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RESEARCH ARTICLE

Expression of u-msf GFP cloning vector of DH 5α strain for Production of Green Fluorescent protein in one liter bioreactor

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Abstract

The present investigations were carried out for the optimization and production of GFP in *Escherichia coli* DH-5α strain. Based on the results obtained through shake flask experimentation, process is forwarded to produce GFP using 1 liter bioreactor (Spectrochem Laboratories Hyderabad). Around 2 to 3 colonies of *Escherichia coli* contain GFP gene was isolated and inoculated on to 650 ml (1 Liter Bioreactor) of LB broth that contain 1000µl of ampicillin. After 12 hr the growth rate was suddenly increased to 1.4 which is measured at 600 nm. Exponential and steady state phases were lasting in between 11.25 hours to 29 hours and found maximum cell density 4.2 with the significant production of protein with 9.6 mg/ml. The doubling time during the exponential phase was noticed approximately is 4.81 hour. Cell density remained almost constant in between 29 to 31.0 hrs.

Introduction

From the bio processes engineering standpoint, the combination of microbial strain and method development plays crucial role in maximizing output of the product. Optimization of recombinant protein production in the cells includes increase in the biomass that can extensively produce the protein of desired interest properly. Over recent years the use of microbial strains and cell-based systems has been drastically increased for the recombinant protein production.

Escherichia coli is generally considered as important hosts in modern research for recombinant protein production. The use of this organism throughout academia and industry are widespread and has been a favorite organism for several metabolic modeling and metabolic engineering projects in the past (Berry, 1996; Cameron, et al., 1998; Koffas, et al., 1999).

The list of recombinant proteins Humulin and Humatrope (Eli Lilly and Co.), forms of human insulin and growth hormone (IGF-1) have been successful production with *Escherichia coli* host system (IGF-1), (Chen et al., 1995 and Cui et al., 2001). In fact *E.coli* is commonly considered as one of the most important production hosts for inhaled form of recombinant insulin.

Green fluorescent protein (GFP) is composed of 238 amino acid residues with molecular weight of 26.9 kDa and exhibits bright green fluorescence by exposure to blue light. Many marine organisms also possess green fluorescent protein but GFP was traditionally refers to the first protein isolated from the jellyfish *Aequorea Victoria*. It has many applications as a reporter of gene expression, to make biosensors.

Potential use of GFP coding genes is to express even in small sets of specific cells which allows the researchers to detect certain specific cell types *in vitro* and *in vivo* optically. Integration of several spectral variants of GFP is a helpful technique to determine the brain circuitry i.e., Brainbow (Livet et al., 2007). Other important functions of these fluorescent proteins include as sensors of neuron membrane potential, tracking of AMPA receptors on cell membranes, detection of viral entry and the infection of individual lenti and influenza viruses etc (Adesnik et al., 2005; Lakadamyali 2003; BJoo et al., 2008). With the above concerned applications the present investigation was carried out for the expression of GFP coding gene and production in to DH_5 α E using 1 liter bioreactor.

MATERIAL AND METHODS

2.1 Recombinant Strain

Recombinant *E.coli* with vector pET (29772) LIC cloning vector (u-msf GFP) made up of 5481 base pairs with ampicillin resistant gene in DH5alpha strain was procured from Add gene Research Laboratories, China has been used in the study.

2. Bioreactor

Bioreactor was purchased from Spectrochem instruments Limited, Hyderabad-India. Total volume of reactor 1 Liter (1000 ml), working volume (600-700 ml)

.2.1 Bioreactor specifications

SCI/FERM/BIOTRON-L1000/VSLO 1L
SCI/FERM/BIOTRON-L1000/EU-2PMP
SCI/FERM/BIOTRON-L1000/Prob-pH/170STER
SCI/FERM/BIOTRON-L1000/Prob-DTMP

Temp. System

SCI/FERM-HTCL/CA/-HT 30L/CL 30L/Tap 30L

2.3 Chemicals

Tris-HCl, SDS, Glycerol, β-mercaptoethanol, Bromophenol blue, Coomassie Brilliant Blue, Methanol, Acetic acid, Acrylamide, Bis-acrylamide, APS, TEMED, ampicillin were purchased from SRL Laboratories, Mumbai, whereas, all other chemicals were purchased were of research grade.

2.4 Reagent Preparation

30% Acrylamide and Bis-acrylamide

7.25 gram Acrylamide + 0.25 gram Bis-acrylamide dissolved in 25 ml of distilled water

1.5 M Tris pH 8.8

4.53 gram Tris-base dissolved in 25 ml of water. The pH of the medium was adjusted to 8.8 with HCl and then sterilized it by autoclaving.

1.0 M Tris pH 6.8

3.025 gram Tris-base dissolved in 25 ml of water. The pH of the medium was adjusted to 6.8 with HCl and then sterilized it by autoclaving.

Running Buffer pH 8.3

3.6 gram Tris-base, 16.8 gram Glycine in 1000 ml d

2.5 Preparation of starter culture

With the help of inoculation loop four to five well isolated colonies of the same morphological type was transferred into 5ml of nutrient broth. The culture was maintained at 37^0C for 24 hrs till a clear visible turbidity is appeared. The turbidity (MC Farland standard ($1x10^8$ to $5x10^8$ cfu/ml)) was used as starting culture for the production of green fluorescent protein.

Following formula is applied for determination of bacterial culture in the sample of inoculums.

$$V_1N_1=V_2N_2$$

Where V_1 = Volume of media in bioreactor/shake-flask
N_1 = Desired initial Optical Density in bioreactor/shake-flask
V_2 = Volume of inoculum to be added
N_2 = Optical density of the inoculum

2.6 Shake flask fermentation

The colonies that contain GFP coding gene are transferred on the LB media in 500 ml conical flask for optimization of various parameters for production of green fluorescent protein. Parameters such as temperature, pH of the medium were optimized and adjusted. The shake flask experiment was carried up to 32 hrs. At end of the experiment the cells were subjected for lysis for the collection and determination of protein.

2.7 Fermentation Using 1 Liter Bioreactor

Colonies of DH_5 *α E.coli* strain with pET (29772) LIC cloning vector (u-msf GFP) during shake flask produced significant amount of GFP was then used for the expression in 650 ml of LB broth (consisting of ampicillin of about 140µl) in 1lit bioreactor. The reactor then under control of all well pre set parameters was started and maintained at 37^0C with constant stirring of about 150 rpm. The samples are collected at intervals for the determination of growth rate (phase of the bacterium) of cells measured at 600 nm.

2.8 Cell Lysis Method

Once the process of fermentation was terminated the cells were harvested and subjected for centrifugation at 14000xg for 2-4 min. Collect the cell pellet and transfer in to 100 micro liters of SDS sample buffer (SDS loading Dye) and followed by heat treatment at 95^{o}C for 5 min. Centrifuge the culture at 14000xg for 5 min. Load 15 micro liters per lane on an SDS polyacrylamide gel for protein determination.

2.9 SDS Page analysis

2.9.1 Sample Preparation

SDS PAGE is carried out after purification of the collected protein by column chromatography. The two glass slides, which were used for gel preparations, were fixed by keeping the spacers in between. The glass slides were tightened by keeping the clips, so that the gel doesn't come out when pored between the glass slides. TEMED was added to the previously prepared resolving gel and poured without any delay. Immediately, stacking gel which was poured into the space provided between the glass slides. Once the gel gets solidified, the water was poured off and the comb is removed. The running buffer was filled up to the mark and the purified protein was loaded onto the wells to commence the set up.

3.0 Result and Discussion

3.1 Shake flask fermentation

During the shake flask studies it was observed that the cells were present in the lag phage during the first 5 hrs of fermentation. There was a sudden and rapid increase of the growth rate after 5.25 hrs. Time course samples were drawn for every one hr and analyzed. The contamination of the culture was also checked every time when sample is taken. LB medium was used for blank and for dilutions. The fermentation process was induced with 1M IPTG (1µl/1ml) and samples were collected for before induction, 6 hrs and 24 hrs of induction. The fermentation was terminated at 33.0 hrs. The maximum optical density was found to be 2.9 after 29 hrs of incubation. Exponential phase was noticed from 5.25 hrs to 28 hrs of fermentation and the specific growth rate was determined by graphical method and was approximately 0.1441 hr^{-1}. The doubling time during the exponential phase was approximately 4.81 hr. After 30.0 hrs, the cell density started decreasing slowly and declining phase was noticed from 31 to 33 hrs. From 28 hr to 30 hrs, the cell density remained almost constant. The maximum amount of protein produced was noticed as 6.9 mg/lit after 31.2 hrs of incubation. The results are shown in Fig 3.

3.2 Fermentation Using 1 Liter Bioreactor

Studies of green fluorescent protein using 1 liter bioreactor revealed that the protein was significantly produced induction after 26 hrs. The cells were lasting in lag phage for about first 4.9 hrs of incubation and there after the growth rate 3.6 was drastically increased from 5.20 to 29.0 hrs (Fig. 4). Cell in the reactor were induced with 1M IPTG (1µl/1ml) and time course samples were measured at 600 nm. After 31.0 hrs, the cell density started decreasing slowly and declining phase was noticed from 31 to 33 hrs. From 29 hr to 31 hrs, the cell density remained almost constant. The fermentation process was terminated at 33.2 hrs, the cells were processed for cell lysis method and collection of crude protein which was purified and estimated. The maximum amount of protein produced was noticed as 9.6 mg/lit after 31.2 hrs of incubation (Fig. 4).

3.3 SDS Page Analysis

The size of GFP is 26.9 KDa. There was no GFP production prior to induction with IPTG as evidenced in lanes 4th and 7th (Fig. 5). After 5 hours of induction, there is production of GFP but not very significant. Thin bands are noticed in lanes 3rd and 6th between 26 and 34 KDa markers. Thick bands were observed in the 2nd and 5th lanes between 26 KDa and 34 KDa markers. These bands are because of GFP and correspond to 24 hours induction. As expected, the longer induction yielded better GFP expression. There effect of centrifugation to remove cell debris has no significant. Overall, the SDS-PAGE results indicate the intracellular expression of GFP up on adding IPTG, further increasing the induction time up to 24 hours can result in increased amounts of the GFP protein production.

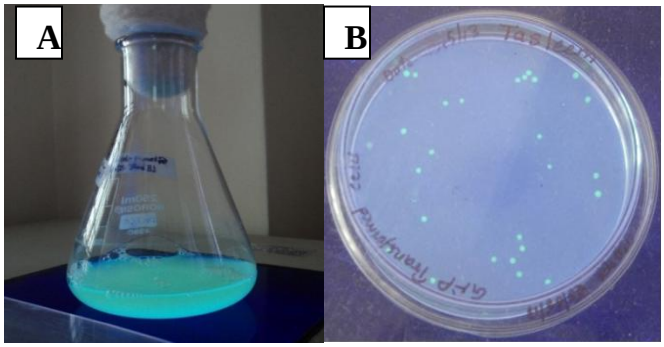

Fig. 1 A- DH-5 α strain exhibiting the Green fluorescence from Shake flask experiment, B-Starter culture used for production of GFP in 1 liter bioreactor

Fig 2 Biotron 1 Liter Bioreactor showing reactor with motor connected to processor unit and compressor

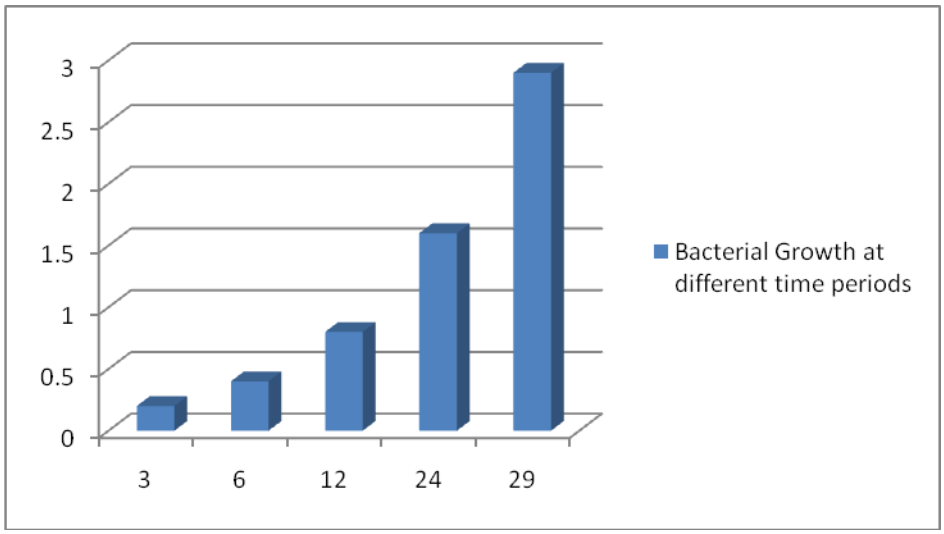

Fig 3 Determination of growth curve of the organism at regular intervals during the Shake flask fermentation of GFP

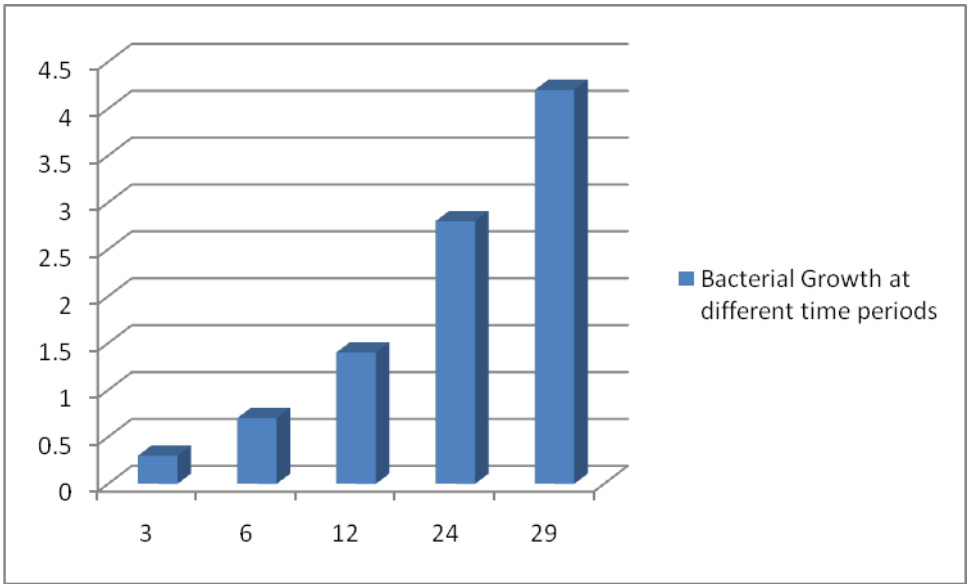

Fig 4 Determination of growth curve of the organism at regular intervals during the fermentation of GFP using 1 liter bioreactor

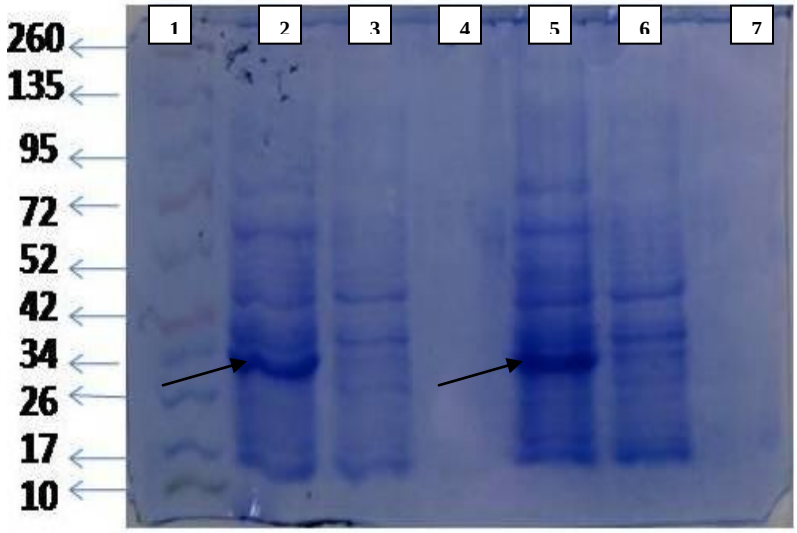

Fig. 5 Lane 4th and 7th with no GFP production prior to induction with IPTG, Thin bands are noticed in lanes 3rd and 6th between 26 and 34 KDa markers. Thick bands were observed in the 2nd and 5th lanes between 26 KDa and 34 KDa markers.

4.0 Conclusion

The present investigation work on optimization of various parameters in shake flask for production of GFP from recombinant E. coli DH5 α strain concludes that the protein production was found more in the reactor compared to the shake flask transformed plasmid into $DH_5\alpha$ *E. coli* produced significant amounts of green fluorescent protein in 1 liter bioreactor at 31.2 hrs.

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