EFFECT OF GROWTH CONDITIONS ON PRODUCTION OF GREEN FLUORESCENT PROTEIN FROM *Escherichia coli* FERMENTATION

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ABSTRACT

Effect of growth conditions (temperature, agitation rate and working volume) on production of enhanced green fluorescent protein (EGFP) from *Escherichia coli* (*E.coli*) fermentation was studied in this research. An improved growth conditions are needed to maximize the functional EGFP production. Preparation of cell culture was done by transferring E. *coli* strain BL21 (DE3) carrying plasmid pRSETEGFP to agar plate by streaking method and incubated at 30°C for 18 hr. Inoculum was prepared from a single colony of *E.coli* from agar plate. Fermentation of batch cultures were carried out in Erlenmeyer flasks inoculated with inocula [5% (v/v)]. The process variables include temperature, agitation rate and working volume were varied throughout the batch fermentation by using one factor at a time method. During the cultivation process, samples were taken from Erlenmeyer flask to measure cell biomass and EGFP concentrations. Cell biomass concentration was determined based on the culture absorbance using spectrophotometer and EGFP concentration was determined using gel-based imaging method. The results obtained shows that EGFP production by *E.coli* decreases as the working volumes was increased from 20 to 50%. However, when the agitation rate was increased from 100 to 250 rpm, it subsequently increases the yield of EGFP and cell concentration. Suitable temperature (30°C) enables *E.coli* to grow well and produce higher amount of functional EGFP. Consequently, functional EGFP production decreased at low temperature (<30°C). At higher temperature (> 30°C) also resulted in a decrease of EGFP and biomass production due to protein aggregation into inclusion bodies. In conclusion, the yield of functional EGFP was highest in shake flask fermentation under condition, working volume of 20%, agitation rate of 200 rpm and temperature of 30°C with 0.04, 0.061 and 0.06 g/L respectively.

ABSTRAK

Kesan keadaan pertumbuhan (suhu, kadar pengadukan dan isipadu ‘medium’) pada pengeluaran ‘green fluorescent protein’ (EGFP) dari penapaian *Escherichia coli* (*E.coli*) telah dikaji dalam kajian ini. Satu keadaan pertumbuhan yang lebih baik diperlukan untuk memaksimumkan pengeluaran EGFP yang berfungsi. Penyediaan sel kultur yang telah dilakukan dengan memindahkan *E.coli* BL21 (DE3) yang mempunyai plasmid pRCETEEGFP kepada bekas agar dengan kaedah ‘streaking’ dan dieram pada 30 °C selama 18 jam. ‘Inoculum’ diperbuat dari koloni tunggal *E.coli* dari bekas agar. Penapaian kultur telah dijalankan dalam kelalang Erlenmeyer yang ditambah dengan ‘inocula’ [5% (v / v)]. Proses pembolehubah termasuk suhu, kadar pengadukan dan jumlah kerja telah diubah sepanjang penapaian dengan menggunakan satu faktor pada satu masa. Semasa proses penapaian, sampel telah diambil dari kelalang Erlenmeyer untuk mengukur kepekatan sel ‘biomass’ dan EGFP. Kepekatan sel ‘biomass’ ditentukan berdasarkan penyerapan kultur menggunakan spektrofotometer dan kepekatan EGFP telah ditentukan menggunakan kaedah pengimejan berasaskan gel. Keputusan yang diperolehi menunjukkan bahawa pengeluaran EGFP oleh *E.coli* berkurang apabila isipadu ‘medium’ meningkat daripada 20 kepada 50%. Walau bagaimanapun, apabila kadar pengadukan telah meningkat dari 100-250 rpm, ia kemudiannya meningkatkan hasil EGFP dan kepekatan sel. Suhu yang sesuai (30°C) membolehkan *E.coli* bertumbuh dengan baik dan menghasilkan jumlah EGFP berfungsi yang lebih tinggi. Manakala, pengeluaran EGFP berfungsi menurun pada suhu rendah (<30°C). Suhu tinggi (>30°C) juga menyebabkan penurunan hasil EGFP dan pengeluaran ‘biomass’ kerana pengagregatan protein. Kesimpulannya, hasil EGFP berfungsi adalah paling maksimum di dalam kelalang penapaian dalam keadaan isipadu ‘medium’ 20%, kadar pengadukan 200 rpm dan suhu 30 °C dengan nilai 0.04, 0,061 dan 0.06 g / L masing- masing.

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LIST OF SYMBOLS

%	Percentage
℃	Degree Celcius
μg	Micro gram
μL	Micro litre
(v/v)	volume/volume
mL	Milli Litre
L	Litre
mg	Milli gram
g	gram
hr	hour
min	min
nm	nano meter

LIST OF ABBREVIATIONS

EGFP	Enhanced Green Fluorescent Protein
OD	Optical Density
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
rpm	Rotation per minute
LB	Luria Bertani
HCl	Hydro chloric acid
NaOH	Sodium hydroxide
E. coli	*Escherichia coli*
A.victoria	*Aequorea victoria*
UV	Ultra violet
n-PAGE	Native Polyacrylamide Gel Electrophoresis

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CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

The aim of presenting this chapter is to present the background of the study together with some others aspects such as the problem statement, objectives and scope of research. All this aspects would be a foundation in order to proceed with analysis in this research.

1.2 BACKGROUND OF STUDY

Green fluorescent protein (GFP) is a type of glowing protein found in photoorgans of *Aequorea victoria (A. victoria)*, a species of jellyfish which has existing for more than one hundred and sixty million years. GFP was first discovered by Osamu Shimomuro in 1960 (Shimomura et al., 1962) and was cloned in 1992 (Prasher et al., 1992). Contemporarily, GFP is cultivated in laboratories as a recombinant protein using bacteria such as *Escherichia coli* (*E. coli), Lactobacilus* and algae. It has been expressed in most known cell types and is used as a non-invasive fluorescent marker in living cells and organisms (Zimmer, 2002). GFP also enable a wide range of applications where they have functioned as a cell lineage tracer, reporter of gene expression and a measure of protein-protein interactions. March and co-workers (2003) have explained that GFP can be used as a transcriptional probe for monitoring non-product information such as temperature, oxygen, pH and nutrient availability in bioprocess technology. Figure 1.1 shows an image of Hydromedusa *A.victoria*, a type of jellyfish and Figure 1.2 shows an image bioluminescence of *A.victoria*.

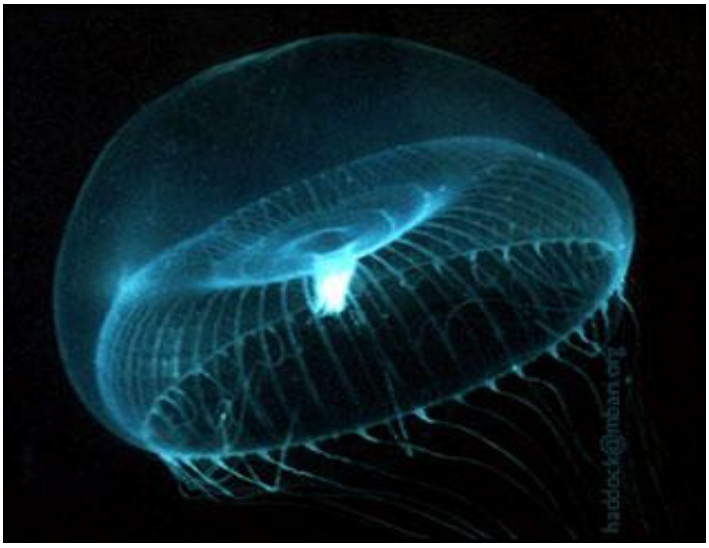

Figure 1.1: Hydromedusa *Aequorea victoria*

(Source: Davenport. D and Nichol. J.A.C, 1995)

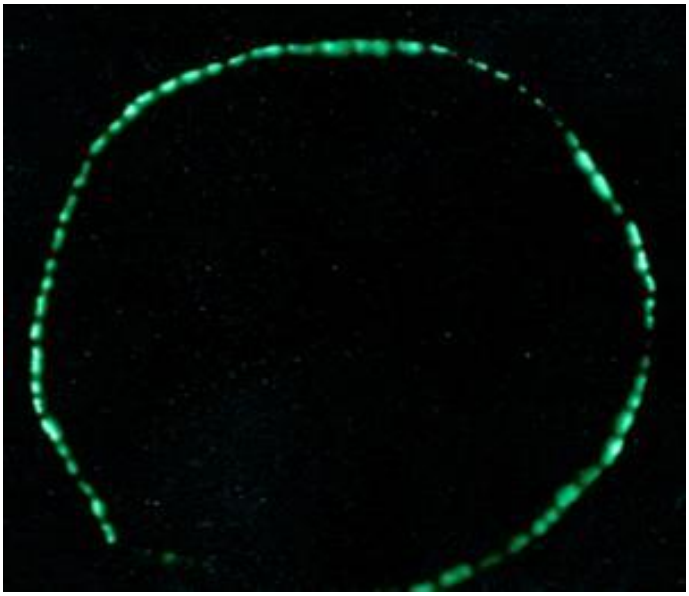

Figure 1.2: *Aequorea victoria* bioluminescence

(Source: Shimomura et al., 1962)

1.3 PROBLEM STATEMENT

Nowadays, the glowing gene revolution has led to significant practical advances in cell biology. It has been estimated that 1 µmol well-folded wild-type GFP molecules are required to equal the endogenous autofluorescence of a typical mammalian cell, where it is double the fluorescence over background noise during the process (Niswender et al., 1995). Patterson et al. (2007) have stated that cultivated protein with improved extinction coefficients can improve its fluorescence by three to tenfold. Therefore, an improved GFP growth conditions are required to maximize its production. The growth of cell and production of GFP can be optimized by varying cultivation conditions such as agitation rate, aeration rate, cultivation period, culture temperature, inducer concentration, pH, time of induction, medium composition, inoculum density and oxygenation (Berlec et al., 2008; Gao et al., 2007; Psomas et al., 2007; Nikerel et al., 2006; Wang et al., 2003; Donovan et al., 1996). This is important in producing high amount of functional EGFP.

1.4 OBJECTIVE

The objective of this research is to study the effect of growth conditions on production of EGFP from *E.coli* fermentation.

1.5 SCOPE

Optimization of various growth conditions for recombinant GFP production was reported in the literature review. Three process variables which include temperature, agitation rate, and working volume were selected to determine how significantly they affect the functional EGFP production in *E.coli* fermentation. EGFP concentration and cell biomass concentration were analyzed to determine the effect of the process variables (temperature, agitation rate, working volume). By varying these variables, the objective of the research was achieved.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

The purpose presenting this chapter is to present a review of past research that related to GFP, its applications and effect of growth conditions which influence the yield of functional GFP. The reviews were done so that this present study attempt can be designed appropriately based on previous literature and achieve the objective of this research.

2.2 GFP

GFP was first isolated from a species of jellyfish *A. victoria* in 1962 where it absorbs ultraviolet (UV) light and gives off longer wavelength green light (Shimomura et al., 1962). In the year of 1992, GFP was successfully cloned from *A. victoria* (Prasher et al., 1992) and used as reporter gene in 1994 (Chalfie et al., 1994). The GFP tertiary structure and its chromophore chemical structure are shown in Figures 2.1 and 2.2 respectively.

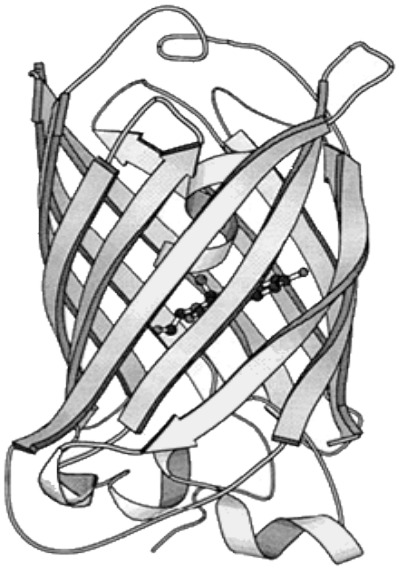

Figure 2.1: The tertiary structure of GFP. (The central darker circles represent the chromophore, while the long flat sheets represent the barrel surrounding it.) (Source: Tsien, 1998)

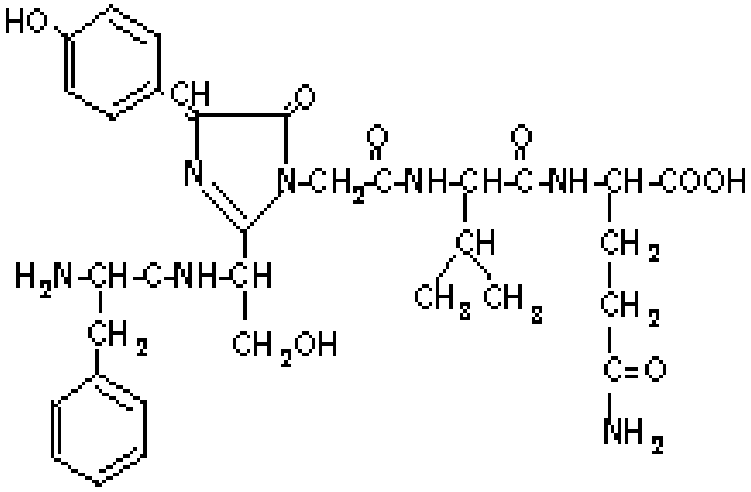

Figure 2.2: The chemical structure of the chromophore in GFP. The cyclized chromophore is formed from the trimer Ser-dehydroTyr-Gly within the polypeptide. (Source: Ward et al., 1989)

Fluorescence of GFP requires no other cofactor except light and oxygen for visualization because the fluorophore is formed from the cyclization of the peptide backbone. This unique characteristic of GFP makes it extremely useful as a biological marker. This feature makes the molecule a virtually unobtrusive indicator of protein position in the cells. Chiu et al. (1996) have stated that GFP fluorescence is species-independent and can be studied in living tissues without cell lysis or tissue distortion. Figure 2.3 shows image of various organism's visualization using GFP without any harm to them.

Figure 2.3: Whole Visualization of Organisms by using EGFP
(Source: Cubitt et al., 1995)

Besides that, Yang et al. (1996) have claimed that EGFP fluorescence is also stable under various conditions, for example temperature up to 65°C, pH range of 3 –12, and in inorganic solvents such as 1% SDS, 8 M urea and glutaraldehyde or formaldehyde.

GFP has been used extensively throughout the biological sciences. One of most common application of GFP is for protein fusion. A fusion between a cloned gene and GFP can be created using standard sub cloning techniques. The resultant single organism (Chimera) can be expressed in a cell or organism. In this way, GFP fusion tags can be used to visualize dynamic cellular events and to monitor protein localization (Lippincott, 2001). EGFP as a tag does not alter the normal function or localization of the fusion partner (Tsien, 1999). March et al. (2003) stated that GFP imparts stability to its fusion partners and allows for facile estimates of protein locale and quantity. Various organisms such as *E.coli* (Patkar et al., 2002), Chinese hamster ovary cells (Hunt et al., 2002) , mammalian cell lines (Kawahara et al., 2002) and Bacillus (Chen et al., 2000) have been studied in researches by using GFP as a host for protein fusion.

GFP was first used as reporter gene in 1994 (Chalfie et al., 1994). A GFP gene which is under the control of a promoter of interest is used to monitor the gene expression. GFP has been extensively used as reporter gene especially in spatial imaging of gene expression in living cells (Sexton et al., 2001).

GFP can be used as transcriptional probe for monitoring non-product information such as temperature, oxygen, pH, and nutrient availability in bioprocess technology (March et al., 2003). Olsen et al. (2002) have showed that a pH-sensitive derivative of the GFP, the designated ratiometric. GFP can be used to measure intracellular pH (pHi) in both gram-positive and gram-negative bacterial cells. In a research by Albano et al. (2001), GFP fusions were constructed with several oxidative stress promoters from *E.coli*.

It has been estimated that 1 µmol well-folded wild-type GFP molecules are required to equal the endogenous autofluorescence of a typical mammalian cell, where it is double the fluorescence over background noise during the process (Niswender et al., 1995). Therefore, an improved GFP growth conditions is required to maximize its production.

2.3 *E. coli* FERMENTATION IN PRODUCTION OF GFP

E. coli is a standard host cell in industrial recombinant protein production bioprocesses. Although, there are many available molecular tools, the easily cultivable, genetically and metabolically well-known *E. coli* still chosen as cultivation host medium. *E. coli* can be grown to high biomass concentrations in the cultures and this can produce high amount of heterologous protein (Makrides, 1996). The characteristics of all *E.coli* strains used in recombinant protein production are reviewed by Waegeman & Soetaert, (2011). Although *E.coli* B and *E. coli* K12 strains are equally used as host for recombinant protein production (47% and 53%, respectively), *E. coli* BL21(DE3) is by far the most commonly used strain (35%) in academic purposes (Waegeman & De Mey, 2012). *E. coli* BL21 (DE3) displays higher biomass yields compared to *E. coli* K12. This results in substantially lower acetate amounts which in return has a positive effect on the recombinant protein production (Shiloach et al., 1996). *E. coli* BL21 (DE3) is used extensively as microbial host for recombinant protein production because it is deficient in the proteases Lon and OmpT. This factor decreases the breakdown of recombinant protein and results in higher yields (Gottesman, 1996). Figure 2.4 illustrate the microscopic image of *E.coli* BL21 (DE3).

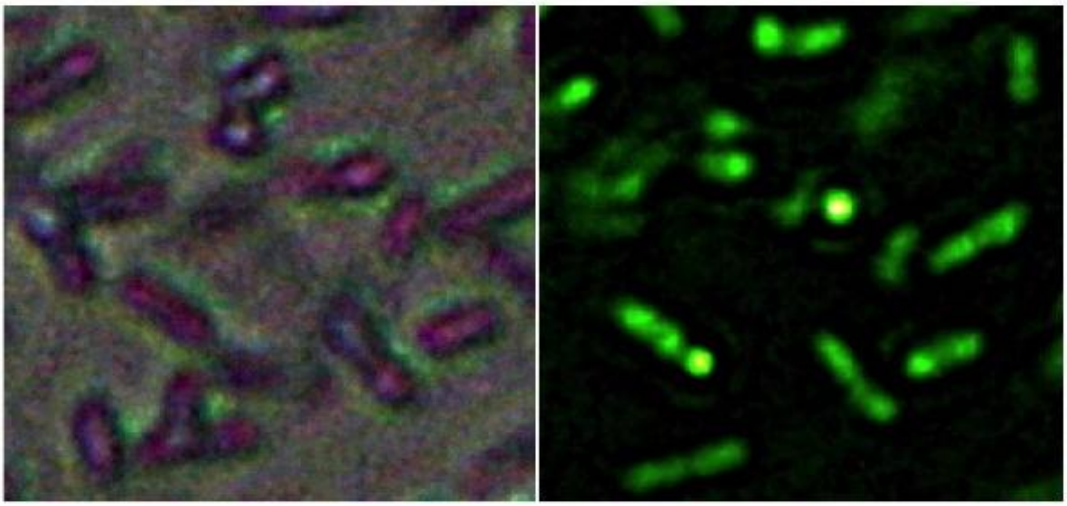

Figure 2.4: Microscopic Image of *E.coli* BL21 (DE3)
(Source: Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology)

2.4 GROWTH CONDITIONS AFFECTING PRODUCTION OF GFP

Many researches have been conducted to study the effect of cultivation conditions on recombinant GFP production. In a research done by Aucoin et al. (2006), culture temperature, induction point, induction duration and the number of induction were considered as factors to maximize GFP production. This research was done by using one factor at a time method. By using the same method, Arellano et al. (2003) have studied the effect of temperature, pH and aeration rate in *Lactobacillus casei*. Oddone et al. (2007) have evaluated the effects of pH, temperature, hemin concentration, concentration of the nisin inducer per cell, and time of induction on GFP production using lactic acid bacteria (LAB) by fed batch fermentation. Chew et al. (2012) have studied the effects of temperature, agitation rate, and time of induction on production of GFP from *E.coli*. In this research, effect of growth parameters (temperature, agitation rate and working volume) on production of EGFP was studied.

2.5 GROWTH PARAMETERS AFFECTING THE FUNCTIONAL GFP PRODUCTION

2.5.1 Temperature

Optimum temperature is important to maximize the production of GFP in *E.coli*. There are several contrast studies about the optimum temperature for GFP production. Low growth temperature which is at 30°C have increased the amount of functional GFP production in *Lactobacillus casei* (Perez-Arellano & Perez-Martinez, 2003) and *Lactococcus lactis* (Oddone et al., 2007). Besides, Aucoin et al. (2006) have concluded that 37°C is an optimal temperature for the maximum production of GFP in *E.coli*. Moreover, Chew et al. (2012) have stated that 31°C is optimized temperature for the GFP production. It has been demonstrated by Waldo et al. (1999) that the GFP can only be emitted when the protein has the correct tertiary structure. According to Webb et al. (1995) and Lim et al. (1995), lower incubation temperature during expression of GFP in bacteria and yeast enhanced GFP fluorescent. Since there was an argument in determining the optimum temperature, a range of temperatures from 25- 40 °C were selected in this research.

2.5.2 Agitation rate

Agitation rate is also an important variable in the production of functional GFP as reported by Wang et al. (2003). Besides improving the mass and heat transfer rate, agitation also provides good mixing efficiency in culture. Chew et al. (2012) have explained that agitation rate could influence the concentration of dissolved oxygen which consequently affects growth of *E.coli* and functional GFP expression. Heim et al. (1994) have reported that oxygen is crucial for the formation of GFP chromophore. Penna et al. (2004) have claimed that highest yield of GFP can be obtained when 100 rpm is used as agitation speed. However, this is contrary to that reported by Chew et al. (2012), where higher agitation speed (206 rpm) can provide better mixing and sufficient oxygen transfer in culture and resulted in higher functional GFP production. Besides that, higher agitation rate can affects the cell growth due to higher shear force applied (Kao et al., 2007). Hence, effect of agitation rates from 100-250 rpm was studied.

2.5.3 Working volume

Working volume of the medium is also one of the significant factors to be optimized for the production of GFP during fermentation. Jin et al., (2004) have stated that working volume of the medium influences the mixing level of culture, and thus affects the growth of the cell and production of particular product. It affects the dissolved oxygen level of the fermentation broth in a shaking flask in which the smaller the medium volume in a shaking flask, the higher the level of dissolved oxygen (Haq & Mukhtar, 2007). Viitanen et al. (2003) and Ekwealor and Obeta (2005) have also optimized 20 and 25 % volume of the medium for the production of lysine and single cell proteins by Bacillus megaterium and *E. coli* respectively. Therefore, working volume of medium of 20-50% was varied in order to study its effect on EGFP production.

2.6 CONCLUSION

The information's obtained from the reviews of different articles and journal could be related to the present research. The facts discovered from these reviews would be helpful in order to compare the obtained results or to produce some new information which is helpful in further research.

CHAPTER 3

METHODOLOGY

3.1 INTRODUCTION

The purpose of presenting this chapter is to present the research materials and methods related to the present study about the effect of growth conditions on production of EGFP using *E. coli*. It revolves around the sample preparations and the procedures. Two types of analysis including EGFP production and cell biomass concentration were done in order to fulfil the scope and objective of this research attempt.

3.2 FLOW DIAGRAM

The overall process including pre laboratory preparations and experimental procedures were shown in Figure 3.1.

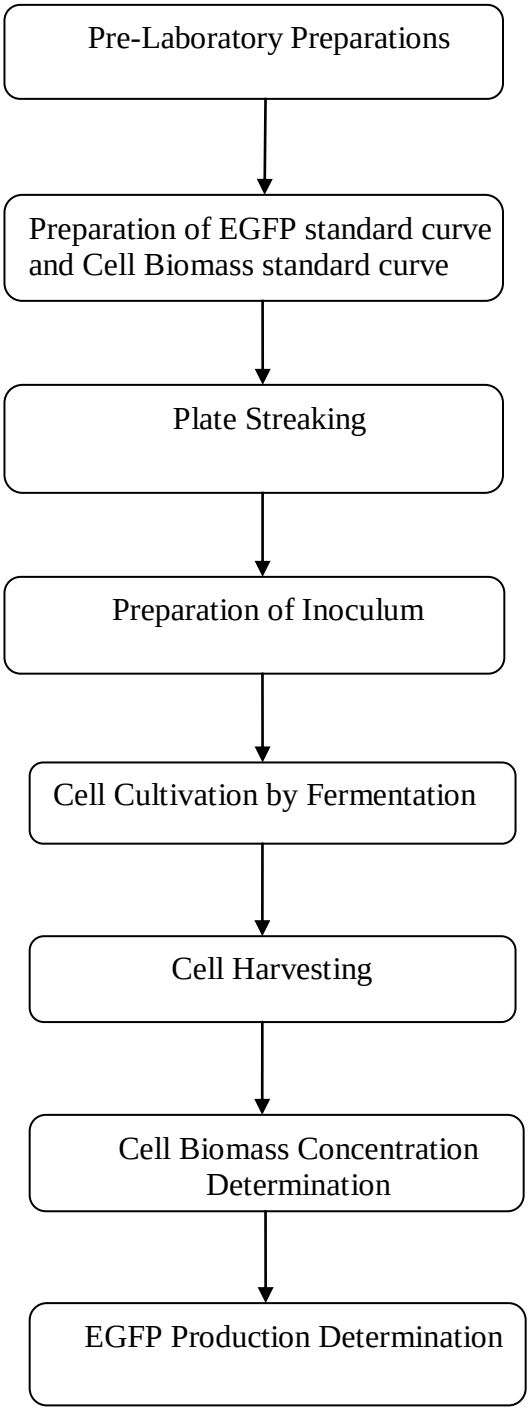

Figure 3.1: Flowchart of research methodology