Transformation

To one 125 µl of the competent cell aliquot ,add 10 µl of isolated plasmid sample,tap and mix the contents, label as TC.Take another 125 µl of competent cell aliquot and label as CC. Incubate the vials (both TC and CC) on ice for 20 minutes. Tap gently and keep the tubes in water bath adjusted to 42°C for 2 minutes,immediately chill on ice for 10 minutes. Add 0.5ml of LB aseptically to both TC and CC andincubate for 60 minutes at 37°C in shaker incubator. Pour plate 100 µl and 150 µl of the transformation culture (TC) to LB agar with ampicillin. Similarly pour plate 100 µl of untransformed competent cells(CC) into LB agar with ampicillin and also LB agar without ampicillin. Incubate the plates overnight at 37°C and observe the results.

K. Gst Induction And Purification

Gst Induction Inoculate an isolated colony from transformed LB ampicillin plates to 5ml of LB broth.
Incubate overnight at 37°C in a shaker incubator at 130 rpm. Reincubate 2ml of overnight culture into 100 ml of LB ampicillin broth in a 1000ml conical flask. Incubate in a shaker incubator at 130 rpm till optical density at 600 nm reaches 0.5. Draw 5ml of culture to sterile test tubes and label as before induction(BI) sample. Add IPTG to remaining culture broth to the final concentration of 0.1mM and mark as after induction (AI) sample. Incubate both BI and AI samples in a shaker incubator for # hours at 30°C at 220rpm. Transfer 5ml of AI sample to sterile test tube and label as AI .Cell Lysis Take 4ml of BI and AI cultures in a sterile eppendorff vial and centrifuge at 8000rpm for 10 minutes.Discard the supernatant. Resuspend the pellet in 150 µl of extraction buffer. Add 250 µl of sample loading buffer to BI and AI cell suspensions and mix gently. Boil the BI and AI to 70°C-100°C in a water bath for 20 minutes. Centrifuge the BI and AI samples at 6000 to 8000 rpm for 10 minutes.Transfer the supernatant into fresh vials and label them respectively and use them for SDS-PAGE.

L. Fast Protein Liquid Chromatography

Prepare buffers for chromatography and vacuum filter/de-gas using 1L Corning bottle and bottletop filter (0.2 µm). Turn on power to AKTA-Prime, place both solvent lines (A and B) into filtered dH2O and perform a system wash (i.e. Template→Application template→System wash). Attach column to AKTA-Prime (injection valve #1 to column inlet and column outlet to UV monitor flow cell). Wash column with 5-10 column volumes of filtered dH2O (flow rate = 5 mL/min for Hi-Trap columns or 2 mL/min for homemade low pressure columns). Place solvent line A into filtered sample buffer and line B into gradient buffer (e.g. 0.5 M imidazole). Purge solvent lines by doing a System Wash (i.e. Template→Application template→System wash).
Wash column with 5 column volumes of gradient buffer (B) followed by 10 volumes of sample buffer (A) at a flow rate of 5 ml/min for Hi-Trap column.
Set-up large-sized Sample Loop (150 mL):
Connect upper end piece of sample loop to injection valve position #6 and lower end piece to injection valve #2. Fill upper chamber of sample loop with buffer and insert end piece (exclude air) and tighten by screwing outer end piece into place. Fill lower chamber with small volume of buffer (exclude air) and insert end piece. Attach an appropriate syringe (filled with sample) at injection valve position #3, select LOAD position on injection valve (see below), and transfer sample from syringe into sample loop. Apply sample to column by selecting INJECT position on injection valve and apply flow through sample loop and onto column at flow rate of 2 mL/min. Wash column with 5-10 column volumes of sample buffer (A) at 5 ml/min until. Elute protein using a gradient (Manual Run)

M. SDS-PAGE

Clean the glass plates with methanol and make them grease free. Clamp the plates and make sure they are leak proof. Pour 12% resolving gel in between the glass plates. Allow the gel to polymerise and wash the top of the gel with buthanol wipe using a filter paper. 5% stacking gel is then poured over the resolving gel and the comb is inserted immediately and allowed to polymerise. Meanwhile , the samples(30µl sample + 20µl 1X gel loading buffer) are denatured at 80°C in water b ath for 5 minutes. After polymerisation,remove the comb and the basal spacer.Clean both basal spacerand wells with filter paper dipped in methanol. Place the plates in the electrophoresis unit with buffer and load 50 µl of the processed protein sample into the wells.
Electrophoresce the sample till the dye reaches the base of the gel. Dissect the gel into two parts,subject one part to commassie brilliant blue staining as in SDS-PAGE.

III. RESULTS AND DISCUSSION

The various protocols for PA-IL gene cloning ,expression and purification of the protein using FPLC was carried out. The results obtained are as follows

A. GENOMIC DNA ISOLATION:

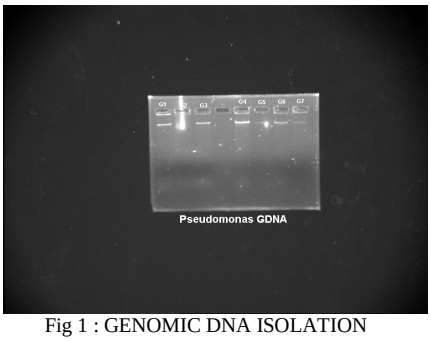

Fig 1 : GENOMIC DNA ISOLATION

Genomic DNA was isolated from Pseudomonas aerugnosa and it was run on agarose gel.UV transilluminator was used to easily visualize the bands.Thick bands was observed in G1,G3,G4 and G6.Whereas the the other wells had only faint bands.Thick bands indicate the presence of genomic DNA.Thus,G1,G3,G4 and G6 has sufficient amount of genomic DNA which are used in further studies.

B. PLASMID DNA ISOLATION:

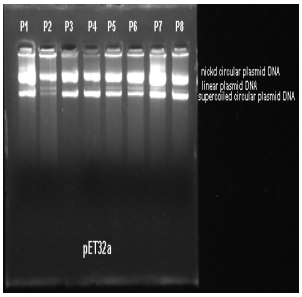

Fig 2 : PLASMID DNA ISOLATION

Isolated plasmid DNA in eight vials was loaded in the wells of agarose gel.After running the gel electrophoresis for about 45 minutes the bands obtaine was observed under UV transilluminator.Three distinct bands was observed.The three bands are nicked circular plasmid DNA ,linear plasmid DNA and supercoiled circular plasmid DNA respectively.Due to different speed of the three different plasmids they separate at different distances as shown in the diagram above.

C. GENE ISOLATION USING PCR:

The isolated genomic DNA underwent PCR to amplify the Lec A gene coding for lectin protein.PCR products was run on agarose gel and the bands was obtained as shown above.DNA marker was also runned along with the PCR products as shown above. A clear distinct band is observed in all four wells (PCR1,PCR2,PCR3 and PCR4).It was also observed that the band has travelled till 369 bp of the DNA marker.This confirms that the Lec A gene was isolated and amplified as the band is thick.

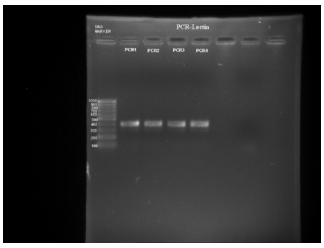

Fig 3 :GENE ISOLATION USING PCR

D. RESTRICTION DIGESTION:

Isolated plasmid was subjected to restriction digestion and it was confirmed by running on agarose gel.Three samples of the plasmid was loaded on the gel and allowed to run.The bands obtained was visualized using UV-transilluminator.Native plasmid (NP) was also runned along with the plasmid samples.The bands obtained for PL1,PL2 and PL3 was compared that of NP.By this comparison it was confirmed that onlt linear DNA is obtained .Thus it indicates that the plasmid has been restriction digested to linear plasmid.Now this linear plasmid can be used for insertion of Lec A gene.

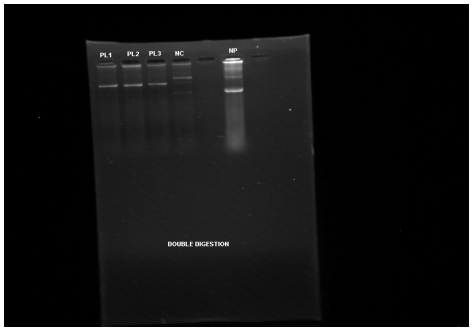

Fig 4:RESTICTION DIGESTION

E. TRANSFORMATION:

The control plate is just the agar medium itself.Since there is no growth in the medium it is inferred that there is no overall contamination due to environment and equipment handling method.

Fig 5 : CONTROL PLATE

F. F.COMPETENT CELLS GROWN IN MEDIA WITHOUT AMPICILLIN:

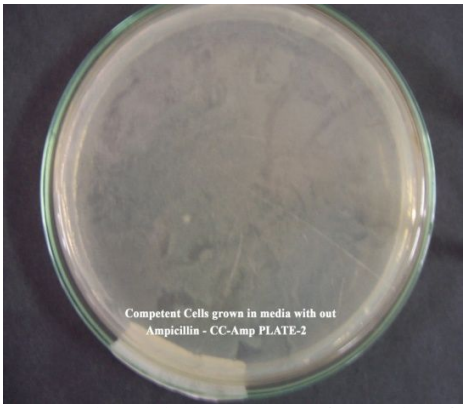

Fig 6 : COMPETENT CELLS GROWN IN MEDIA WITHOUT AMPICILLIN

Competent cells do not have the ampicillin resistant gene ,thus, when it is grown in media without ampicillin the competent cells are expected to grown evenly over the surface of agar.This is observed in the picture above.This indicates that only competent cells grow in the media and there is no external contamination.

G. G.TRANSFORMED CELLS GROWN ON MEDIA WITH AMPICILLIN:

Transformed cells have the ampicillin resistant gene .The picture shows that there are colonies of transformed cells growing on the media.It is confirmed that only transformed cells are growing on the media as only transformed cells can survive in a media rich in ampicillin.

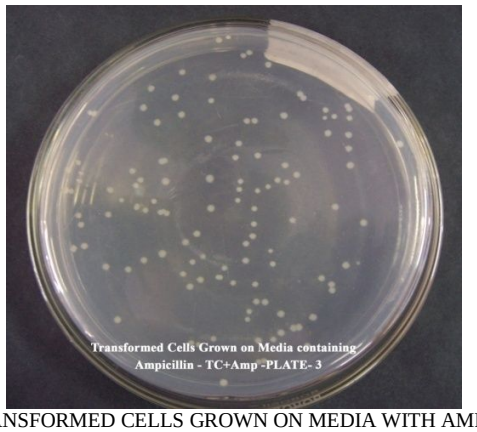

Fig 7: TRANSFORMED CELLS GROWN ON MEDIA WITH AMPICILLIN

H. H. FAST PROTEIN LIQUID CHROMATOGRAPHY

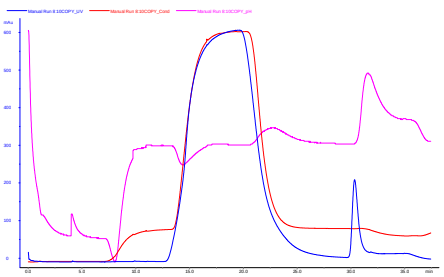

Fig 6 :FPLC CHROMATOGRAM

The above is the chromatogram for FPLC.Two peaks are shown by the pink graph .The first peak represents the proteins in the host cell being eluted out after about 20 minutes after the start of the operation of the column.The second peak represents the lectin protein being eluted out after 30 minutes of start of operation.About 150ml of lectin is obtained.

I. SDS-PAGE:

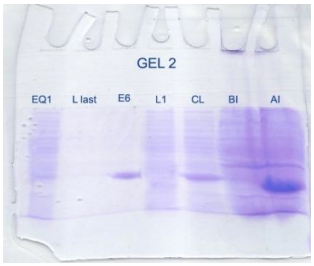

Fig 7 : SDS-PAGE

IV. CONCLUSION

Thus the Lec A gene from Pseudomonas aeruginosa have been isolated and cloned in the cloning host E.coli DH-5α. Positive clones of the transformed cells have been selected and the plasmid isolated. This plasmid have been transformed into the expression host E.coli BL-21. Lec A gene have been induced and over expressed using IPTG. Over produced lectin protein have been purified using FPLC and it was detected using SDS-PAGE. Lectin protein can be used to produce subunit vaccine which can be used to treat many diseases caused by Pseudomonas aeruginosa. Production of subunit vaccine is the future aspect of this project.

REFERENCES

[1] Anne Imberty , MichaelaWimmerová , Edward Mitchell P. , Nechama Gilboa-Garber (2004) "Structures of the lectins from Pseudomonas aeruginosa: insights into the molecular basis for host glycan recognition", Microbes and Infection Vol.6,pp. 221–228.

[2] Bruno Miroux and John Walker E. (1996) "Over-production of Proteins in Escherichia coli: Mutant Hosts that Allow Synthesis of some Membrane Proteins and Globular Proteins at High Levels", J. Molecular Biology ,pp. 289–298.

[3] Chanez Chemani, Anne Imberty, Sophie de Bentzmann, Maud Pierre, Michaela Wimmerová, Benoît Guery P. and Karine Faure (2009) "Role of LecA and LecB Lectins in *Pseudomonas aeruginosa*-Induced Lung Injury and Effect of Carbohydrate Ligands", Infection and Immunity Vol. 77, no. 5,pp. 2065-2075.

[4] Chris Duronio (2012) "Production of a Recombinant Vector to Enable the Study of Thioredoxin Function as a Bound or Detached Solubilizer of Proteinase Inhibitor 2 in a Bacterial Protein Overexpression System", Journal of Experimental Microbiology and Immunology Vol. 16,pp. 79 – 84.

[5] Clara Restrepo I. , Qun Dong, Jordan Savov, William Mariencheck I. and Jo Rae Wright (2002) "Surfactant Protein D Stimulates Phagocytosis of Pseudomonas aeruginosa by Alveolar Macrophages", Am. J. Respir. Cell Mol. Biol. Vol. 21, pp. 576–585.

[6] David Sheehan and Siobhan Sullivan (2004) "Fast Protein Liquid Chromatography", Methods in Molecular Biology Vol 244,pp. 253-258.

[7] Denis Tielker, Stephanie Hacker, Remy Loris, Martin Strathmann ,Jost Wingender, ,Susanne Wilhelm, Frank Rosenau and Karl-Erich Jaeger (2005) "Pseudomonas aeruginosa lectin LecB is located in the outer membrane and is involved in biofilm formation", Microbiology Vol 151,pp. 1313–1323.

[8] Diggle S. P., Stacey R. E., Dodd C., Cámara M., Williams P. and Winzer K. (2006) " The galactophilic lectin, LecA, contributes to biofilm development in *Pseudomonas aeruginosa*", Environmental Microbiology Vol 8,pp. 1095–1104.

[9] Gerald Bodey P., Ricardo Bolivar, Victor Fainstein, and Leena Jadeja (2000) "Infections Caused by *Pseudomonas aeruginosa"*, Oxford Journals, Clinical Infectious Diseases Vol. 5, Issue 2,pp. 279-313.

[10] Licheng Wu, Oscar Estrada, Olga Zaborina, Manjeet Bains, Le Shen, Jonathan Kohler E., Nachiket Patel, Mark Musch W., Eugene Chang B., Yang-Xin Fu, Michael Jacobs A., Michael Nishimura I., Robert Hancock E. W., Jerrold Turner R. and John Alverdy C. (2005) "Recognition of Host Immune Activation by Pseudomonas aeruginosa", Science Vol 309,pp. 774-777.

[11] Licheng Wu,Christopher Holbrook, Olga Zaborina, Emelia Ploplys, Flavio Rocha, Daniel Pelham, Eugene Chang, Mark Musch, and John Alverdy (2003) "*Pseudomonas aeruginosa* Expresses a Lethal Virulence Determinant, the PA-I Lectin/Adhesin, in the Intestinal Tract of a Stressed Host", Annals of Surgery Vol. 238(5),pp. 754–764.

[12] Marbach A and Bettenbrock K (2012) "lac operon induction in Escherichia coli: Systematic comparison of IPTG and TMG induction and influence of the transacetylase LacA", J Biotechnol Vol.157(1),pp.82-88.

[13] Michaela Wimmerova, Edward P. Mitchell and Nechama Gilboa-Garber (2004) "Structures of the lectins from Pseudomonas aeruginosa: insights into the molecular basis for host glycan recognition", Microbes and Infection Vol 6, Issue 2, pp. 221–228.

[14] Gilboa-Garber N. (1997) "Multiple aspects of Pseudomonas aeruginosa lectins", Nova Acta Leopoldina NF 75 301, pp. 153–177.

[15] Gilboa-Garber N., Mizrahi L. and Garber N. (1980) "Purification of the galactose-binding hemagglutinin of Pseudomonas aeruginosa by affinity column chromatography using sepharose", FEBS Lett Vol.28,pp.93-95.

[16] Notani N. K., Joshi V. P. and. Kanade R. P. (1990) "Genetic transformation in bacteria", Journal of Bioscience Vol. 6, Number 4,pp. 525–533.