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Cloning, over-expression and purification of *Escherichia coli murC* encoding UDP-N-acetylmuramate--L-alanine ligase

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ABSTRACT
Gene *murC* encodes Uridine diphosphate N-acetylmuramate–L-alanine ligase, a 53 kDa enzyme involved in synthesizing the bacterial cell wall. The present study is designed to clone, overexpress and purify the recombinant murC using *Escherichia coli* as a model system. *murC* nucleotide sequence was obtained from the NCBI Gene database. The murC ORF from the genomic DNA of E. coli was amplified using primers containing restriction sites and a poly his-tag included in the pET 28b expression vector and was cloned into it. In E. coli BL21(DE3), the recombinant plasmid was transformed and overexpressed. The overexpressed recombinant His-tagged protein was purified using an affinity nickel column in a single chromatographic step. Since MurC ligases are a crucial enzyme involved in the synthesis of the bacterial cell wall and since it is absent in vertebrates including humans, this enzyme could be a desirable target for the development of antibacterial drugs. This method could be used for the production of MurC proteins that could help in identifying and screening small molecule inhibitors against pathogenic bacteria.
Keywords: Bacterial cell wall, Peptidoglycan, *murC*, Expression, purification, pET28b, His tag.
Abbreviations: bp, base pairs; kb, kilobases; kDa, kiloDalton; ORF, open reading frame; PCR, polymerase chain reaction; Kan, kanamycin; IPTG, isopropyl L-D-thiogalactopyranoside; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

1. INTRODUCTION
In both Gram-positive and Gram-negative bacteria, the cell wall polymers are made up of a peptidoglycan network that protects the cell against osmotic pressure[1]. A crucial element of the bacterial cell wall is peptidoglycan[2]. The peptidoglycan is a heteropolymer made up of glycan strands that have been crosslinked by peptides. Bacterial peptidoglycan is produced by a two-stage biosynthesis process. The production of the disaccharide peptide monomer unit constitutes the first stage, while the polymerization events and insertion of the freshly produced peptidoglycan material into the cell wall constitute the second stage. Various cytoplasmic and membrane processes, each very specialized, are used to assemble the peptidoglycan unit[3]. The first step in the route is the conversion of UDP-N-acetylglucosamine to UDP-N-acetylglucosamine-enol pyruvate, which can be thought of as the initial precursor. This process is carried out by the catalytic activity of UDP-N-acetylglucosamine enolpyruvyl transferase (MurA). The second step involves UDP-N-acetyl enol pyruvoyl glucosamine reductase (MurB), which converts the enolpyruvate moiety to D-lactate and produces UDP-N-acetylmuramate. A set of ATP-dependent amino acid (aa) ligases catalyze the successive attachment of the pentapeptide sidechain to the D-lactyl group of UDP-N-acetylmuramate (MurC, MurD, MurE, and MurF)[1].
The current evolution and dissemination of different resistance mechanisms among bacterial pathogens significantly reduces or eliminates the therapeutic effectiveness of antibiotics used in clinical settings. This makes it more important than ever to identify novel targets that can be suppressed. Due to their extraordinary specialization and unique occurrence in eubacteria, the amino acid ligases that build the peptide moiety of peptidoglycan are prospective targets of significant interest. The mechanism by which these enzymes work is the same: carboxyl activation of the nucleotide substrate to an acyl phosphate intermediate, followed by a nucleophilic assault by the

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amino group of the condensing amino acid, with the elimination of phosphate and peptide bond formation[4-7].

The murC gene coding for the L-alanine- adding enzyme has been identified on the chromosome of E. coli, in a large cluster of genes between pbpB and envA that code for proteins involved in peptidoglycan biosynthesis or cell division. The nucleotide sequence of murC has been determined. It contains 1473 nucleotides which are translated into 491 amino acids corresponding to a protein with a molecular mass of 53kDa[8]. Here we report on cloning, overproduction, and homogeneity through purification of murC from *E. coli*. Production of a pure form of the MurC protein may aid in developing therapeutic targets against pathogenic bacteria.

2. MATERIALS AND METHODS

Sigma Aldrich chemicals (MERCK) provided all of the chemicals, reagents, and kits.

2.1 Gene sequence and protein structure -(https://www.ncbi.nlm.nih.gov) *murC* gene sequence (EGT67291.1) of *Escherichia coli* was retrieved from the NCBI Gene database. The *murC* gene encoding UDP-N-acetylmuramate-L-alanine ligase protein structure (10.2210/pdb2F00/pdb) retrieved from PDB (www.rcsb.org).

2.2 Selection of restriction sites and primer designing:

Snapgene (https://www.snapgene.com/) was used to find the restriction sites in the *murC* gene and pET28 b vector[9]. Nco 1(R3193S) (5′-C/CATGG-3′) and Xho 1(R0146S) (5′-C/TCGAG-3′) were selected for restriction digestion in the vector because these sites were not present in the *murC* gene. A model vector was designed in Snap gene including the *murC* gene which already contained Nco 1 and xho 1 restriction sites.

Forward primer (ATACCATGGGCATGAATACACAACAATTGG) and reverse primer (GTGCTCGAGGTCATGTTGTTCTTCCTCC) were designed to incorporate restriction sites Nco 1 and Xho 1 both within the murC gene.

2.3 Retrieving restriction sites from the vector:

Restriction sites in the Multiple Cloning Sequence region in the pET28 b expression vector were retrieved using the NEB cutter[10] (https://nc3.neb.com/NEBcutter). The information about Nco 1 and Xho 1 restriction sites, their mutual compatibility of the two restriction enzymes, cut smart buffer used to digest, and incubation temperature of 37°C and Tm°C of the enzymes were obtained from NEB.

2.4 Sigma oligoevaluator (http://www.oligoevaluator.com/LoginServlet) was used to evaluate the total base count, molecular weight, Tm°C, µg/OD, GC%, and secondary structure in the primers.

2.5 PCR - Polymerase Chain Reaction (PCR) reactions were done using a forward primer containing Nco1 restriction site ATACCATGGGCATGAATACACAACAATTGG and reverse primer containing Xho1 restriction site GTGCTCGAGGTCATGTTGTTCTTCCTCC. PCR conditions were optimized as follows: initial denaturation at 95°C for 3 minutes, denaturation at 95°C for 10 seconds, annealing at 55°C for 20 seconds, extension at 72°C for 1 minute, and final extension for 72°C for 7 minutes. No of cycles being 35. The total reaction mixture is 50µl containing the following components - 5µl of PCR buffer (10x), 2µl of dNTPs mM, 5µl of F – primer NC, 5µl of F – reverse XH, 1µl of Taq DNA polymerase (D1806), 1µl of Template DNA (*Escherichia coli* genomic DNA), 31µl of nuclease-free water.

2.6 Cloning of *Escherichia coli murC* into pET28 b expression vector, transformation to SG10 strain, and confirmation of positive clone by restriction digestion.

The PCR amplified *murC* gene and pET28 b (Novagen) expression vector was digested with the Nco 1 and Xho1 restriction enzymes and the murC gene was cloned into the pET28 b vector under the control of the bacteriophage T7 promoter. The recombinant plasmid (pET28b + murC) was transformed into SG10 *Escherichia coli.* The transformation protocol used is as follows: 50 ng of a plasmid containing recombinant DNA was added into 50µl thawed chemically ($CaCl_2$) competent

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SG10 cells and mixed, and the transformation mixture was incubated for 10 min on ice and then heat shocked for 2 minutes in 42 °C water bath then snap chill on ice for 2 minutes. After that, the cells were recovered in 700µl plain LB broth (L3522-250G) and incubated for 1 hour at 37 °C in a shaker incubator, after that centrifuged at 5000 rpm for 3 minutes and the supernatant was discarded and the pellet was resuspended in 50µl LB broth (L3522-250G) then plated on an LB agar (L3147-250G) plate containing 50 ug/ml kanamycin and incubated overnight at 37°C. The selection of positive clones was done by double digestion of recombinant plasmids. Double digestion protocol for 10µl reaction is as follows: recombinant plasmid 5µl, 1µl Nco 1, 1µl of Xho 1, 1µl of cut smart buffer (10x), 2µl nuclease-free water, and incubation 37°C for 30 minutes. The insert release was observed in agarose gel electrophoresis using 0.8% agarose gel.

2.7 Transformation of positive clones back into BL21 *E. coli* strain.

The recombinant plasmid was transformed into a competent *E. coli* strain BL21(DE3) (CMC0014) by the heat shock transformation method which is a strain used for high-level protein expression and easy induction[11]. The transformation protocol used is as follows: 500 ng of a plasmid containing recombinant DNA was added into 50µl thawed chemically ($CaCl_2$) competent BL21(DE3) cells and mixed, and the transformation mixture was incubated for 10 min on ice and then heat shocked for 2 minutes in 42 °C water bath then snap chill on ice for 2 minutes After that recover the cells in 700µl plain LB broth (L3522-250G) and incubate for 1 hour at 37 °C in a shaker incubator, after that centrifuged at 5000rpm for 3 minutes and the supernatant was discarded and the pellet was resuspended with 50µl LB broth (L3522-250G) then plated on LB agar (L3147-250G) plate containing 50 ug/ml kanamycin and incubated overnight at 37°C[12].

2.8 Overexpression of *E. coli MurC*

BL21 (DE3) with a recombinant plasmid (pET28b + *murC*) was inoculated into LB + kan agar and grown overnight at 37 °C to isolate a single colony. Then single colony from LB + kan agar was inoculated into 5 ml LB + kan broth and grown overnight at 37 °C, 250 rpm to prepare a starter culture until OD 600 was reached.

Then an expression of UDP-N-acetylmuramate-L-alanine ligase was induced by adding IPTG (Isopropyl β-D-1-thiogalactopyranoside) at different concentrations. These concentrations varied from no IPTG, 0.1mM, and 1mM. The culture was then incubated on a shaker incubator at room temperature for 3 hours. The cells were harvested by centrifugation at 4000 rpm for 10 minutes. Then the pellet was stored in a −20 °C freezer until further purification.

Inclusion bodies were purified from the frozen cell pellets as described below. The frozen pellets were thawed, mixed with 1 ml of Bugbuster (70584-3), 1µl of 0.1% Twin, and 10µl of 1% protease inhibitor, and then incubated at room temperature for 10 to 20 minutes. Then the cells were broken by sonication for 5 cycles (1 min per cycle), on ice with 30 seconds of cooling between the cycles. The mixture was centrifuged at 12000 rpm for 5 min then the supernatant was collected and the pellet was resuspended with 200µl bugbuster (70584-3). Parallelly whole cells were taken without breaking down the cell walls by using lysate buffer in case the produced protein was confined inside the cell. These samples were then run in 15% resolving and 4% stacking gel SDS - PAGE. (https://www.sigmaaldrich.com/IN/en/technical-documents/protocol/protein-biology/gel-electrophoresis/SDS-page) to confirm the overexpressed protein.

2.9 Purification of recombinant protein, *E. coli MurC*

Recombinant *murC* protein was purified using affinity chromatography since the recombinant protein contained an inbuilt His tag and Nickel has a high affinity to His tag.

Note: All centrifugations were done at 1000 rpm for 3 - 5 minutes.

Cell lysate supernatant (His tagged protein) was added with the 10 mg Nickel NTA agarose beads (57356000) stored in 50% ethanol. During this time His-tagged protein will bind the beads. After that, it was centrifuged and the supernatant was collected (flow-through), 500µl of the (200mM phosphate buffered saline(P7059) was added, 1mM DTT (1,4-dithiothreitol) and 5% glycerol) buffer

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was added to the beads and centrifuged. 500µl of supernatant (wash 1) was collected, then again 500µl of buffer was added with 100mM of Imidazole (I5513) because imidazole has a higher affinity towards nickel beads than His tags. 500µl of supernatant (wash 2, Elution 1) was collected after centrifugation, and 500µl of buffer was added with 250mM of Imidazole. The mixture was centrifuged supernatant (wash 3, elution 2) collected, and then a 500µl buffer with 500mM of Imidazole was added. Then centrifuged and the supernatant (wash 4, elution 3) was collected. SDS - PAGE was run to confirm purified recombinant protein.

HiTrap column purification:

In this method nickel beads are packed in a 1 ml column the sample was passed through the column using the syringe following the above protocol. (Except instead of centrifugation the sample is run through the column)

3. RESULTS AND DISCUSSION

The sequence of *murC* was retrieved from the NCBI Gene database. The length of the sequence is 1473 base pairs. *murC* encodes UDP-N-acetylmuramate--L-alanine ligase **(Fig 1)**

***Fig 1: UDP-N-acetylmuramate--L-alanine ligase* the protein has 2 chains (dimeric) and the length of the molecular weight of the protein is 108.83 kDa [13]**

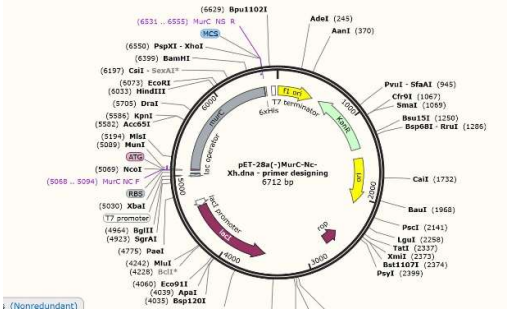

Fig 2: Modeled vector (pET28 b vector with *murC* gene) the total vector including the murC is 6712bp

The pET 28 b (Fig 2) is the expression vector [14] with a length of 5368 base pairs and the *murC* gene includes many restriction sites in the sequence then the Nco 1 and Xho1 restriction sites are not there in the *murC* sequence so these sites can be used to incorporate the *murC* gene into the vector. The primers were designed to include these two restriction sites (**Fig:2**). Forward primer used is ATACCATGGGCATGAATACACAACAATTGG and the reverse primer is GTGCTCGAGGTCATGTTGTTCTTCCTCC.

The primers are evaluated in a sigma oligo evaluator as the forward primer - length 30bp, µg/OD at 260 nm is 30.1, Tm(°C) is 72.9, GC % is 40, GC clamp is 2, and had weak secondary structure. Then the reverse primer - length is 28bp, µg/OD at 260 nm is 34.5, Tm(°C) is 74.6, GC %is 53.6, GC clamp is 2, and strong secondary structure.

E. coli genomic DNA was used as a template for the PCR reaction to amplify the *murC* gene. The PCR amplified (**Fig 3**) *murC* gene was 1.47 kb and it is confirmed by comparing it with the DNA ladder in agarose gel. This DNA containing the inserted primers was then used in further cloning procedure.

The DNA extracted was purified in a silica column, washed and eluted, cleaned, and concentrated in a buffer solution using Gel Extraction Kit (NA1111-KT) [15], Quantification of the total DNA by using Nanodrop Concentration was 48.9 ng/µl, Absorbance ratio of A_{260}/A_{280} = 1.82 and A_{260}/A_{230} =

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2.09. The quantification of the vector pET28b Concentration was 49.9ng/µl, A_{260}/A_{280} = 1.85, A_{260}/A_{230} = 2.92.

3.1 Cloning and transformation

The PCR product was double digested using Nco1 and Xho1 restriction enzymes to create restriction sites in the primer region and to remove other sequences of the primer. The same procedure was done with the pET28b vector to create the same restriction sites. The product was recovered by using the PCR clean-up method (PLN70-1KT) and quantified the recovered products. The double digested pET28b concentration is 10.6 ng/µl, A_{260}/A_{280} = 1.73, A_{260}/A_{230} = 1.19 and double digested concentration of *murC* – 21.4ng/µl, A_{260}/A_{280} = 1.77, A_{260}/A_{230} = 1.55. The cloning was done with two reactions. One with an insert and another without an insert as a control. In gene cloning, purified DNA ligase (enzyme) is used to covalently join the ends of restriction fragments and vector DNA that have complementary ends. The ideal ratio for ligation inserts to vector is 3:1. After cloning the product was amplified using PCR with T7 primers. T7 promoter and the terminator are present at both the ends of the MCS region. Pre-cloned recombinant *murA* with pet28a vector (1.7Kb) used as a positive control to the PCR reaction without the insert (negative) the product amplified will be 385 bp and with insert (positive) the product will be 1.7 kb.

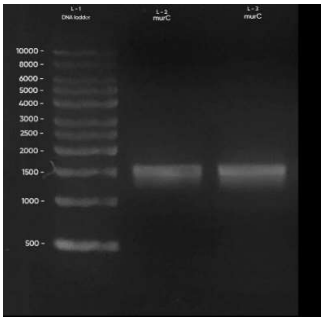

Fig:3 PCR amplified product the gene *murC* showing a length of 1.47kb compared with DNA ladder Lane 1: DNA ladder, Lane 2 & 3 *murC* replica 1 and 2.

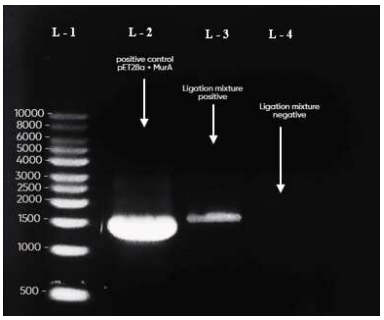

Fig:4 A PCR reaction using T7 primers. The gene amplified in lane1 is a DNA ladder, and Lane 2 vector containing the *murA* gene is used as a positive control. Lane 3 a is the vector with *murC* insert. Lane 4 is the vector without *murC*

The transformation was done to the SG10 *E. coli* strain by the heat shock transformation method

Fig 5 Negative control (without *murC*). No colonies grown after transformation.

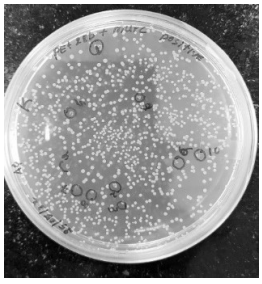

Fig 6 Positive control (with *murC*). Colonies have grown after the transformation.

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3.2 election of positive clones and transformation of positive clones into BL21 (DE3)

Three clones (2, 3, 6 in Fig 6) were randomly selected for the screening of positive clones. after plasmid isolation of three clones, quantification of the plasmids

Quantification of three clones

Colony number	2	3	6
Concentration ng/µl	93.1 ng/µl	85.1 ng/µl	111.6 ng/µl
A_{260}/A_{280}	1.86	1.85	1.88
A_{260}/A_{230}	2.32	2.78	2.42

After quantification of the plasmids run the agarose gel electrophoresis to confirm the positive clone and pre-cloned and purified recombinant pet28b with *murA* used as positive control and then double digest the plasmid to confirm the insert release. Clone number 3 and 6 are the recombinant plasmids confirmed by comparing the positive control **(Fig 7)** and also these two plasmids have to insert release compared to clone number 2 plasmid **(Fig 7)**

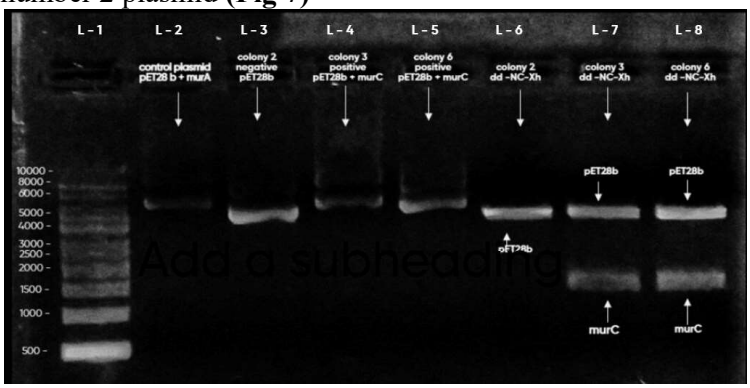

Fig:7 Lane:1 protein marker, Lane 2 pre cloned recombinant pET28b with *murA* used as a positive control, Lane 3,4,5 are the whole plasmids of three clones, Lane 6,7,8 are the double digested plasmids of three clones.

Colony no.6 recombinant plasmid was used to transform by the heat shock transformation method to the BL21(DE3) *E. coli* strain.

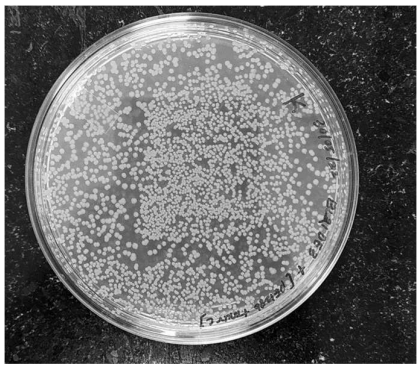

Fig:8 Transformation results of positive clone (recombinant plasmid) into BL21(DE3)

3.3 Overexpression of *E. coli MurC*

A culture containing the recombinant plasmid (pET28 b with *murC*) was grown and induced with IPTG (Isopropyl β-D-1-thiogalactopyranoside) at 0.1mM and 1mM concentration. The transformants

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synthesized an inducible protein of the expected size of *E. coli MurC* (53 kDa) [16]. The ratio of the quantity of *murC* protein present in a supernatant form is more than in pellet form. The estimation of protein was done by comparing the band brightness in 53kda **(Fig 9)** on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

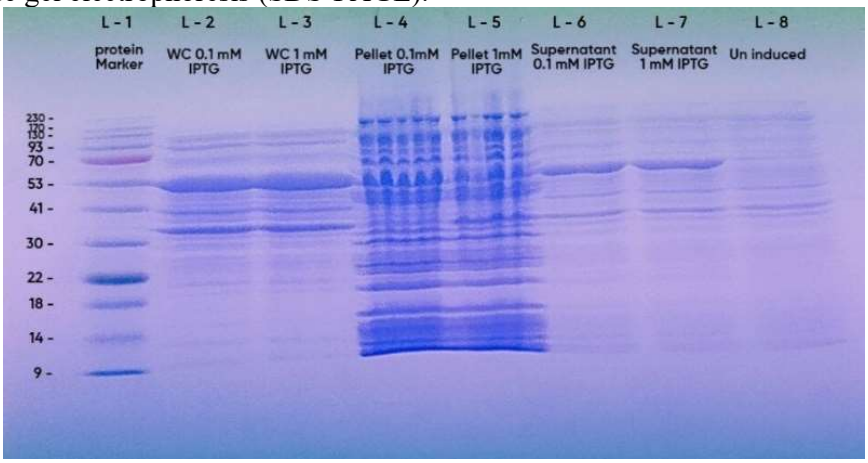

Fig:9 SDS-PAGE comparing the protein yield from three induced forms with uninduced cells. Lane:1 protein marker, lane:2&3 are the extract of whole cells induced with IPTG, Lanes 3 & 4 contain pellet after breaking down the cell wall and centrifugation, lanes 5 & 6 contain supernatant of the cell of which the cell wall was broken down, and Lane: 7 is uninduced. protein in all the cases was overexpressed except uninduced. And the protein expressed was 53 kDa.

3.4 Purification of recombinant protein, *E. coli MurC*

The purification was done in a single chromatographic step on an affinity nickel column. The recombinant protein MurC has a histidine tag in the C terminal of the protein which has an affinity towards Nickel NTA beads. The beads themselves have high affinity towards imidazole than histidine, so imidazole is used at different concentrations ranging from 100mM, 250mM, to 500mM concentration to remove MurC protein from nickel beads. Most of the proteins are purified in 250-500mM concentration **(Fig 10)**.

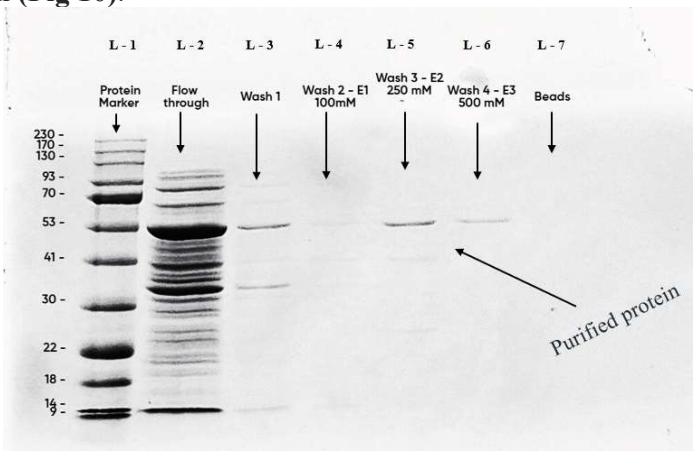

Fig:10 Lane 1 protein marker. lane 2 flow through protein washed with buffer (without imidazole), lane 3 washed with buffer and 10mM imidazole, lane 4 washed with buffer and 100mM imidazole, lane 5 washed with buffer and 250mM imidazole, lane 6 washed with buffer and 500mM imidazole, lane 7 empty nickel beads - most of the purified protein is seen in lane 5 (250mM).

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3.4 HiTrap Column Purification

a sample was passed through the Nickel complex pre-packed FPLC column for purification using a syringe.

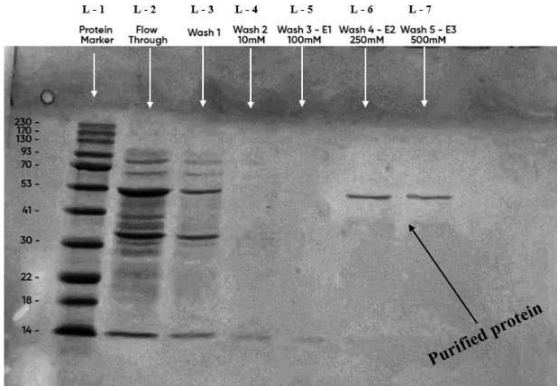

Fig:11 Lane 1 protein marker, lane 2 flow through protein washed with buffer (without imidazole), lane 3 washed with buffer and 10mM imidazole, lane 4 washed with buffer and 100mM imidazole, lane 5 washed with buffer and 250mM imidazole, lane 6 washed with buffer and 500mM imidazole, lane 7 empty nickel beads.

MurC protein could be a potential target for developing new monoclonal antibodies to inhibit bacterial growth. Not only *Mur C*, but other genes involved in the same pathway *MurD, E, and F* can also prove to be potential target sites common ATP binding site is a characteristic of these enzymes. More studies are needed to block the Mur proteins and decrease the possibility of resistant mutants. by targeting the particular epitope of that protein.

4. CONCLUSION:

The functional MurC enzyme of E. coli was obtained by gene cloning, intracellular expression, and protein purification. The enzyme was characterized as done. This enzyme is a validated drug target and future work is focused on deducing the three-dimensional structure of the enzyme and helping in identifying and screening small molecule inhibitors against pathogenic bacteria. which could be used as a drug target.

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