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Research Article

MOLECULAR CLONING, EXPRESSION AND FPLC PURIFICATION OF LECTIN A FROM *PSEUDOMONAS AERUGINOSA*

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Abstract

Pseudomonas aeruginosa (PA) as an opportunistic pathogen infects the pulmonary tract, bladder, cystic fibrosis patients and burn victims. PA infections treatment is challenging because of its ability to rapidly develop resistance to multiple classes of antibiotics. Lectin is protein that is expressed in cell of PA and cause of infection by attaching to the host cells. Lectin A gene coding lectin protein so we cloned and expressed this gene then purified of related protein, that can be used in preparation of vaccine to treat PA infections.

Keywords: Molecular Cloning; Lectin A; *Pseudomonas aeruginosa*

Introduction

Pseudomonas aeruginosa is a Gram-negative rod-shaped bacterium that grows in soil, marshes and coastal marine habitats, as well on plants and animal tissues (Poole, 2004). It forms biofilms on wet surfaces such as those of rocks and soil (Walker *et al.*, 2004). The emergence of *P. aeruginosa* as a major opportunistic human pathogen during the past century may be a consequence of its resistance to the antibiotics and disinfectants that eliminate other environmental bacteria. *P. aeruginosa* is now a significant source of bacteremia in burn victims, urinary-tract infections and hospital-acquired pneumonia in patients. It is also the predominant cause of morbidity and mortality in cystic fibrosis patients (D'argenio *et al.*, 2001; Miyata *et al.*, 2003; Cornelis, 2008).

P. aeruginosa is naturally resistant to a large range of antibiotics and may demonstrate additional resistance after unsuccessful treatment (Mcvay *et al.*, 2007), in particular, through modification of a porin. It should usually be possible to guide treatment according to laboratory sensitivities, rather than choosing an antibiotic empirically (Wright *et al.*, 2009). If antibiotics are started empirically, then every effort should be made to obtain cultures, and the choice of antibiotic used should be reviewed when the culture results are available (Nadeem *et al.*, 2009a; Nadeem *et al.*, 2009b).

Lectins are a type of receptor protein of non-immune origin that specifically interacts with sugar molecules (carbohydrates) without modifying them. These proteins recognize and bind specifically to monosaccharides and are classified by which sugar they recognize. While the function of lectins in plants is believed to be the binding of glycoproteins on the surface of cells, the role in animals also includes the binding of soluble extracellular and intercellular glycoproteins (Diggle *et al.*, 2006). For example, there are lectins found on the surface of mammalian liver cells that specifically recognize galactose residues. It is believed that these cell-surface receptors are responsible for the removal of certain glycoproteins from the circulatory system (Bajolet-Laudinat *et al.*, 1994; Imberty *et al.*, 2004).

Pseudomonas aeruginosa DNA fragment contains the structural gene coding for the galactophilic PA-1 lectin (pa-1L , 369 bp) that can be used as a subunit vaccine (Sudakevitz *et al.*, 2004).

Materials and Methods

Materials

Plasmid isolation

Overnight grown bacterial Culture (pGEX-3X), Solution I, Solution II, Solution III, Chilled ethanol, 70% ethanol, 1X TE Buffer, Antibiotic (Ampicillin), LB Broth, 0.8% Agarose

Genomic DNA isolation

Overnight grown bacterial Culture (*Pseudomonas aeruginosa*), LB Broth, 10% SDS, 1X TE Buffer (10mM Tris HCl +1mM EDTA), 5M Potassium acetate, RNase A enzyme, Isopropanol, 70% ethanol, 1X TE Buffer, 0.1X TE Buffer, 0.8% Agarose

Agarose gel electrophoresis

0.8% Agarose, 1X TAE Buffer, Gel loading dye, Ethidium bromide, Samples (Genomic DNA & Plasmid DNA)

Polymerase chain reaction

Double distilled water, Buffer, dNTP's, Samples (Genomic DNA), Forward primer, Reverse primer, Taq polymerase

Restriction digestion

Sterile distilled water, NEB buffer 4, Samples (PCR Product & Plasmid DNA), Enzyme 1 (Bam H1), Enzyme 2 (E.CoR1), 1% Agarose.

Ligation

Samples (Insert & Vector), Sterile distilled water, T4 DNA Ligase Buffer, T4 DNA Ligase

Transformation

Samples (Insert & Vector), Competent cell strain, Sterile distilled water, L B Broth, 0.1 M CaCl2, Ampicillin 50 mg/ml

Polymerase chain reaction Screening

Samples (Cloned pGEX, native pGEX & *Pseudomonas aeruginosa*), Double distilled water, Buffer, dNTP's, Forward primer, Reverse primer, Taq polymerase

GST tag protein induction

Bacterial culture bearing GST gene and ampicillin resistant gene, LB broth, Ampicillin, 0.1mM IPTG, Extraction buffer, 140mM Nacl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, Alumina, Triton x 100, sample loading buffer, 50mM Tris HCl(pH- 6.8), 2%SDS, 0.1%Bromophenol blue, 10%Glycerol, 100mM 2-mercapthoethanol

Purification of GST tag protein (Lec A)

Column –Glutathione CL agarose, Sample load –cell lysate-18 ml

Equilibration buffer (pH-7.4)

15mM NaH_2PO_4, 0.15mM Nacl

Elution buffer (pH-8)

50mM tris HCl, Reduced glutathione (152mg for 100ml elution buffer)

SDS PAGE

***10% Resolving Gel Reaction Mixture (5ml)*:** Double distilled water- 2.10ml,30% Acrylamide mix- 1.78ml, 1.5M Tris (pH 8.8)- 1.35ml, 10%SDS- 53µl, 10%APS- 53µl, TEMED- 10µl were used.

5% Stacking Gel Reaction Mixture (2.5ml): Double distilled water- 1.7ml, 30% Acrylamide mix- 0.42ml, 1.5M Tris(pH 8.8)- 0.32ml, 10%SDS- 20µl,10%APS- 25µl, TEMED- 15µl was taken.

Protein samples –purified GST, BI, AI, cell lysate, standard marker were used. 100ml of staining solution contained Methanol-50ml, Glacial acetic acid-10ml**,** Water-40ml and Commassie brilliant blue -200mg. Similarly, 100ml of destaining solution was prepared by using Methanol-50ml, Glacial acetic acid-10ml, Water- 40ml.

Methods

Plasmid DNA Isolation

The E.coli culture containing **pGEX-3X i**s centrifuged first. This is done to sediment and concentrates the bacterial cells and pellets. The pellets are then suspended in ice cold solution I. Solution I contains glucose which acts on an osmoticum, TRIS-HCL buffering agent which maintains stable pH conditions and EDTA which acts as a chelating agent, that is EDTA removes Mg^{2+} ions from the cell wall most effectively. Mg^{2+} ions help in the rigidity of the cell wall and hence removal will loosen the cell wall. Net freshly prepared solution II was added, which contains SDS and NaOH. The SDS removes lipid molecules from the cell membrane and cause cell-wall degradation. The NaOH acts as a strong base, increasing pH sharply causing the chromosomal and plasmid DNA to denature. Hence this method is referred to as alkali lysis method. After the ice cold solution III is added, acetic acid brings about a pH drop. At the lower pH the plasmid DNA undergoes renaturation and chromosomal DNA and protein undergo precipitation. Potassium acetate reacts with SDS to give KDS which is a heavy precipitate and also a flocculant. Renaturation and precipitation takes place only in ice cold conditions. Next centrifugation is carried out to remove all cell debris. RNase is added to remove the RNA content. Chilled absolute alcohol, a dehydrating agent is added to precipitate DNA. Following this, the vials are centrifuged to sediment the DNA and the pellets so obtained are washed with 70% ethanol again by centrifugation. This is done to remove the excess salts and impurities. It is also dehydrates the DNA. The pellets are then suspended in TE buffer after air drying. This maintains the pH and prevents the DNase activity.

Genomic DNA Isolation

Pseudomonas aeruginosa was cultured in 2% LB broth overnight at 37°C, then harvested and centrifuged in a 1.5ml microfuge tube at 5000 rpm for 5 min to pellet the cells . After re suspending the pellet in 1X TE buffer (575µl) and adding 10% SDS (150µl) , the cells were mixed thoroughly and incubated at 37°C for 1hr. Afterward, potassium acetate (150µl) was added, mixed gently and incubated for 15 min on ice. The mixture is centrifuged at 10,000 rpm for 10 min and then the supernatant was transferred in to a fresh vial. After adding RNase A(1µl) and incubating at 37°C for 30 min, isopropanol (0.6ml) was added and mixed well and precipitated at -20°C for 20 min. The mixture was centrifuged for 8 min, at 12,000 rpm and at 4°C, the supernatant was discarded, 70% ethanol (400µl) was added and centrifuged for 5 min at 12,000 rpm at 4°C. After discarding the supernatant, the pellet was dried on a tissue paper. The achieved pellet was then dissolved in TE buffer

(40µl) and kept at 4°C. Electrophoresis was performed on a 0.8% agarose gel to observe the DNA bands.

Polymerase Chain Reaction

PCR reaction mixture was prepared with water- 16.6 µl, Buffer- 2.5 µl, dNTP's- 1.0 µl, Forward primer- 1.0 µl, Reverse primer- 1.0 µl, DNA (lectin PA1L)- 2.5 µl and Taq Polymerase- 0.4 µl. The thermal profile for PCR contained initial denaturation at 94 ºC for 2 minute, denaturation at 93 ºC for 1 minute, annealing at 45 ºC for 1 minute, extension at 72 ºC for 30 seconds and Go to for 2 cycles; thereafter denaturation at 93 ºC for 1 minute, annealing at 65 ºC for 1 minute, extension at 72 ºC for 30 seconds, Go to 19 cycles, final extension at 72 ºC for 3 minutes and 4 ºC for Forever. The samples were electrophoresed and electrophoresis was performed on 1.2% agarose gel and samples were visualized under UV Transilluminator.

Purification and Concentration of PCR product

Samples were divided in 2 vials and after adding double volume of chilled alcohol the samples were kept in for 2 hours at -20 ºC. The samples were centrifuged for 10 minutes, at 12,000 rpm and 4 ºC. In the next step, supernatant was removed and 70% ethanol (400 µl) was added to the pellet, centrifuged for 5 minutes at 5,000 rpm and 4 ºC. Supernatant was removed and achieved pellet was completely air-dried and then dissolved in 0.1 X TE buffer (50 µl) and kept at 4 ºC.

Restriction digestion

Table 1 shows the components used for restriction digestions to digest plasmid DNA and PCR product utilizing E.CoR1and Bam H1.

The vials were incubated at 37 ºC for 1 hour and followed by heat inactivation for 10 min at 80ºC in a water bath and for 5 min in fridge. Electrophoresis was performed on 1% agarose gel and samples were visualized under UV Transilluminator. E.CoR1 and Bam H1 will lead to linearization of the vector pGEX as all the nicked and supercoiled forms are converted to linear form.

Expression of Lec A gene in E. coli utilizing an expression vector

A prokaryotic expression vector (pET 32-a, Novagen) was utilized to sub-clone the genes. Bam HI and Eco RI restriction enzymes were used to digest PCR amplified DNA fragment and the vector (sites included in primers). The complete restriction was confirmed by electrophoresis. The ligation reaction with 1:3 molar ratio was carried out, the ligation mixture components were incubated for 12 hours at 16°C, transformed to E. coli BL 21, plated on Luria agar comprising Amp (100 mg/ml) and finally incubated for 12 hours at 37°C. The recombinants were verified via Colony PCR.

GST INDUCTION

Formed Colonies in transformed LB ampicillin plates were inoculated an isolated to 5ml of LB ampicillin broth and incubated at 37°C overnight and at 130rpm in a shaker incubator. Next, 2ml of the overnight culture re incubated into 100 ml conical flask and in a shaker incubator at 130rpm to achieve the O.D 0.5 at 600nm. 5ml of culture was taken and labeled as before induction sample. IPTG was added to the remaining culture broth to achieve 0.1mM and marked as after induction sample. Both samples were incubated at 220 rpm overnight. 5ml of both cultures were centrifuged for 10 min at 8000 rpm, after discarding the supernatant, the pellet was re suspended in extraction buffer (150 µl), gel loading dye (20 µl) was added to the cell suspensions and gently mixed. Both samples were centrifuged for 10 min at 10000rpm.They could be directly utilized for PAGE or stored at 4º C.

In the other Cell lysis procedure, Cells were pelleted for 10 min at 5,000 rpm at 4°C. After discarding the supernatant, tubes containing the cells were weighed and pellet was transferred in to a chilled mortar and the tubes were weighted again. Each presented pellet was transferred to the mentioned chilled mortar and alumina (2.5x) and equilibration buffer (16.4ml) were added. After transferring the whole contents to a fresh tube and centrifuging at 5000rpm for 50 min, transferring the supernatant in to other fresh tubes and adding triton x 100, the contents were again centrifuged for 50 min at 5000 rpm. The achieved supernatant was decanted into a fresh sterile tube and then stored at 4°C.

Table 1: Components used for restriction digestion

	pGEX (µl)	**PCR Product** (µl)	**pGEX**(-ve) (µl)	**PCR**(-ve) (µl)
Water	34.4	34.4	35.0	40.0
Buffer	5.0	5.0	5.0	5.0
DNA	10.0	10.0	10.0	5.0
BamH1	0.4	0.4	---	----
E.CoR1	0.2	0.2	---	----

Expression of GST tagged protein (Lec A)

Plasmid containing isopropyl beta Thiogalactoside (IPTG) inducible promoters are capable of expressing proteins at level that exceed 30%of total cell mass of bacteria proteins. Expression of inserts cloned in to a pGEX vector is under the control of IPTG inducible lac promoter. This IPTG – inducible expression vector carries a lac I^q allele. The lac I gene product is a repressor protein that binds to the operator region of the lac promoter preventing the expression until the induction by IPTG, thus maintaining tight control over the expression of the insert .The concentration of IPTG to induce lac repressor regulated promoters can dramatically influence the expression . Glutathione-S-Transferase tag protein should be visible 30 min after the induction at 37° C. The amount of GST tag protein should increase through the induction.

Purification of GST tag protein (Lec A)

sCell lysate was applied to an immobilized Glutathione CL agarose column and the sample was allowed to flow through the gel bed completely. The samples were collected and labeled as washed and flow through, the column was also washed with equilibration buffer to achieve O.D reaches 0.05. Elution was carried out using freshly prepared elution buffer and monitored by absorbance at 280nm. In the last step, the eluted GST fusion protein was assayed using SDS PAGE.

Results and Discussion

Agarose gel showing pGEX-3X bands

The super coiled form is the most condensed form of the plasmid and can traverse through the pores in the gel most easily in comparison to the linearised and nicked forms. The linear form exists in two alternating conformation; linear and coiled. Since the nicked plasmid is cut only on one strand, it cannot coil as much as the linearised form. Hence it is the slowest moving form of plasmid.

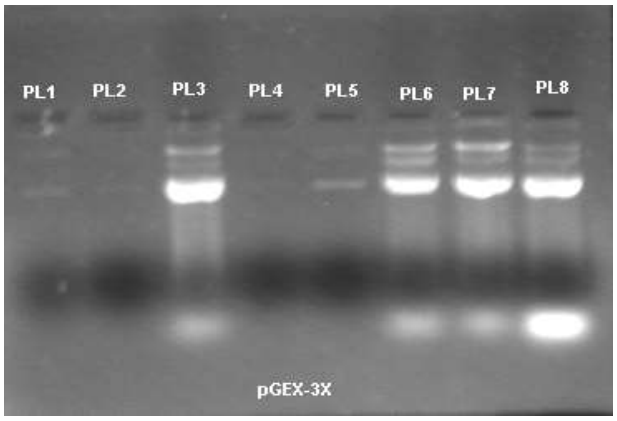

Fig 1: Agarose gel photograph of pGEX-3X plasmid DNA isolated from E.coli using the alkali lysis method.

The **pGEX-3X** plasmid DNA was isolated from E.coli using the alkali lysis method. The pellet was seen adhering to the vial. This was then further analyzed on agarose gel. On electrophoresis bright bands were observed for only PL3, PL6, and PL7 & PL8 under UV Transilluminator as shown in the Fig. 1.

Genomic DNA Isolation

Genomic DNA was isolated from E.coli. A white creamy pellet was formed in the sample vial.

This was further analysed on agarose gel. On electrophoresis a thick bright band of DNA was observed for all three G1, G2, G3, G4 and G6 except G7 in which a faint band was observed under UV Transilluminator as shown in Fig 2.

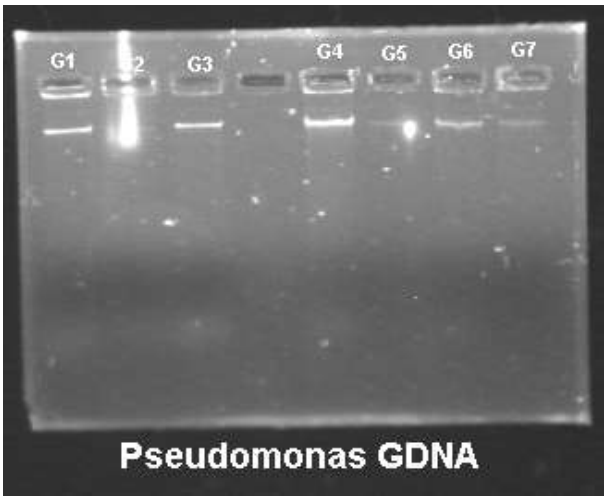

Fig 2: Agarose gel showing *Pseudomonas* DNA bands

The bacterial pellet obtained after centrifugation is suspended in TE buffer. The EDTA present chelates the Mg^{2+} ions and TRIS-HCL regulates pH. The SDS added to this mixture disrupts the cell and releases the cellular contents onti the solution. The solution is then treated with potassium acetate. This reacts with SDS to form KDS which helps in precipitation an act as flocculant. The cell debris is removed through centrifugation and RNase is added to remove the RNA. Isopropanol is added to precipitate and renature the DNA. Rinsing with 70% alcohol is done by centrifugation to remove excess salts and rehydrate the DNA. The pellets are then suspended in TE buffer after air drying. This maintains the pH and prevents the DNase activity.

Polymerase Chain Reaction

On electrophoresis single bright band was seen corresponding to all four wells PCR1, PCR2, PCR3 and PCR4 indicating the amplification of the desired gene as shown in Fig 3. When compared with marker it was found to be in between 300 & 400 bp.

In pGEX, E.CoR1 is located near the promoter; so for PA1L gene (369bp) cloning E.CoR1 (TATCGGACGGGATCCATGGCTTGGAAAGGTGAG) was used as forward primer and Bam H1 (CGGAATTCTTATCAGGACTGATCCTTTCCAATA) was used as reverse primer.

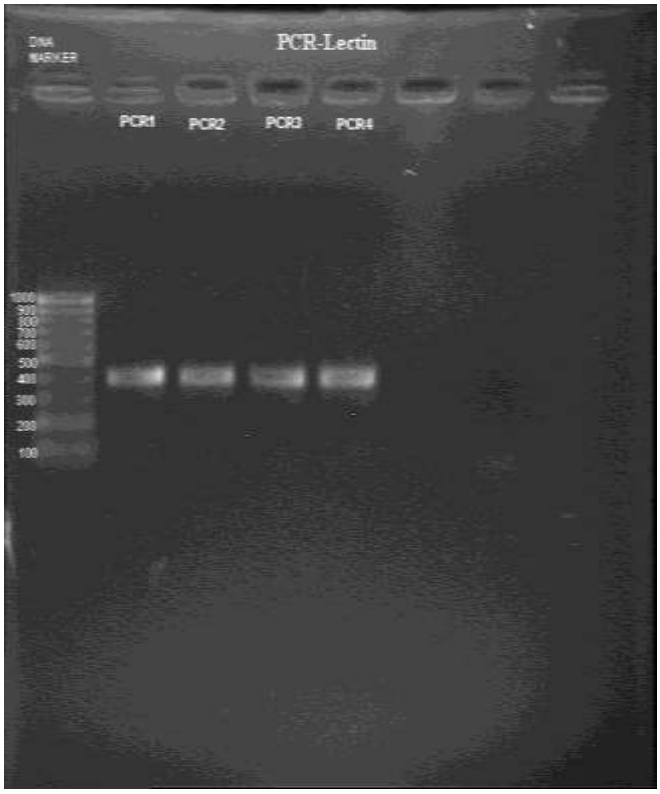

Fig 3: Agarose gel showing PCR amplified product of lectin gene

Restriction digestion

Restriction digestion of PCR product, PCRRD1, PCRRD2 and PCRRD3 can be seen as a band ahead of the negative control (NC) (Fig 4).

Restriction digestion of pGEX was seen as a single band corresponding to PL1,PL2,PL3 with the molecular weight in between linear and supercoiled form (more towards linearised) of native pGEX as shown in Fig. 5.

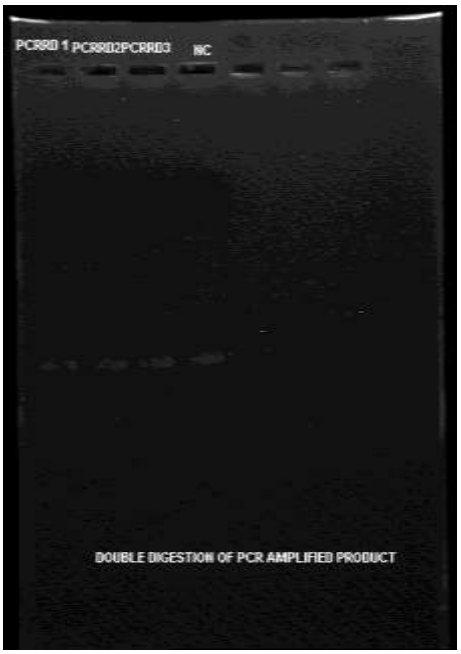

Fig. 4: Agarose gel showing digested lectin bands.

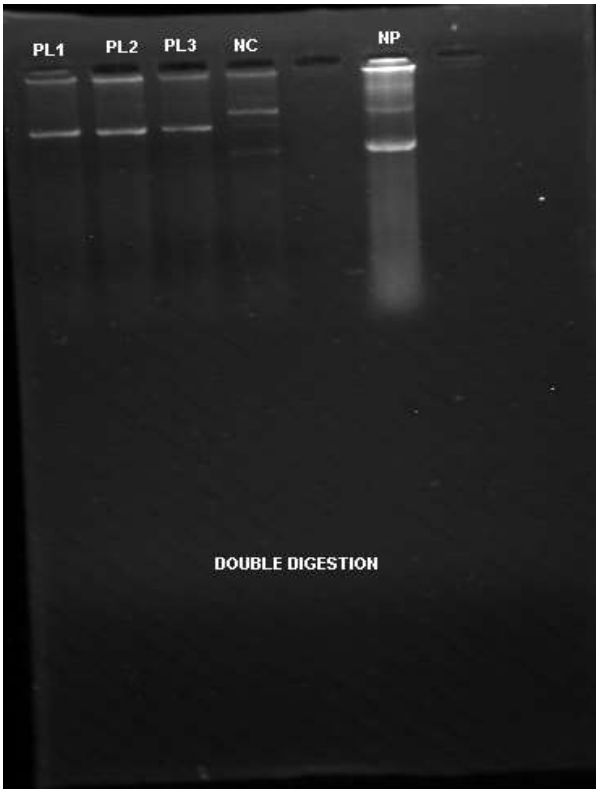

Fig 5: Agarose gel showing linearised pGEX 3x bands

Transformation

In transformation, the recombinant plasmid was transferred into a competent host cell, DH5 α and allowed to multiply. During the multiplication of the cells, the recombinant plasmid also will get multiplied and so that we can have enormous number of recombinant plasmid.

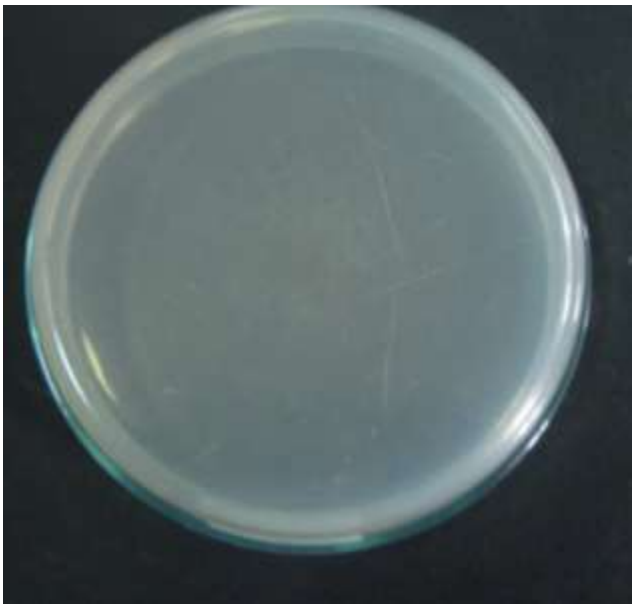

Fig 6: LB agar (control)

A control plate was prepared without any antibiotic and inoculum to ensure that the plating is done under aseptic conditions. As expected the plate did not show any growth after overnight incubation as shown in Fig. 6.

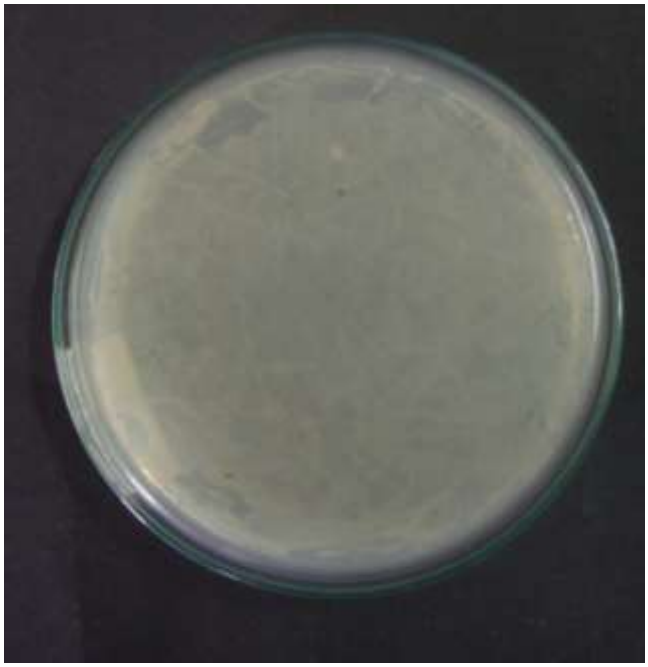

Fig 7: Competent cells in LB agar

Competent cells (100µl) are plated in LB agar to ensure its growth in the supplied conditions. As expected the plates showed crowded growth after overnight incubation (Fig. 7).

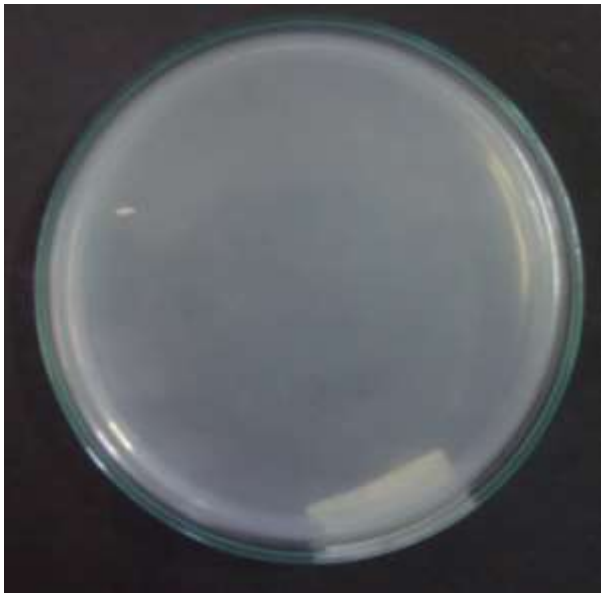

Fig 8: LB agar with Ampicillin showing no growth of competent cells

Competent cells (100µl) are plated into LB agar with Ampicillin (50mg/ml) and incubated overnight to ensure that whether it having any natural resistance to the Antibiotic, ampicillin. As expected there was no growth in the culture plates. From this it is clear that the natural competent cells cannot grow in ampicillin containing medium since it does not have ampicillin resistant gene (Fig. 8).

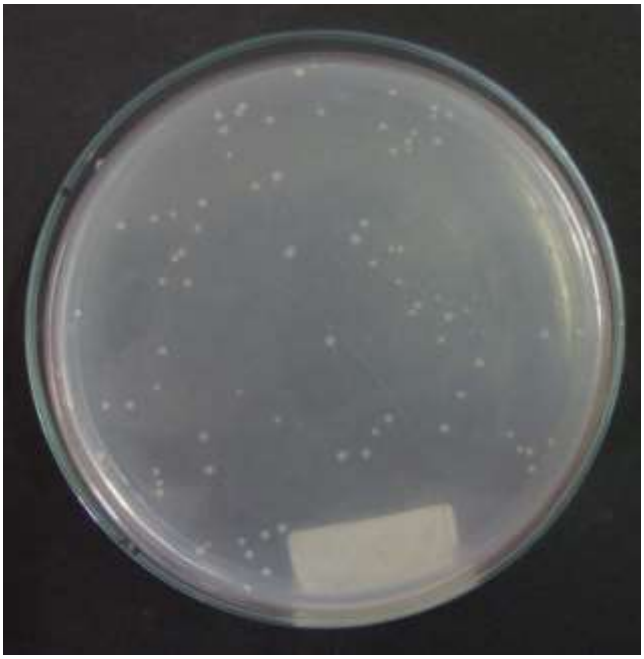

Fig 9: LB agar with Ampicillin showing transformed colonies

Growth was seen on the plates containing the transformed cells as they have gained resistance to the ampicillin present in the media (Fig. 9). The plasmid pGEX has been incorporated into the cell. It has the genes that code for β–lactamase that detoxify ampicillin. The plates containing non-transformed cells, but having ampicillin in its media did not show any growth since the cells are sensitive to the antibiotic. But the non-transformed cells plate and the media with no ampicillin showed growth, since there was no need for the cells to have resistance against any antibiotics.

Fig 10: PCR screening of the transformed colonies

PCR screening of clones (transformed pGEX-3X)

On electrophoresis a single band was observed for all the clones (C2, C4, C5) indicating the amplification of the desired gene. When compared with marker it was found to be in between 300 & 400 bp. It was also observed that the desired gene does not contain any flanking regions as a result the molecular weight is reduced and hence ahead of the genomic DNA amplification band, confirming that the clones contains the desired gene (Fig. 10).

Expression of the protein

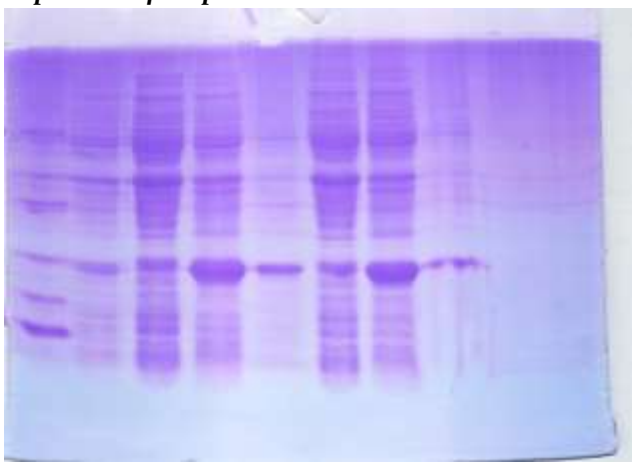

Fig 11: SDS gel showing protein bands

The purification of protein is done by affinity column chromatography.

Detection of purified protein by SDS:

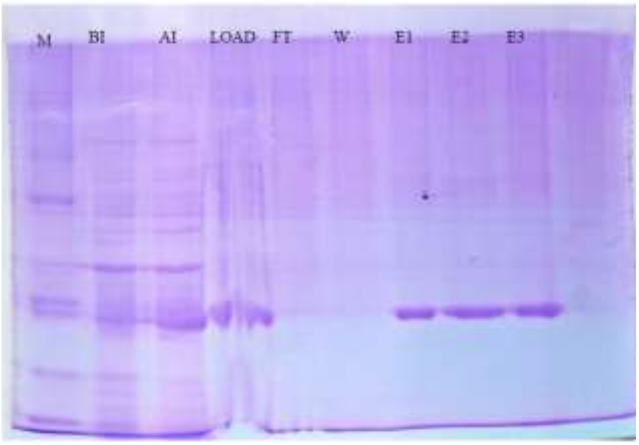

Fig 12: SDS gel showing protein bands.

M– Marker, BI– Before induction, AI– After induction, LOAD– Cell lysate, FT– Flow through, W –Wash, E1, E2, E3 –Elution samples at different concentrations

SDS PAGE is carried out to confirm whether our desired protein is present or not. The eluted samples showed bands corresponding to the molecular weight of the GST tagged protein (Fig. 11 and Fig. 12).

Fusion proteins expressed from pGEX vector contain a Glutathione-S-Transferase moiety (GST) and can therefore be purified to near homegencity by affinity chromatography on glutathione agarose. GST' is a class of enzymes that utilize glutathione (gamma-glutamylcysteinylglycine) as a substrate to inactivate small toxic molecules via the formation of mercapturic acids. Because the affinity of GST for its substrate is in the submillimolar range, immobilization of glutathione on an agarose matrix makes a highly efficient affinity chromatography resin .Bound GST fusion proteins are readily displaced from the column by elution with buffers containing free glutathione.

The chromatography column is equilibrated with equilibration buffer to remove unwanted protein sticking to the column .The cell lysate being run down the column filters the GST tag protein that bind to the glutathione. Repeated use of equilibration buffer removes all the other protein and when the O.D reaches 0.05 indicates that only GST tag protein is sticking to the column .The elution buffer with 38mg of reduced glutathione reduces the bonds between GST tag protein and glutathione beads and hence the eluted samples contain only the GST tag protein .The concentration of the GST tag protein reduces with the addition of elution buffer.80% of GST tag protein will be present in first three elution samples .The concentration of GST tag proteins can be estimated by measuring the absorbance at 280 nm.

Conclusion

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. As *Pseudomonas aeruginosa* is a very pathogenic organism the part of the genomic DNA coding for the galatophilcic lectin is isolated and maintained as a clone (Cioci *et al.*, 2003; Mewe *et al.*, 2005).

This study aim is focusing on the expression of Lectin A protein from *Pseudomonas aeruginosa* in E. coli bacteria. In this project, the Lec A gene was isolated and expressed in E. Coli utilizing an expression vector (pET-32a). The recombinant E. coli cells containing the Lec A gene have been then cultured in L.B media. The Lectin A protein generation has been induced by addition of inducer IPTG. The separation and purification of the Lectin A expressed protein was carried out using affinity chromatography and confirmed via SDS PAGE. The standard concentration of IPTG was shown by identification of maximum production of Lectin A protein. After that the rate of RPM, pH, temperature can be standardized to get maximum production of Lectin A protein. More studies are needed to distinguish the structure, physical and biochemical characteristics of Lectin A protein. These results are important for development of a safe *Pseudomonas aeruginosa* vaccine using recombinant DNA techniques, thus avoiding contamination with toxic products of this bacterium.

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