Research Article

Production of red fluorescent protein (mCherry) in an inducible *E. coli* expression system in a bioreactor, purification and characterization

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ABSTRACT

New and improved genetic engineered variants of fluorescent proteins (FPs) have become useful tools for bioimaging in biomedical researches. Red fluorescent proteins (RFPs) first derived from the sea anemone *Discosoma* show high performance *in vivo* labeling and imaging. mCherry is a member of RFPs which has very high photostability, resistant to photo bleaching and rapid maturation. These advantages ensure that mCherry can be successfully fused to many proteins and widely used for quantitative imaging techniques. In this study, the constructed recombinant plasmid pBADCherry was expressed in *Escherichia coli* BL21(AI) then culture conditions, inducer concentration and induction time were optimized. Results of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis demonstrated that 5 hours induction at 0.04% of arabinose concentration was optimal for the highest mCherry yield. The expression of hexa histidine-tagged (6xHis) recombinant mCherry was induced by arabinose and purification performed using nickel (Ni^{2+}) affinity chromatography. High throughput expression of 81 mg fluorescent protein from a liter of *E. coli* culture carried out in bioreactor.

1. Introduction

The story of fluorescent proteins began with discover of green fluorescent protein (GFP) from the bioluminescent jellyfish *Aequorea victoria* [1]. After cloning of GFP and demonstration applications in living cells it has become an important tool for fluorescent labeling in molecular and cell biology [2]. GFP and its variants in different colors have developed into a unique tool for visualization of living cells and organisms. Fluorescent proteins (FPs) are used in a variety of applications for structural studies especially in labeling of proteins, nucleic acids, organelles and organisms. On other respects functional studies such as protein-protein interactions, development of biosensors, production of reactive oxygen species (ROS), drug screening can be carried out using FPs. Mutagenesis studies on production of new versions FPs have led to improve properties of

these proteins as brightness, photostability, maturation rate, oligomerization and performance in fusion labeling [3].

Since discovery of original GFP from jellyfish the broad range of FPs characterized from other organisms (*e.g.* hydrozoans, corals, anemones) that emit in the yellow-orange to far-red regions of the visible light spectrum [4-6]. Fluorescent proteins with red, orange and far-red emitting fluorescence are assessable quality for multicolor imaging experiments. These proteins have been isolated mostly from corals of class Anthozoa and have longer excitation wavelengths that give advantages for lower phototoxicity and deeper penetration in tissue.

Oligomerization, non-specific localization, interactions with undesired proteins and aggregation are main restrictive results for fusion applications of oligomeric FPs. Non-*Aequorea* FPs are not naturally monomeric,

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they have mostly dimeric or tetramer structure in nature and are not suitable intracellular expression and labeling. Artificial monomerization of these proteins has resulted decrease in performance so hydrophobic surfaces engineered monomeric FPs has been interested recently [7-9].

The first monomeric red fluorescent protein mRFP1 derived from the Indo-Pacific sea anemone, *Discosoma sp.* tetrameric red fluorescent protein, DsRed (Figure 1) has limitations including brightness, chromophore maturation and fast photo bleaching [10,11]. Direct mutagenesis targeting chromophore residues has yielded a series of mRFP1 known as 'mFruits'. mCherry is the best red member in mFruit family which has brightness level is ~75% of EGFP, respectively. mCherry is a 28.8 kDa monomer with 256 amino acids and derived from DsRed. The protein exhibits emitting light in the range of wavelength 610 nm that makes it suitable for multiple labeling. It gives results in a short time during imaging experiments due to the rapid maturation period after transcription. High photostability and other advantages make mCherry presently the most widely used monomeric RFP [11,12].

Recent studies reported that the *araBAD* promoter system in *Escherichia coli* (*E. coli*) performs to manipulate the yield of desired protein induced by L-arabinose added in culture medium [13-16]. The *araBAD* promoter enables to manipulate of the transcriptional rates of the soluble recombinant protein expression levels [17,18].
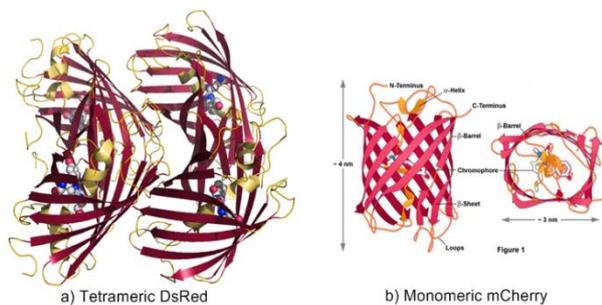

Figure 1. Structure of wild-type tetrameric RFP, DsRed (a) and monomeric RFP, mCherry (b) [9].

Expression systems based on *araBAD* promoter (P$_{araBAD}$) use for control of expression in *E. coli* which can be induced by external inducer [19]. pBAD system has been designed for high level expression of interested gene(s) that can be induced by arabinose [20,21]. pBAD offers tightly regulated expression resulted in high protein yields, dose-dependent induction for modulating expression levels and optimization of soluble protein expression [14] Besides expression under an effective promoter, cultivation of recombinant *E. coli* cells in bioreactor gives the yield in high level with maximum

cell productivity [22,23].

In this study, we investigated the use of pBAD promoter system to express of red fluorescent protein mCherry. We optimized the inducer concentration and induction time to maximize expression level. The performance for the highest productivity studied in bioreactor. Besides the yield of recombinant mCherry had been purified and characterized. This method provides rapid, practical, cost-effective and high efficient production of soluble mCherry that can be used for further biomedical researches.

2. Material and Method

2.1. Recombinant mCherry vector backbone

The backbone of the mCherry plasmid was pBAD expression system and had ampicillin resistance gene (*bla*). pBADCherry was a gift from Michael Davidson & Roger Tsien (Addgene plasmid #54630). The mCherry coding sequence was cloned into N-terminal backbone with a hexa histidine-tag is shown in Figure 2. The pBADCherry vector contains 708 nucleotides that encoded insert mCherry was transformed into competent *E. coli* DH5α. The transformed cells were grown overnight at 37°C in LB plates containing 100 µg/mL ampicillin. Plasmid purification from 5 mL LB culture performed using EZ-10 Spin Column Plasmid DNA Miniprep Kit (Biobasic).
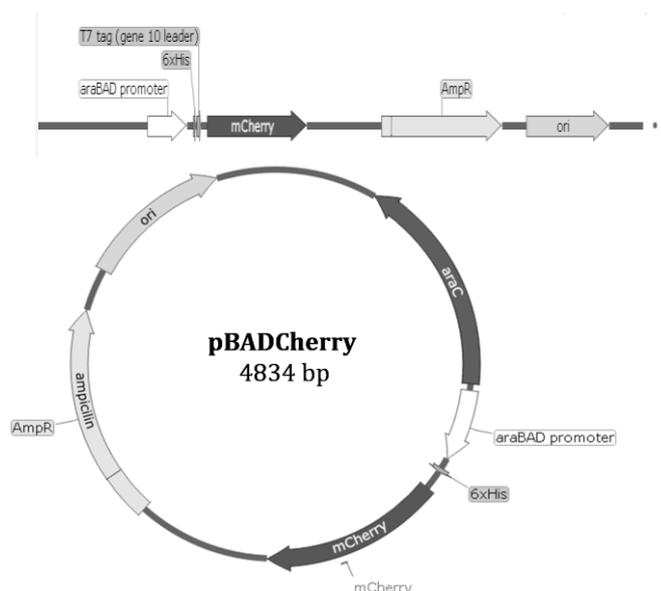

Figure 2. The construction of the pBADCherry plasmid

2.2. Expression of mCherry

High efficiency chemically competent *E. coli* BL21 (AI) cells transformed with recombinant plasmid pBADCherry by heat shock. LB agar containing ampicillin (100 µg/ml) was utilized as a selective medium for transformants of *E. coli* cells. A single colony from the agar was inoculated into 5 mL LB and cultured at

37°C and 200 rpm overnight in shaking incubator. Glycerol stocks of the transformed cells were made by mixing 1:1 ratio of the overnight culture with 30% (w/v) sterilized glycerol solutions and stored at −80°C. *E. coli* cells carrying any insert were grown as negative control. The preculture was centrifugated at 5000 rpm for 5 min and the pellet was resuspended in fresh medium.

2.3. Optimization of expression of mCherry

Culture parameters (inducer concentration and induction time) were varied to investigate optimal conditions. Initially, small scale in flask contains 50 mL LB used for optimization before large scale protein production. The resuspended culture was inoculated in LB flasks at a ratio of 1:100 and was grown at 37°C until OD_{600} was reached 0.6. Bacterial cultures were then induced with varying concentrations (0.002, 0.004, 0,006, 0.008, 0.01, 0.02, 0.04 and 0.2 %) of arabinose and grown for another 5 hours at 37°C.

The effect of induction time on mCherry expression was investigated. Cultures that induced with the optimum inducer concentration were withdrawn induction at different time (1, 2, 3, 4, 5 and 6 h) intervals. Equal volume from each sample (20 μl) was analyzed by 12% SDS-PAGE.

2.4. Cultivation in bioreactor

The overnight culture of *E. coli* strain BL21(AI) that transformed with a pBADCherry plasmid was inoculated (1%) in 50 mL LB medium in a 250 mL shake flasks and incubated overnight at 37°C with shaking at 200 rpm. Production of mCherry in large scale was performed in 'Sartorius Biostat®A plus' bioreactor that has 5L working volume. 3 L LB medium (containing 100 μg/mL ampicillin and 1% olive oil as a foam breaker) in bioreactor was inoculated with the fresh overnight culture. Cultivation was carried out under controlled conditions at 37°C and pH 7.0. The pH was kept constant at 7.0 by the controlled addition of 5M NaOH. The dissolved oxygen concentration (DO) was maintained at 30% saturation by increasing agitation speed (500–2,000 rpm) and O_2-enrichment if required. The optical density of culture was measured at 600 nm with spectrophotometer. Expression of recombinant mCherry was induced at $OD_{600} = 2.0$ with optimum arabinose concentration that decided from experimental concentration data for optimization. Batches were finished when no further growth was detected with optical density.

2.5. Purification of target protein

After induction and expression of target protein in high cell density, cells were harvested by centrifugation at 5000 rpm for 5 min. Cell pellets of 3L *E. coli* culture resuspended in 100 mL of lysis buffer (100 mM sodium phosphate, 100 mM NaCl, pH 7.0). Cell suspension was lysed by sonication (10 cycles of 15s pulse with intervals of 15s each, at 90% amplitude) for 2 hours on ice bath. The lysate was pelleted at 8000 rpm 5 min to separate undisrupted cell contents. Ultra-centrifugation was performed at 30000 rpm for 1 hour to get soluble protein solution.

Preparation of column for purification 10 mL of Ni-NTA resin in column was pre-equilibrated with 5 column volumes (CVs) of washing buffer (100 mM NaH_2PO_4, 100 mM NaCl, pH 7.8). Supernatant that transferred to a fresh tube after ultra-centrifugation was loaded to column. The flow through was collected and all untagged proteins were removed from column using 10 CVs of wash buffer 1 (100 mM sodium phosphate, 100 mM NaCl, 10 mM imidazole pH 7.8) and wash buffer 2 (100 mM sodium phosphate, 100 mM NaCl, 20 mM imidazole, pH 7.8). His tagged mCherry that bound to column was recovered with elution buffer (100 mM sodium phosphate, 100 mM NaCl, 300 mM imidazole, pH 7.8). Purified mCherry fractions were dialyzed overnight in buffer (20 mM Na_2HPO_4, 50 mM NaCl, pH 7.8) at 4°C.

2.6. Protein analysis

Optimal induction time and inducer concentration dependent expression levels, purity and molecular mass of purified mCherry samples were determined by SDS-PAGE. Whole cell lysate samples and collected protein purification samples were mixed 1X sample buffer and denatured at 95°C for 3 min, subjected to a 12% denaturing SDS-PAGE and stained with Coomassie brilliant blue. SDS-PAGE was performed essentially by the methods of Laemmli [24].

The concentration of the purified mCherry by the absorption spectra using a UV-VIS spectrophotometer (Varian Cary® 50). Absorbance was read at 280 nm.

2.7. Fluorescence measurements

The spectral properties of the purified mCherry were measured in a fluorometer (PTI Quanta Master™). The excitation and emission wavelength of mCherry were reported.

3. Results and Discussion

3.1. Construction of pBADCherry

The gene encoding red fluorescent protein mCherry is commercially available cloned in *E. coli* vector (pBAD). The pBADCherry was gift from Dr. Tsien (University of California, San Diego, CA) that used in this study. The gene is 708 nucleotides in size and encodes a start codon, with N-terminal 6XHistidine and was placed under

control of the arabinose-inducible *araBAD* promoter (P_{BAD}) (Figure 1).

3.2. Cytoplasmic expression of mCherry

E. coli BL21(AI) cells was transformed using the constructed pBADCherry expression vector. The mCherry was expressed as a His-tagged protein. A single clone of transformed *E. coli* cells was cultivated for detection of protein expression. The effects of inducer concentration and induction time on soluble mCherry expression were carried out in lab scale shake flasks contain LB medium. The degree of purity of recombinant mCherry was analyzed by 12% SDS-PAGE under denaturing conditions, stained by Coomassie brilliant blue. The molecular size of mCherry with Histidine tag was approximately 28 kDa as expected (as estimated in 12% SDS-PAGE gel) (Figure 3). The purity of the final protein product analyzed by Coomassie-stained gels was greater than 95% .
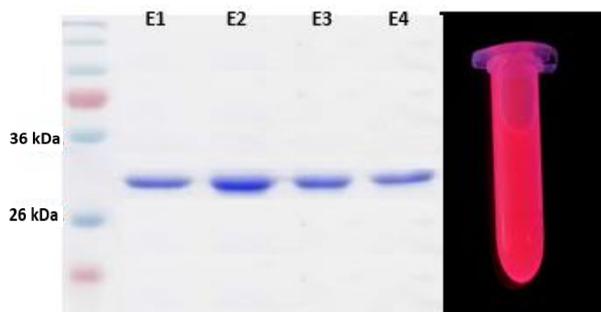

Figure 3. SDS-PAGE analysis of mCherry purified fractions.

Optimum arabinose concentration was decided by working at eight different concentrations at 37°C. As shown in Figure 4 expression levels were increased with arabinose concentrations and the expression was highest at 0.04% arabinose concentration. In addition the optimal induction was performed at 5 hours after addition of 0.04% arabinose shown in Figure 5. Taken together, the optimal culture conditions for highest expression of mCherry was established as 0.04% of arabinose at 37°C for 5 hours. Whole cell lysates of the optimization studies were analyzed by 12% SDS-PAGE.
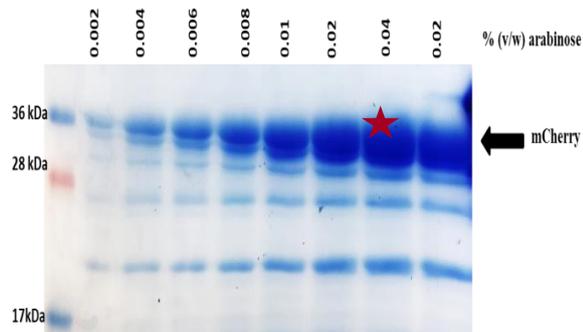

Figure 4. Arabinose induction of the expression of mCherry at different arabinose concentrations. Proteins in the whole cell lysates were analysed by 12% SDS-PAGE.
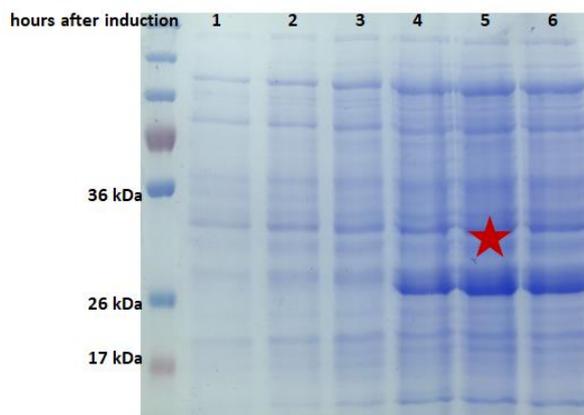

Figure 5. Effect of the induction time on mCherry expression with 0.04% arabinose concentration. Proteins in the whole cell lysates were analyzed by 12% SDS-PAGE.

3.3. Overexpression of mCherry

After determining the mCherry overexpression in *E. coli* in shaking medium the performance was observed under bioreactor conditions. The effects of induction parameters on protein expression were optimized with small culture of *E. coli* in flasks before performing large culture in a bioreactor. All processes in bioreactor were have been carried out in batch culture conditions. Optimized parameters (inducer concentrations and induction time) were applied to bioreactor. In order to determine optimal arabinose concentration for maximum yield of mCherry, induction was performed between 0.002-0.2% arabinose concentrations and analyzed by 12% SDS-PAGE (Figure 4).

Maximum yield of protein was observed at %0.04 arabinose concentration. The optical density of bacterial culture at 600 nm reached to a maximum of 12 after 5 hours of induction. Absorbance measured at 280 nm is used to calculate the concentration of purified mCherry. The yield measured for the fluorescent protein concentration was 81 mg/L from 3 L bacterial culture medium in a bioreactor while the concentration was 15 mg/L in shake flasks.
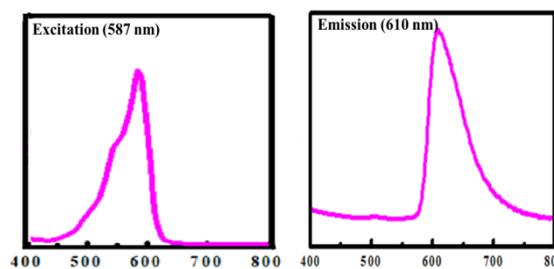

Figure 6. Excitation and emission spectra of mCherry

Figure 6 indicates the fluorescence excitation (587 nm) and emission (610 nm) spectra of purified mCherry. Spectral results were same as the ones reported before in the literature [25,27].

4. Conclusion

The advancement in FPs experimental studies makes FPs very practical tools for broad applications in biosciences, especially *in vivo* imaging. GFP and its variants used as a multicolor labeling of different compartments of cells, tissues or organisms. It is believed that because of increasing in diversity of FPs, they will become favorable tools for clinical, preclinical or therapeutic studies, such as drug screening, tumor monitoring and photodynamic therapies of cancer [7, 8].

Expression and purification of new generation FPs that improved by genetic engineering techniques represent variety of undiscovered opportunities for basic investigations. Production of engineered pure monomeric mCherry protein for structural studies is very important. mCherry has a monomeric structure unlike ancestor of RFPs and the palette of monomeric FPs enables multicolor labelling experiments [3].

Isolation and purification of proteins from organisms such as plant, animal or microorganisms is quiet challenging and very expensive process besides contamination risk of human and animal diseases derived from isolated hosts. Recombinant DNA technology helps to overcome these restrictions and gives product of protein with improved features that close to their natural structures [26].

For overproduction of recombinant proteins both eukaryotic and prokaryotic expression systems are used. Bacterial host systems have many advantages for achieving a very high yield of recombinant proteins. *E. coli* is the most preferred system because of its typical features such as capability to produce large amounts of recombinant protein in a short time at low cost with its well-known genome. Also *E. coli* expression systems are very convenient for the production of proteins such as FPs that don't require specific posttranslational modifications.

The *E. coli* pBAD system based on *araBAD* operon is a tightly regulatable expression system that allows turning expression on/off. Strain BL21(AI) is also the most widely used host for target gene expression. In this study pBADCherry in *E. coli* BL21(AI) cells were grown in bioreactor gives a very high yield of recombinant mCherry protein about 6 times higher than flask culture at 0.04% optimum arabinose concentration. The results of study indicated that by varying the concentration of arabinose, expression of proteins can be regulated at different levels. The expression level of mCherry at different time was shown in Figure 5. It is observed that the expression level of mCherry remained unchanged after 5th hours of induction and there was no significant difference between 5th and 6th hours of induction. Consequently induction with 0.04% arabinose concentration for 5 hours was decided as optimum condition of induction.

In this study expression of recombinant mCherry fluorescent protein in *E. coli* was optimized by an inducible system, overexpressed in bioreactor and also purified in a very high purity. It is hope that expressed pure recombinant FPs will be a control model for further studies to improve their biophysical properties such as brightness, photostability, maturation and tolerance for physical conditions. Taken together, our given method demonstrates that a high-throughput to produce FPs that usable for biophysical, structural and functional studies for instance, mechanisms of maturation, behavior of their chromophore and investigating of FPs for potential use in dye-sensitized solar cells.

In conclusion, we demonstrated an effective method for producing recombinant mCherry. We suggest that soluble mCherry protein yield can be further increased by optimization of vector, strain and bioreactor conditions.

Acknowledgment

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