Cloning green fluorescent protein gene in pQE-80L vector and charecterisation of transformed protein in *E.Coli*

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Abstract
Green fluorescent protein (GFP) role in different applications of biotechnology such as protein fusions, imagining whole organism, and transcriptional reporters created an interest to probe the possibility of its production. Cloning the gene responsible for fluorescence using recombinant DNA technology and its expression in prokaryotes like Escherichia coli is very useful in many biotechnological applications. The gene encoding Green Fluorescent Protein highly stable (GFPhs) was cloned into the pQE80L vector and the cloned gene was transformed into E.coli strains of DH5α and Tyrosine auxotroph. The plasmid has been isolated from the respective colonies of DH5α containing pQE80L vector.

The isolated plasmid was double digested using BamHI and HindIII and the digested plasmid was confirmed with the Agarose gel electrophoresis. The expression profile of GFP has been confirmed by SDS-PAGE. The purification of GFP protein was carried out through Nickel His-tag affinity chromatography using Akta purifier. The purified protein was characterized using UV-Visible spectroscopy and fluorescence spectroscopy. The secondary structure of the purified GFP was confirmed by CD spectroscopy. The synthesised can be harvested to be used in different biotechnological applications.

Keywords: GFP, GFPhs, Cloning, Vector, *E. coli*, pQE80L, DH5α.

Introduction

The application of green fluorescent protein (GFP) in different biotechnological applications is expanding. It is used as a reporter molecule to monitor gene expression, protein localization and protein-protein interaction. The use of this marker protein in bio imaging to visualize specific organelles, cells, tissues and organs enhanced the scope of its application in immunology, neurobiology, development, and carcinogenesis.[6,7] The discovery of green fluorescent protein (GFP) from the bioluminescent jellyfish *Aequorea victoria* has revolutionised the exploration in molecular and cell biology.[9]

As the Gfp gene is heritable, the descendants of labelled entities also exhibit green fluorescence The discovery of GFP mutants of several colored variants and from other species had intensified the application of GFP technology in the study of cellular, molecular, and drug discovery. Using GFP fluorophore, the DNA and protein localization in cell cycle are well understood.[1] GFP acts as a biological tracer that allows understanding of the colonization, proliferation, tenacity, and spread of pathogen in live animals and helps to monitor the efficiency of gene transfer in transgenic animals and embryos.[18] The co-expression of GFP with a specific antibody or cytokine gene, its mutant variants and fluorescent fusion protein expression have helped to understand many intricacies in molecular biology.[14]

GFP is also useful to understand protein-protein interactions, to design biosensors, to produce reactive oxygen species as anti-oxidants and in drug screening.[10] GFP molecule is made of 238 amino acids with a molecular weight of about 27 kDa. As GFP can withstand denaturation well and can remain stable at a high pH range of <4.0 or >12.0, it is well used in biotechnological applications.[11] In the present study the gene encoding Green Fluorescent Protein was cloned into the pQE80L vector and the cloned gene was transformed into *E. coli* strains of DH5α and Tyrosine auxotroph for GFP production. Although the proposed study is traditional, it provides a scope for mass production of GFP.

Material and Methods

Materials: The bacterial strains used for cloning and transformation are *E. coli* DH5α and *E.coli* Tyrosine auxotroph (JW2581). The vector used for cloning is pQE-80L expression vector (Qiagen). Plasmid purification and gel extraction were done by using extraction kit. (QIAGEN®). The extraction kits used were Plasmid purification kit (QIAGEN®), QIAquick® gel extraction kit. Ampicillin stock solution (100mg/ml) and IPTG stock solution (100mM) were prepared and sterilized using 0.2μm syringe filter membrane.

Growth medium: To prepare growth medium, 40g of Luria Bertani broth was weighed and it was dissolved in 1L of distilled water. LB agar was prepared and autoclaved at 121^{0}C for 20 mins at 15psi. The medium was allowed to reach the hand bearable warm (50^{0}C) and ampicillin was added to the final concentration of 100μg/ml.

Agarose gel electrophoresis: The stock solution of 50X TAE (Tris acetate-EDTA) was prepared by adding 242g of Tris base, 57.1ml of glacial acetic acid and 100ml of 0.5M EDTA (pH 8). DNA gel loading dye stock solution of 6x

was prepared using the composition of 50% glycerol, 0.2M of EDTA (pH 8) and 0.05% Bromophenol Blue.

Tris Glycine SDS polyacrylamide gel electrophoresis: Resolving/Separating gel (5ml) was prepared by adding 1.1ml of water, 2.5ml of 30% acrylamide, 1.3ml of 1.5M tris pH 8.8, 0.05ml of 10% SDS, 0.05ml of 10% APS and 0.002ml of TEMED. The stacking gel was prepared by adding 1.4ml of water, 0.33ml of 30% acrylamide, 0.25ml of 1.0M tris pH 6.8, 0.02ml of 10% SDS, 0.02ml of 10% APS, 0.002ml of TEMED.

The running buffer 10X SDS (1L) was prepared by the mixing of 30g of tris base, 144g of glycine and 10g of SDS. The running buffer is to maintain the pH of the gel. The 3X SDS gel loading dye of 10ml was prepared by adding 2.4ml of 1M Tris Cl with pH 6.8, 3ml of 20% SDS, 3ml of 100% glycerol, 1.6ml of β- Mercaptoethanol and 0.006g of bromophenol blue. Staining solution of SDS-PAGE was prepared by the following chemicals of 0% methanol, 10% acetic acid and 0.1% Coomassie brilliant blue R250. Aforementioned components were filtered using Whatmann® cellulose filter paper and finally made up to 50% water.

It was used to stain the gel to view the band. Destaining solution I for the SDS-PAGE was prepared by adding 50% water, 40% methanol and 10% glacial acetic acid. Destaining solution II for the SDS-PAGE was prepared by adding 5% methanol, 7% glacial acetic acid and 88% water. Dialysis tubing cellulose membrane (10kDa) was purchased from Sigma Aldrich®. Dialysis membrane activation buffer was prepared by the combination of 100mM sodium bicarbonate and 10mM EDTA (pH 8). For dialysis, 10 mM of phosphate buffer was prepared from the stock solution of 0.2 M phosphate buffer which is dissolved in water.

Cell lysis buffer was prepared to lyse the cells to release the protein out of the cell. It was prepared by adding 1M tris, 0.5M EDTA, 10% SDS and 10mg/50ml of lysosyme. Protein purification was made using His 6 Ni Superflow™. Resin column was purchased from Clon Tech, India.

GFPhs gene was cloned into pQE-80L vector by restriction digestion and ligation. Reaction condition for double digestion of plasmid was carried out at 37^0C for 3 h with 50 µl of plasmid DNA, 2 µl of BamH1, 6 µl of HindIII, 10 µl of 1X BamH1 Buffer, 32 µl of Nuclease free water. The double digested pQE-80L was ligated with GFPhs by following reaction condition. Ligation reaction condition (20µl)was done at 22^0C for 1 h' Reaction condition for ligation was 2µl of Vector, 6µl of Insert, 2µl of 10X T4 DNA ligase buffer, 1µl of T4 DNA buffer and 9µl of nuclease free water. The ligated product was obtained.

Transformation of ligated product into *E. coli* DH5α
Competent cell preparation using $CaCl_2$: Single colony of desired strain i.e. DH5α was inoculated in LB Broth medium and incubated at 37^0C for overnight. 1% of pre inoculum was inoculated in LB Broth media and incubated for several hours to reach an OD of 0.6 at 600nm. And place it in ice for 30mins and centrifuge for 10mins at 6000rpm at 4^0C. Further steps should be carried out in ice.

Pellet was suspended in 25ml volume of $CaCl_2$ and chill on ice for 15mins and centrifuge for 10mins at 6000rpm at 4^0C. Pellet was again suspended in 12.5ml volume of $CaCl_2$ and chill on ice for 15mins and centrifuged for 10mins at 6000rpm at 4^0C and the pellets were again suspended in 10ml volume of $CaCl_2$ and chilled on ice for 15minutes and centrifuged with the same conditions. Pellet was again suspended with 1ml volume of $CaCl_2$. Then 100µl of cells were aliquoted in 1.5ml sterile Eppendorf tube. And this tube was transferred and stored in -80^0C immediately.

Transformation into DH5α: Ligation mixture was added to the 100µl competent cells and plunged the tube on ice for 30mins. After incubation, sample was subjected to heat-shock treatment at 42°C for 90sec. immediately tubes were plunged into ice for 5mins. The final volume of competent cells was made to 1ml by the addition of LB broth. This was incubated at 37^0C for 45mins and centrifuged at 8000rpm for 3mins. After the incubation, the samples were spread onto LB agar plates with suitable antibiotics.

Plasmid DNA isolation: For plasmid DNA isolation, 5ml overnight culture was harvested by centrifuging at 10000 rpm for 10mins. Pellet was suspended in 4ml Buffer P1 (50mM TrisCl pH 8, 10mM EDTA,100 µg/mlRNase A). 0.3ml of buffer P2 (200mM NaOH, 1% SDS) was added and mixed by inverting the tubes 4-6 times and incubated in room temperature for 5mins. 0.3ml of buffer P3 (3M potassium acetate pH5.5) was added and mixed by inverting the tubes and incubated in ice for 5mins and centrifuged at 13,500rpm for 10mins. This supernatant was applied to the equilibrated column and centrifuged. Flow through was discarded and column was washed by Buffer QC (1M NaCl, 50Mm MOPS pH 7, 15% isoproponal).

Table 1
Buffers used in protein purification using His 6 Ni superflow™ Resin column

Components	Binding buffer	Wash buffer	Elution buffer I	Elution buffer II	Elution buffer III
$NaH_2PO_4.2H_2O$	50mM	50Mm	50mM	50mM	50mM
NaCl	300mM	300mM	300mM	300mM	300mM
Imidazole	10mM	20Mm	100mM	150mM	200mM

Plasmid DNA was eluded with Buffer QF (1.25M NaCl, 50mM TrisCl pH8.5, 15%isoproponol) into a clean microfuge tube. Plasmid DNA was precipitated by isoproponal and 70% ethanol. Plasmid bearing GFPhs gene was confirmed with Agarose gel electrophoresis. Confirmed plasmid bearing GFPhs gene was transformed into *E. coli* tyrosine auxotroph (JW2581). The protein expression profile was confirmed by SDS PAGE.

SDS-PAGE (Bio-Rad Mini Protean): Short plate was placed on the top of a spacer plate and inserted into the casting frame on flat surface. Casting frame was placed on casting stand. Separating gel (15%) was prepared and vortexed slowly and it was poured between the glass plates in casting chamber (~3/4th inch). Small layer of butanol was added on the top of the gel to straighten the level of gel and to remove unwanted air bubbles. After polymerization, butanol was removed and top layer washed with distilled water.

Now stacking gel was prepared and poured on the top of the separating gel and comb was placed. Once the stacking gel polymerized, the comb was gently removed and plate was placed in electrode assembly with short plate inside. Electrode assembly was placed in electrophoresis tank with 1X SDS tank buffer.

Sample was prepared by adding loading dye and heated at 100^0C for 5mins. Prepared sample was loaded in gel cassette. Now electrophoresis tank was connected to the power supply at 100V. When the dye reaches the bottom of the gel, gel was disassembled and stained by staining solution for one hour. Then staining solution was drained and destained for overnight.

Protein Purification: Pellets were suspended in cell lysis buffer and centrifuged at 20,000 rpm for 10mins.

His tag purification: Protein was purified by using His-tag binding nickel affinity column. Column was washed with 100ml distilled water with the flow rate of 3.6 to 4.2ml per min and washed with 30ml binding buffer. Sample was loaded into column. Column was washed with 5/3rd sample volume of wash buffer. Protein was eluted by linear gradient elution buffer. Again column was washed with wash buffer and binding buffer. Finally, column was washed with 10% ethanol and stored at 4^0C.

Dialysis: Purified protein was further dialyzed by 10kDa dialysis tubing to reduce the salt concentration. Dialysis membrane was activated by membrane activation buffer at 60^0C for 2hrs and then washed with distilled water (two times) and milli Q water. Sample was poured in dialysis membrane. Both the ends were tied and placed in dialysis buffer for overnight at 4^0C. Dialysis buffer was changed periodically until maximum removal of salts was reached (mostly three times, for every 12hrs). Purified protein was confirmed with SDS-PAGE.

Biophysical characterization

UV spectrometry: UV spectrum analysis was carried out in spectral region of 200 to 700 nm at room temperature. The concentration of each protein was kept constant at 1mg/ml.

Fluorescent spectrometry: Emission wavelength of each protein was identified by fluorescent spectrum by applying excitation wavelength at room temperature with the constant protein concentration.

CD spectroscopy: The CD measurements were carried out in the spectral region of 200 to 250 nm with a scan rate of 50nm/min using Jasco J-715 spectro polarimeter at room temperature. The band width, response time and data pitch were 1nm, 1sec and 0.1nm respectively. Each spectrum represents the average of three individual scans. The concentration of each protein was kept constant at 0.2mg/ml. For base line correction, CD spectrum of buffer alone was subtracted from CD spectrum and resulting spectra were smoothened.

Results and Discussion

The digested GFPhs were ligated with pQE-80L and transformed into *E. coli* DH5α and plated on LB ampicillin plate. Transformed colonies were screened for the presence of GFPhs using double digestion.

The digested plasmid was confirmed with the length of the digested gene and the vector. Length of the digested gene is 760 bp and the length of the vector is 4751 bp. The confirmed plasmid bearing GFPhs gene was transformed into *E. coli* tyrosine auxotroph (JW2581) using calcium chloride method. The transformed cells were plated onto LB/Ampicillin plates for clone selection. The clones were patched onto fresh LB-amp plate. The transformed colonies were confirmed with the negative control plate.

Single colony of *E. coli* tyrosine auxotroph with bearing GFPhs gene was selected and its protein expression was visualized by SDS-PAGE. Expression of the single colony from the transformed plates was analyzed by the IPTG induction. This expression profile (Fig. 5) clearly shows that our clone is pQE-80L with GFPhs. The band intensity clearly indicates that GFPhs expression was high enough upon IPTG induction. Thus, this clone was selected for the further studies.

The expression of the protein was confirmed by the molecular weight of the GFP. GFP has the molecular weight of 27 kDa. All the clones were showing the same expression level after induction with IPTG. Then the expression of the protein is confirmed by SDS-PAGE.

Purified protein and its expression: Purified protein was obtained from the His-tag affinity purification method. Purified proteins were further purified by dialysis to remove salt impurities and confirmed by SDS PAGE. Protein

expression was induced at 20ºC or 25ºC with 0.1Mm or o.4 isopropyl-β-d-thiogalactosidase (IPTG).

Biophysical characterization

UV Spectrometry: Excitation wavelength was calculated by UV-Visible spectrometry. Protein absorbs maximum light at their excitation wavelength. GFPhs has the excitation wavelength at 503nm (λmax = 503nm).

Fluorescence Spectrometry: Emission wavelength of the GFPhs is calculated by fluorescence spectrometry. It shows the emission wavelength at 520nm (λmax = 520nm).

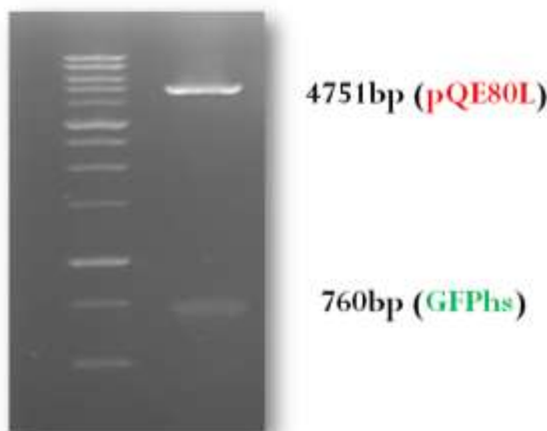


Figure 1: Agarose gel electrophorosis image of Double digested plasmid

(a) **(b)**

Figure 2: (a) Transformed colonies of GFPhs Tyrosine auxotroph, (b) patched clones of transformed colonies of GFPhs Tyrosine auxotroph.

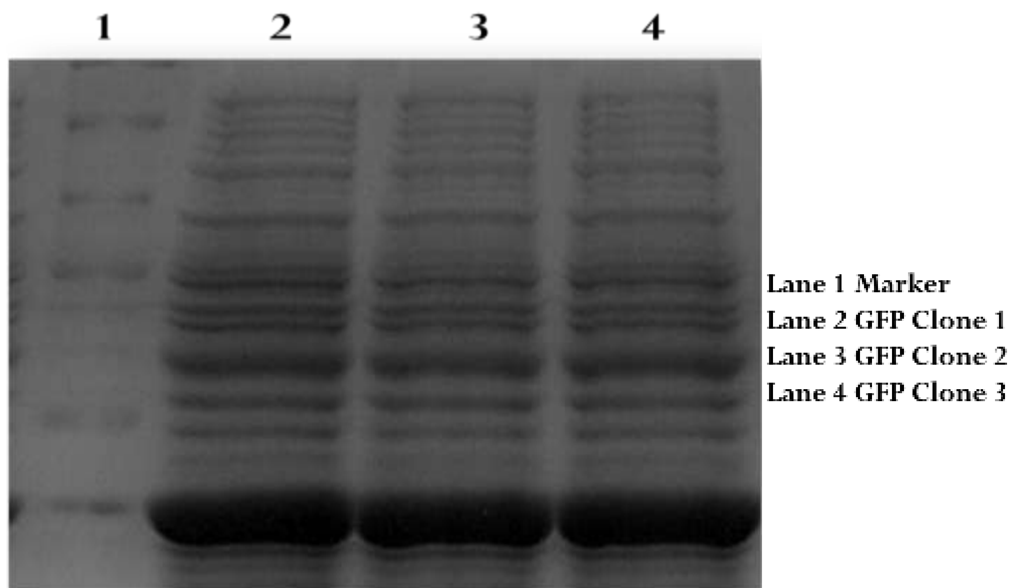
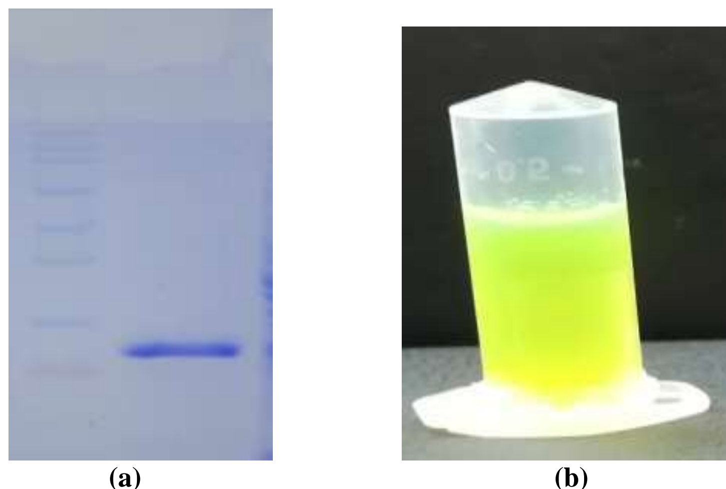


Figure 3: Protein expression profile of GFPhs Tyrosine auxotroph

CD spectrum: Circular dichroism (CD) is a well-known method to characterize the secondary structure of proteins as it provides a unique spectral pattern for various secondary structures in solution. Here, CD was measured for GFPhs. As can be seen from figure 8, CD spectrum of protein showed a negative band around 215nm which is the characteristic feature of GFP as well as proteins with high β-structures.



(a) **(b)**

Figure 4: (a) Expression profile of purified GFP protein (b) Purified GFP protein from Akta purifier

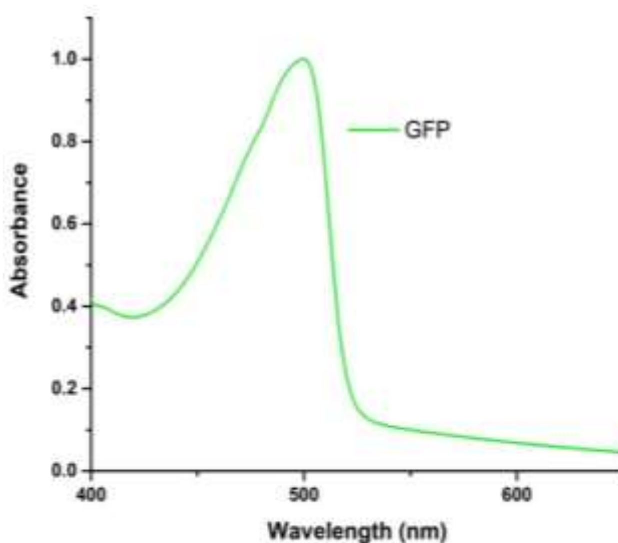


Figure 5: UV-Visible spectrometry of GFPhs

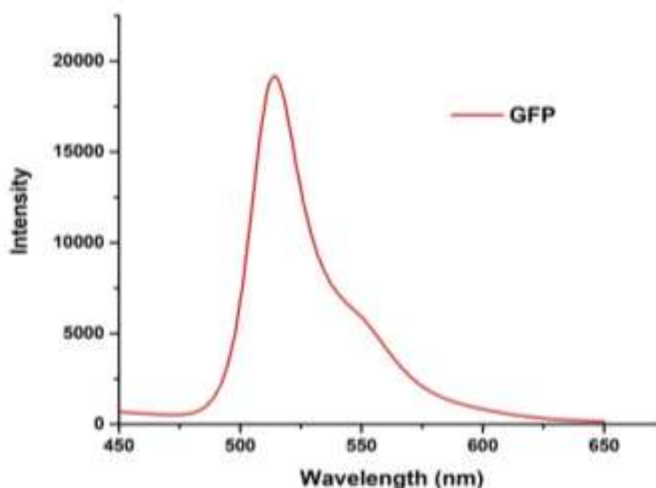


Figure 6: Fluorescent spectrometry of GFPhs

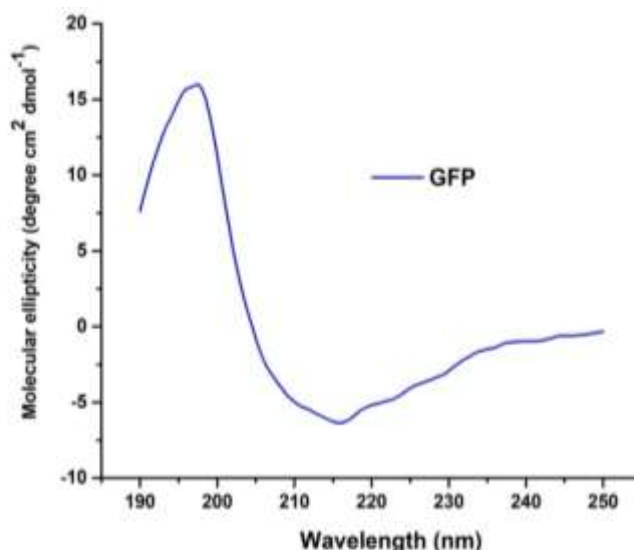


Figure 7: CD Spectrum Analysis of GFPhs

Discussion

Previously, cloning of his-tag GFP in the vector was carried out using polymerase chain reaction (PCR).[4] Here, we used the traditional method of cloning to check the stability of the protein. Cloning of GFP gene into the vector using traditional method gives more number of transformed colonies.

Stability of the protein is confirmed by the colour change of the protein. After purification, if the colour of the protein changes from green fluorescent colour to colourless, it indicates the stability of the protein. In this work, protein colour did not change. Due to the stability, several studies have employed GFP as a reporter for membrane protein expression and detergent selection.[4] With the modern advances in genomics, proteomics and bioinformatics, the number of proteins being produced using recombinant techniques, is exponentially increasing. High throughput screening approaches are being performed to rapidly identify proteins with a potential application as therapeutic, diagnostic or industrial enzymes.[5]

Additionally, genomic approaches are being pursued to solve the structure of numerous proteins.[2] The above-mentioned screening approaches would not be feasible if specific purification procedures were to be developed for each individual protein. Here, the use of affinity tags enables different proteins to be purified using a common method as opposed to highly customized procedures used in conventional chromatographic purification. A literature survey reveals that affinity tags have been observed to (i) improve protein yield,[16] (ii) prevent proteolysis,[17] (iii) facilitate protein refolding,[15] (iv) protect the antigenicity of the fusion protein[8] and (v) increase solubility.

Conclusion

The gene encoding Green Fluorescent Protein highly stable (GFPhs) was cloned into the pQE80L vector and the cloned gene was transformed into *E. coli* strains of DH5α and tyrosine auxotroph. The plasmid has been isolated frsom the respective colonies of DH5α containing pQE80L vector.

The isolated plasmid was double digested using *BamHI* and *HindIII* and the digested plasmid was confirmed with the Agarose gel electrophoresis. The expression profile of GFP has been confirmed by SDS-PAGE.

The purification of GFP protein was carried out through nickel His-tag affinity chromatography using Akta purifier. The purified protein was characterized using UV-Visible spectroscopy and fluorescence spectroscopy. The secondary structure of the purified GFP was confirmed by CD.

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