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

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MALINI A/P SUBRAMANIAM

Thesis submitted in partial fulfilment of the requirements
for the award of the degree of
Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical & Natural Resources Engineering
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JANUARY 2014

SUPERVISOR'S DECLARATION

I hereby declare that I have checked this thesis and in our opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Bachelor of Chemical Engineering (Biotechnology).

Signature :
Name of supervisor : DR. CHEW FEW NE
Position : SENIOR LECTURER
Date : 24 JANUARY 2014

STUDENT'S DECLARATION

I hereby declare that the work in this thesis is my own except for quotations and summaries which have been duly acknowledged. The thesis has not been accepted for any degree and is not concurrently submitted for award of other degree.

Signature :
Name : MALINI A/P SUBRAMANIAM
ID Number : KE10052
Date : 24 JANUARY 2013

Special dedication to my parents, Mr & Mrs Subramaniam Rajasegari

For their endless love, support and encouragement

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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

LIST OF APPENDICES

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*.

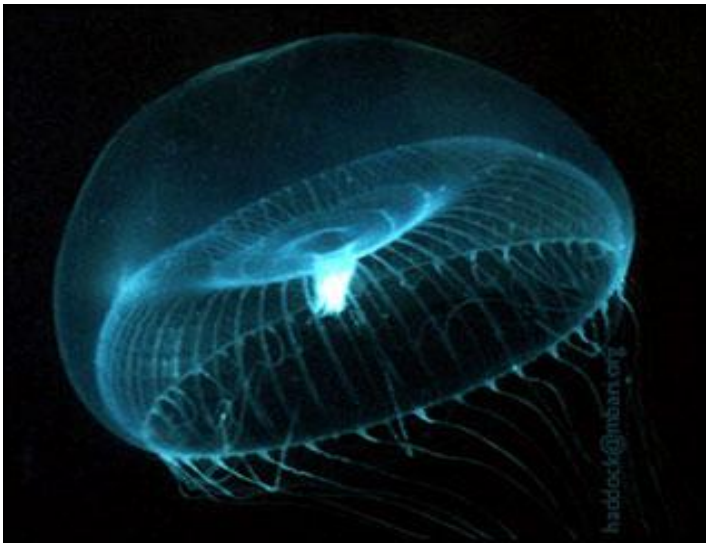

Figure 1.1: Hydromedusa *Aequorea victoria*

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

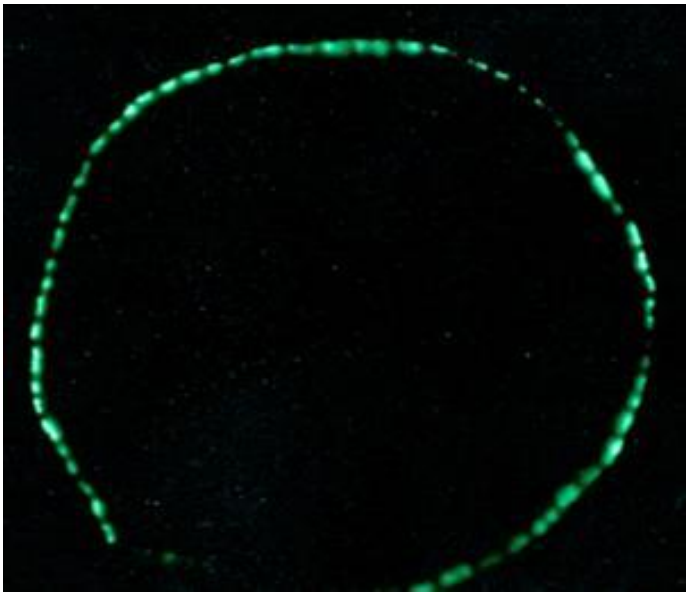

Figure 1.2: *Aequorea victoria* bioluminescence

(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.

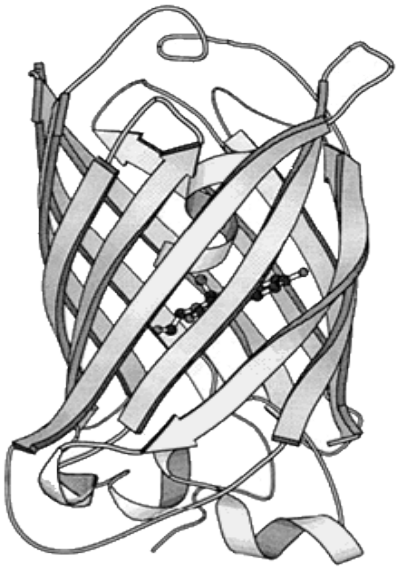

Figure 2.1: The tertiary structure of GFP. (The central darker circles represent the chromophore, while the long flat sheets represent the barrel surrounding it.) (Source: Tsien, 1998)

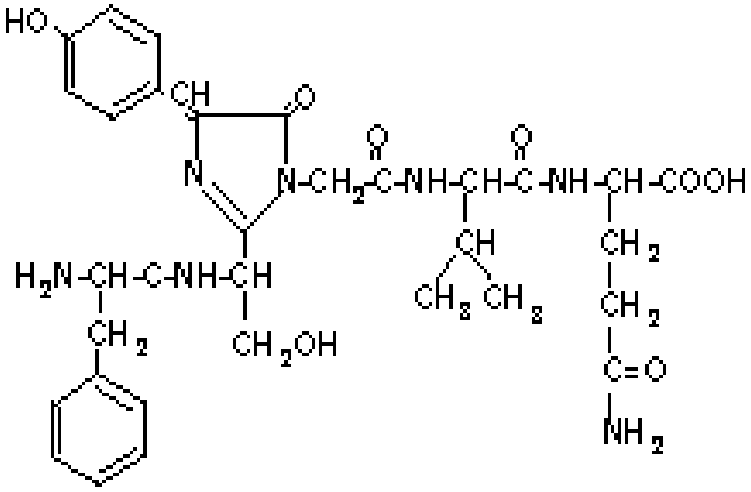

Figure 2.2: The chemical structure of the chromophore in GFP. The cyclized chromophore is formed from the trimer Ser-dehydroTyr-Gly within the polypeptide. (Source: Ward et al., 1989)

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).

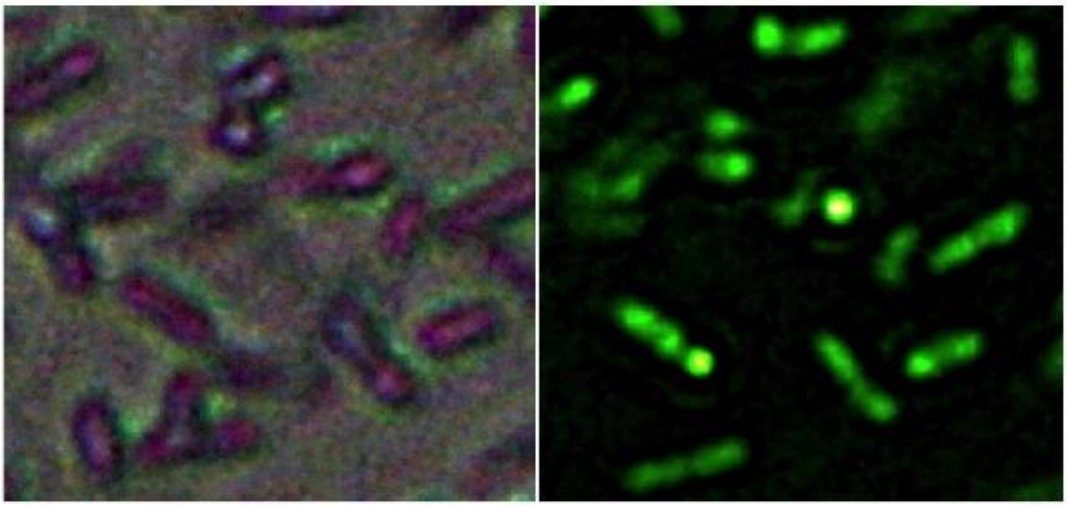

Figure 2.4: Microscopic Image of *E.coli* BL21 (DE3)
(Source: Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology)

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.

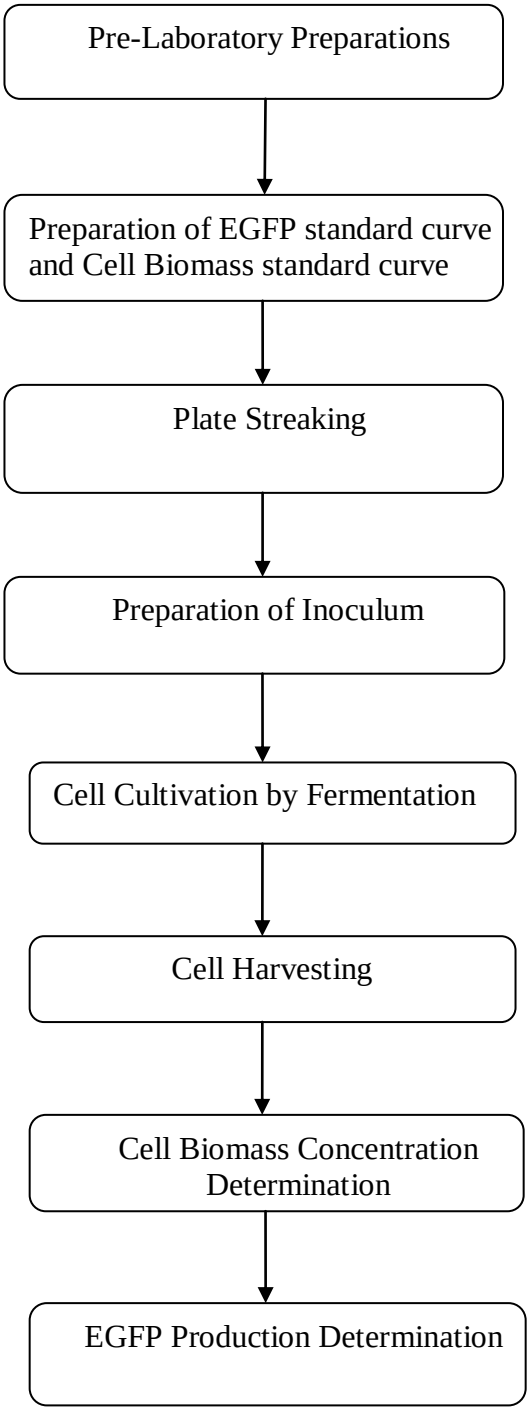

Figure 3.1: Flowchart of research methodology

3.3 PRE-LABORATORY PREPARATION

3.3.1 Apparatus

All the glass apparatus were washed using detergent and dried in the oven. Then, the apparatus were autoclaved at 121°C for 20 minutes. This procedure is crucial to prevent any form of contamination that may affect the fermentation process and lead to deviation in the result obtained.

3.3.2 Agar Plates

10 g of Luria-Bertani (LB) agar powder was weighed and added into 500 mL beaker. Next, 200 mL of distilled water was added into the beaker. Then, the solution is well mixed by using magnetic stirrer for 5 minutes. The well mixed LB agar was poured into measuring cylinder and distilled water was added to 500 mL. The LB agar was poured into clean 500 mL Schott bottle which was then autoclaved at 121°C for 20 minutes. After sterilization process, the autoclaved agar was brought to laminar flow hood and left to cool to about 40°C. 500 µL of 100 µg/mL ampicilin was added into the agar. Ampicilin is an antibiotic to inhibit the growth of other microorganisms. Thus, ampicilin acts as antibiotic for *E. coli* BL21 (DE3) strain. The warm agar was poured into 20 sterile petri plates and was allowed to solidfy. Finally, all the agar plates were sealed with parafilm and stored in 4°C chiller.

3.3.3 Broth Medium

20 g of Luria-Bertani (LB) broth powder was weighed and added into 1000 mL beaker. Next, 500 mL of distilled water was added into the beaker. The solution was well mixed by using magnetic stirrer for 5 minutes. The well mixed LB broth was poured into measuring cylinder and distilled water was added to 1000 mL. The broth was poured into a 1000 mL Schott bottle and autoclaved at 121°C for 20 minutes. The Schott bottle containing broth was cooled to about 50°C and tightly sealed with parafilm. The broth is stored in 4°C chiller.

3.4 EGFP Production

3.4.1 Plate Streaking

The laminar flow hood was sprayed with 70% ethanol and wiped cleanly. Then, all the apparatus was placed under ultraviolet (UV) light in laminar flow hood 30 minutes before starting the plate streaking process. The UV light was turned off and the bunsen burner was lighted up with laminar air flow on. The sterile agar plate was opened in the laminar flow. A loopful of *E.coli* stock culture was obtained aseptically using inoculating loop. It was then streaked on the agar plate. Finally, the petri plate was sealed with parafilm and incubated at 30°C for 18 hrs. Figure 3.2 shows the appropriate streaking technique used.

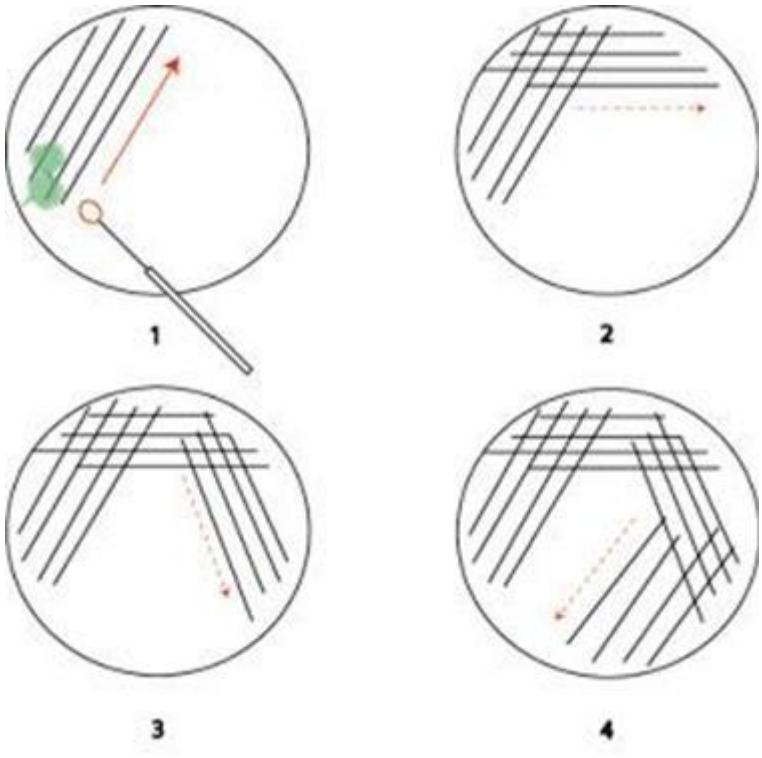

Figure 3.2: Streaking technique

3.4.2 Preparation of Inoculum

20 mL of autoclaved broth was added into a 100 mL Erlenmeyer flask. Then, 20 µL of 100 µg/mL ampicilin was added into the Erlenmeyer flask by using micropipette. Next, single colony of *E.coli* BL21 (DE3) strain was picked from the streaked agar plate by using the tip of the micropipette. The tip was dropped into the flask and its mouth was covered with sterile cotton wool. The flask containing culture was placed in shaker incubator at 200 rpm and 30°C for 18 hours. Figure 3.3 shows the preparation of inoculum throughout the fermentation.

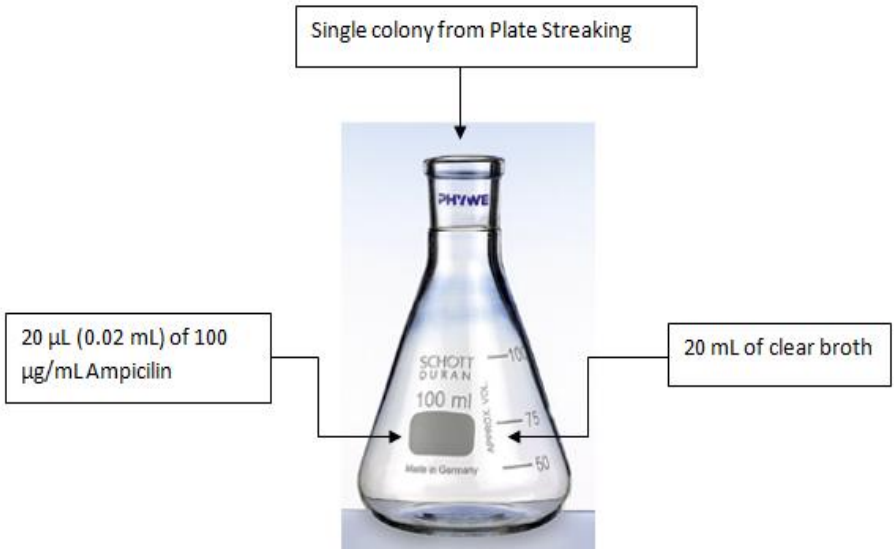

Figure 3.3: Preparation of inoculum

3.4.3 Cell Cultivation by Fermentation

E. coli BL21 (DE3) strain carrying the pRSETEGFP plasmid encoding the EGFP was grown in Luria- Bertani broth with 100 µg/mL ampicilin. 200 mL of clear broth was added into 1000 mL Erlenmeyer flask. Next, 10 mL of inoculum (5% v/v) was also added into the flask. The Erlenmeyer flask was covered with sterile cotton wool. Culture was grown under batch mode operation for 12 hr in a shaking incubator (Ecotron, Infors HT, Bottmingen, Switzerland). The pH of the culture medium was adjusted using HCl and NaOH before inoculation and was not studied throughout the fermentation. Each

experiment was carried out under different conditions as described in Table 3.1 for 12 hr fermentation. For each experiment, isopropyl ß-D-1- thiogalacto pyranoside was added at OD_{600nm} of 0.08-0.1 (after 10x dilution of samples) to induce the expression of EGFP. The experiments was conducted by using one factor at a time method which was started by varying working volume , followed by agitation rate and lastly temperature. All the response values is the mean of duplicate measurements.

Table 3.1: Different conditions applied for 12- hr fermentation

Growth conditions	Range
Temperature (°C)	25, 30, 35, 40
Agitation rate (rpm)	100, 150, 200, 250
Working volume (%)	20, 30, 40, 50

After 12 hours of fermentation in shake flask, culture medium was harvested and continued with analyses of cell biomass concentration and EGFP production rate

3.5 SAMPLE ANALYSIS

3.5.1 Biomass Concentration Determination

3.5.1.1 Preparation of Cell Biomass Standard Curve

The process was started with plate streaking (Section 3.4.1) and followed by preparation of inoculum (Section 3.4.2). Next, 200 mL of sterilized broth was placed in a 1000 mL Erlenmeyer flask. Then, 200 µL of 100 µg/mL ampicilin was added into the flask followed by 10 mL of inoculum (5% v/v). The culture was placed in shaker incubator at 200 rpm and 30°C for 5 hours. After 5 hours, the fermentation product was distributed into six 50 mL centrifuge tubes and is centrifuged at 5000 × g for 30 minutes. The cell pellet was collected from six centrifuge tubes and placed in a 100 mL beaker. Then, 78 mL of autoclaved broth was added into the beaker and a stock solution of cell suspension was obtained. Several dilutions (1x, 2x, 5x, 10x, 15x, 20x) was prepared as shown in Table 3.2 to obtain samples with different concentration of cell suspension.

Table 3.2: Dilutions of the cell pellet stock solution

	1x	**2x**	**5x**	**10x**	**15x**	**20x**
Volume of cell suspension (% w/v)	13	6.5	2.6	1.3	0.87	0.65
Volume of sterilized broth (mL)	0	6.5	10.4	11.7	12.13	12.35
Total Volume (mL)	13	13	13	13	13	13

Figure 3.4 shows the distribution of total volume of each dilution factor.

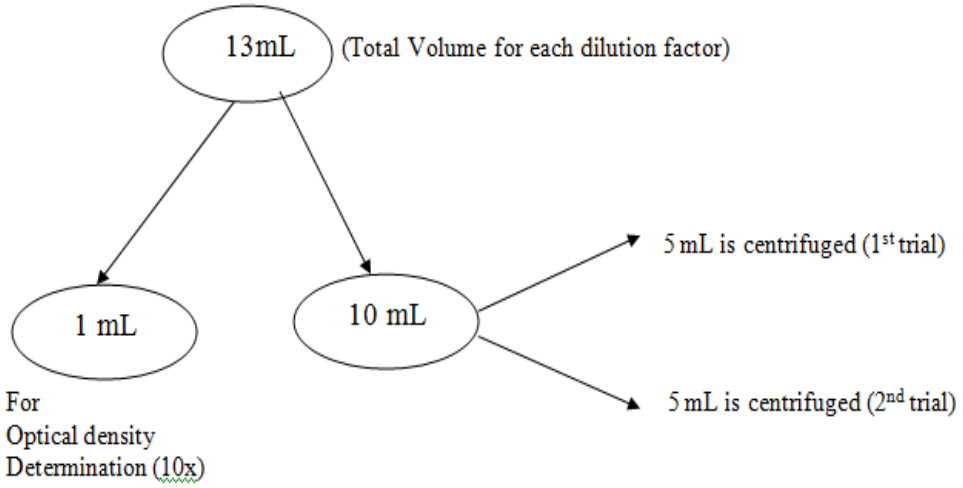

Figure 3.4: Distribution of total volume for each dilution factor

From the 10 mL, 5 mL was centrifuged as first trial at 6000 x g for 25 minutes and the cell pellet was obtained after removing the broth. The centrifuge tube was dried in oven for 48 hours. The dry weight of cell was obtained. The same process was repeated to another 5 mL as second trial. Then, 1 mL from each dilution was taken to determine the optical density (OD) using spectrophotometer (U-1800, Hitachi) at 600 nm. Each dilution was ten times diluted with ten times diluted with sterilized broth before the OD measurement. A standard curve (Figure 3.5) was constructed. Linear equation, $y = 295.83x - 0.0822$ with correlation coefficient, $R^2 = 0.99$ was applied for determination of cell biomass concentration. The raw data were shown in Appendix B.

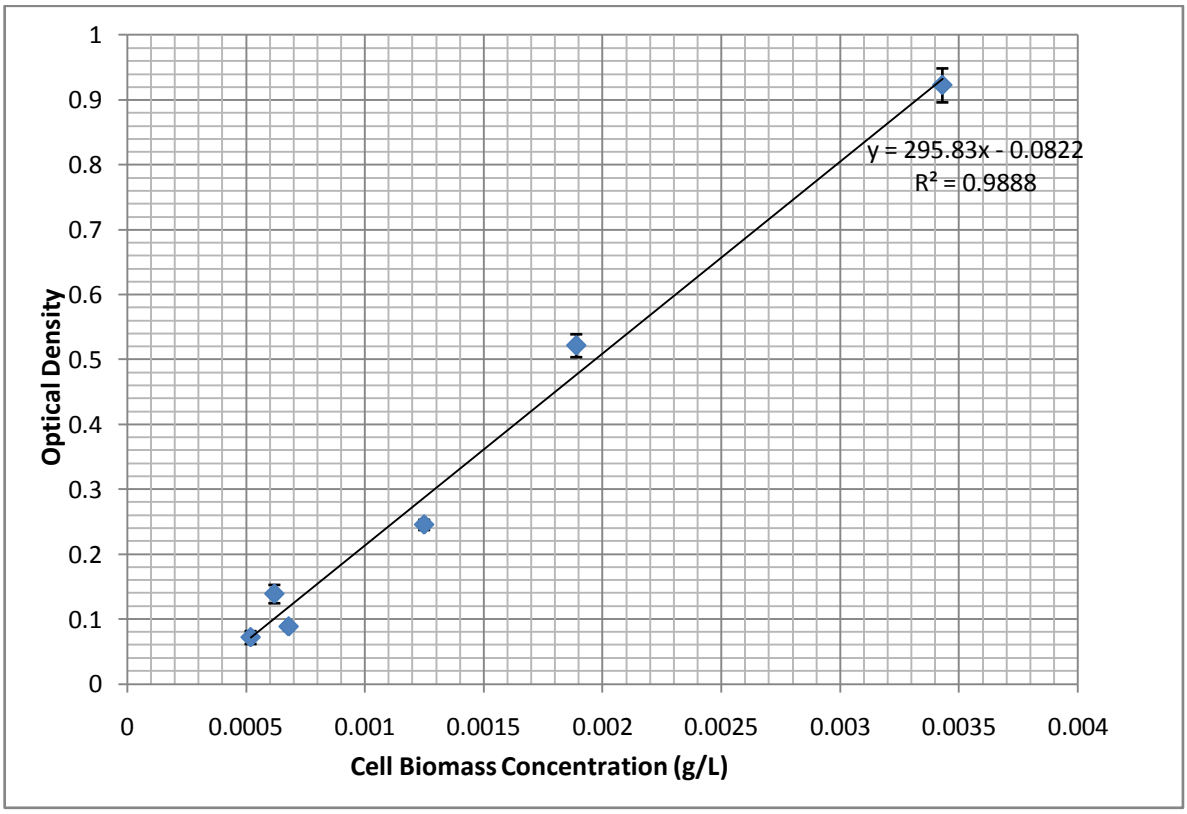

Figure 3.5: Standard curve of optical density versus cell biomass concentration (g/L). Error bars: ± SD of duplicate measurements.

3.5.1.2 Cell Dry Weight Determination

400 µL of samples were diluted (10x) and OD reading were measured with UV-spectrophotometer (U-1800, Hitachi) at wavelength of 600 nm. Cell biomass concentration of samples was determined from standard curve (Figure 3.5).

3.5.2 EGFP Concentration Determination

3.5.2.1 Gel-Based Imaging Method

EGFP concentration was determined based on gel-based imaging method as described in Chew *et al*., (2009). Briefly, native polyacrylamide gel [10 x 10 cm (length x width), 0.20 cm spacer set] with a 4% (w/v) of stacking gel and a 15% (w/v) of resolving gel was prepared by using a OmniPage Mini vertical system (Cleaver Scientific). The composition of chemicals in 15% (w/v) resolving gel and 4% (w/v) stacking gel for native polyacrylamide gel electrophoresis (n-PAGE) is shown in Table 3.3.

Table 3.3: Resolving and stacking gel formulation

	15% (w/v) resolving gel (μL)	4%(w/v) stacking gel (μL)
Acrylamide mix [30% (w/v) acrylamide and 0.8% (w/v) bisacrylamide]	4687.5	622.5
Distilled water, H_2O	2350	2190
4x native Lower buffer [1.5M Tris hydrochloric (pH=8.8)]	2350	-
4x native Upper buffer [0.5M Tris hydrochloric (pH=6.8)]	-	937.5
10% Ammonium PerSulfate (APS)	58.75	25.05
N,N,N',N'- Tetramethylethylenediamine (TEMED)	9.5	5.25

After the polyacrylamide gel was set, EGFP dilution samples were mixed with equal volumes (10 µL) of 2x sample dye (0.125 M 4x native upper buffer, 20% glycerol and 0.01% (w/v) bromophenol blue) and loaded into the well. The equipment was placed into the electrophoresis tank and running buffer (10x Tris- glycine buffer) was poured slowly into the tank until the buffer level was covered the gel. The EGFP samples were electrophoresed at a constant current of 15 mA until the bromophenol blue ran off from the gel.

A Bio imaging system (Alpha Innotech Fluorochem, D.I Scientific) under the same lens control and 48 s of exposure time was used to capture fluorescent image of EGFP on the gel. Quantitation of the fluorescent bands on the gel was done using FluorChem SP quantitation software. Triplicate readings were collected to test the precision of measurements.

3.5.2.2 Preparation of EGFP Concentration Standard Curve

Pure EGFP (Accession number: AAA27722) was diluted into several different concentrations. The formulation of dilutions is showed in Table 3.4.

Table 3.4: Dilutions of Pure EGFP

Dilution	**2x**	**4x**	**6x**	**8x**	**10x**
Pure EGFP	25	12.5	8.3	6.25	5
Sodium Phosphate Buffer	25	37.5	41.7	43.75	45
Total (µL)	50	50	50	50	50

The method used to determine the EGFP intensities were previously described (Section 3.5.2.1). For the purpose of quantitation, a standard curve (Figure 3.6) was constructed. Linear equation, y = 7020.9x + 938.29 with correlation coefficient, R^2 = 0.997 was applied for determination of EGFP production. The raw data were shown in Appendix A.

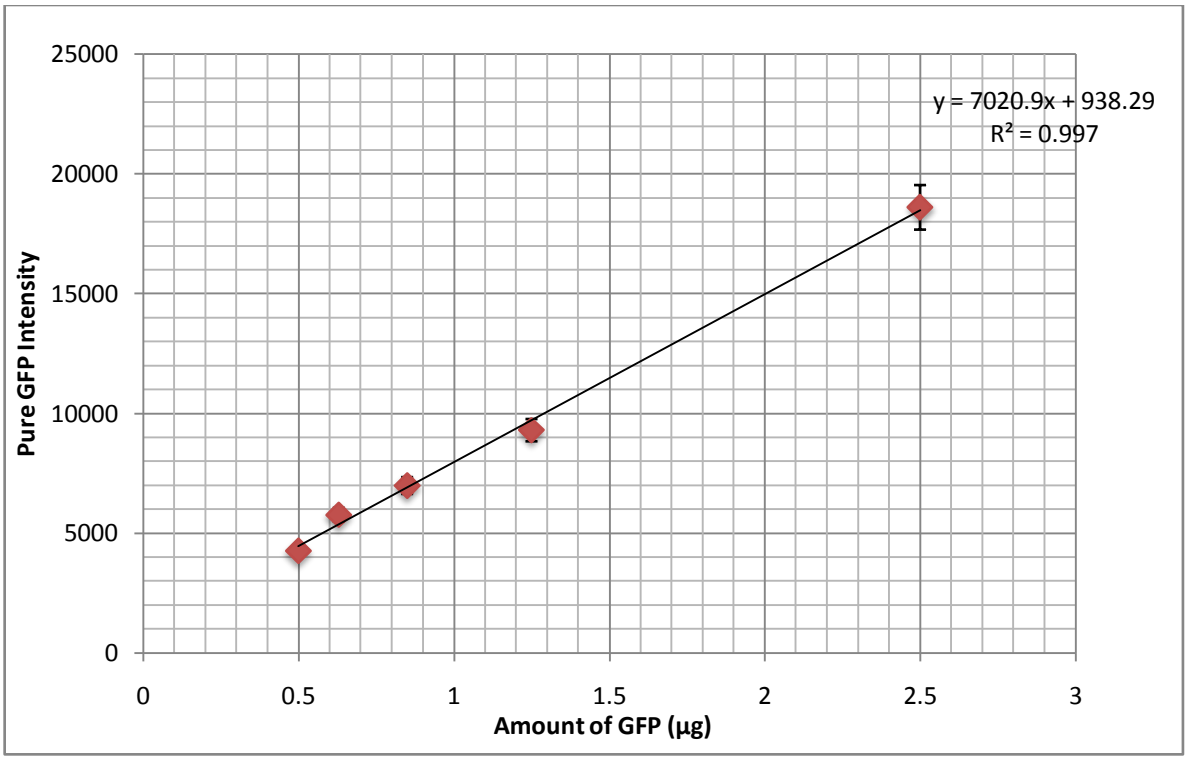

Figure 3.6: Standard curve of EGFP intensity versus amount of EGFP (µg). Error bar: ± SD of duplicate measurements.

3.5.2.3 EGFP Concentration Determination of Samples

For every fermentation process, 1.5 mL of samples were taken and placed in micro-centrifuge tubes which were centrifuged at 10,000 x g for 10 minutes in order to collect cell pellets. The cell pellet was then re-suspended in 200 µL sodium phosphate buffer solution (20 mM sodium phosphate and 0.5 M sodium chloride). EGFP intensity quantitation can be

done by using gel-based imaging method as described in Section 3.5.2.1. The amount of EGFP production was determined from the EGFP standard curve (Figure 3.6).

3.6 CONCLUSION

The information's obtained from the analysis of several materials and methods would be an effective way to conduct the research. The procedures discussed in this chapter were based on the well established method in order to produce an effective result.

CHAPTER 4

RESULT AND DISCUSSION

4.1 INTRODUCTION

The fermentation process was conducted by varying three growth parameters which are working volume, agitation rate and temperature. After 12 hours of cultivation, samples were collected from duplicate runs to determine the cell biomass concentration and green EGFP production rate. EGFP intensity was analyzed using gel-based imaging method (Chew et al., 2009) and the production rate was determined by based on the standard curve of EGFP (Figure 3.2). The optical density (OD) of samples was measured using spectrophotometry. Cell dry weight concentration of samples was determined from standard curve of optical density versus cell dry weight concentration (Figure 3.5).

4.2 WORKING VOLUME

The fermentation process was conducted by using 20, 30, 40 and 50% of working volume where other parameters, agitation rate and temperature were maintained at 200rpm and 30°C respectively. Figure 4.1 shows the EGFP production rate (g/L) and cell biomass concentration (g/L) at different working volumes of medium. The functional EGFP production rate decreased when a higher working volume was applied. The EGFP production at 20% of working volume was the highest (0.04 g/L) and at 50% was the lowest (0.024 g/L) compared to other working volumes. For working volume ranging, from 20 to 30%, the cell concentration was decreased. The biomass concentration was increased

as the working was increased from 30 to 50%. The cell concentration at 50% of working volume was slightly higher compared to 20% with concentration of 0.02 g/L and 0.018 g/L respectively. The raw data is shown in Appendix C.

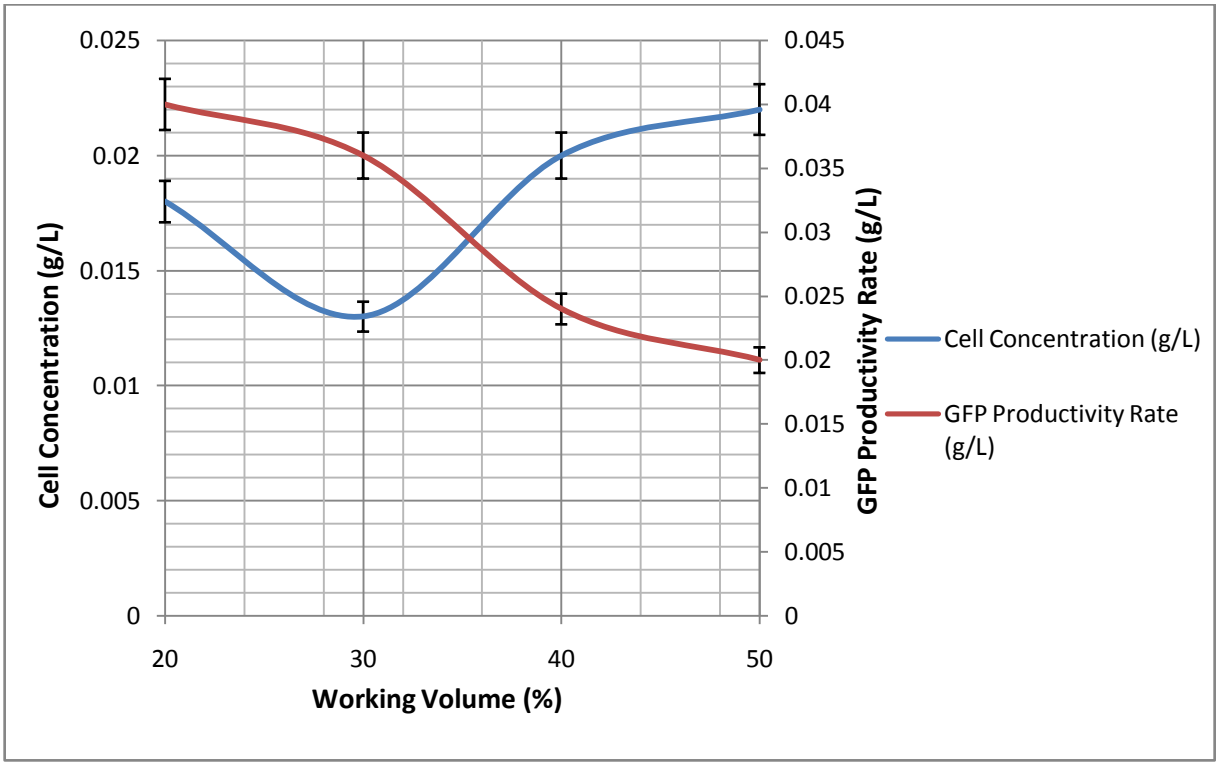

Figure 4.1: Cell biomass concentration and EGFP production rate (g/L) as a function of working volume. The duplicate runs for fermentation were performed at 30°C and 200 rpm.

Roza et al. (2002) explained that working volume of the fermentation flask is one of the significant factors which can directly affect the agitation rate of the culture. Besides that, Jin et al. (2004) have also discussed that working volume of medium affects the growth of the cell and product productions. In this study, maximum EGFP production was obtained when 20% (20ml/100ml flask) volume of the medium was used. Haq and Mukhtar, 2007 had showed similar result for the production of alkaline protease using *Bacillus subtilis*. They have discussed that maximum product was optimal at 20% working volume due to a greater oxygen supply. Working volume can affect the dissolved oxygen level of the fermentation broth in a shaking flask. Oxygen is necessary for formation EGFP

chromophore (Heim et al., 1994). Therefore the smaller the medium volume in a shaking flask, the higher the dissolved oxygen which lead to better EGFP production. EGFP production by *E.coli* decreases as the working volumes was increased (Figure 4.1). Higher volumes of medium such as 50% cause improper agitation (oxygen supply) and affect the EGFP production.

From Figure 4.1, high cell concentration (40 and 50% of working volume) fermentation flask will lead to low functional EGFP production. *E.coli* BL21 (DE3) strain is used as host for recombinant EGFP production in this research. The strain displays higher cell biomass yield in the fermentation (Shiloach et al., 1996). Mansi et al. (1989) have explained that high cell biomass yield does not only imply to a higher conversion to EGFP but sometimes it also accompanied by reduced by-product formation. As the cell biomass concentration increases, the EGFP production decreases may due to the by-product formation during the fermentation. According to Jensen et al. (1990), by-products such as acetate may inhibit the expression of heterologous proteins and consequently decrease protein yield. Besides that, some of the cells are unable to express the EGFP at higher working volumes because of the low dissolved oxygen level. The result obtained is may due to different induction time since the culture was induced once the OD reading reaches 0.08-0.1. Hence, culture in 20% working volume was induced first and followed by 30, 40 and 50%. Induction of IPTG will burden the cell and causes the cell to stop growing or slow growth.

4.3 AGITATION RATE

The fermentation process was conducted at different agitation rate of 100, 150, 200 and 250 rpm by using incubator shaker. From Figure 4.1, 20% of working volume can produce highest amount of functional EGFP. Therefore, fermentation was continued with 20% working volume and the temperature was maintained at 30°C. Figure 4.2 shows the EGFP production rate (g/L) and cell biomass concentration (g/L) at different agitation rates.

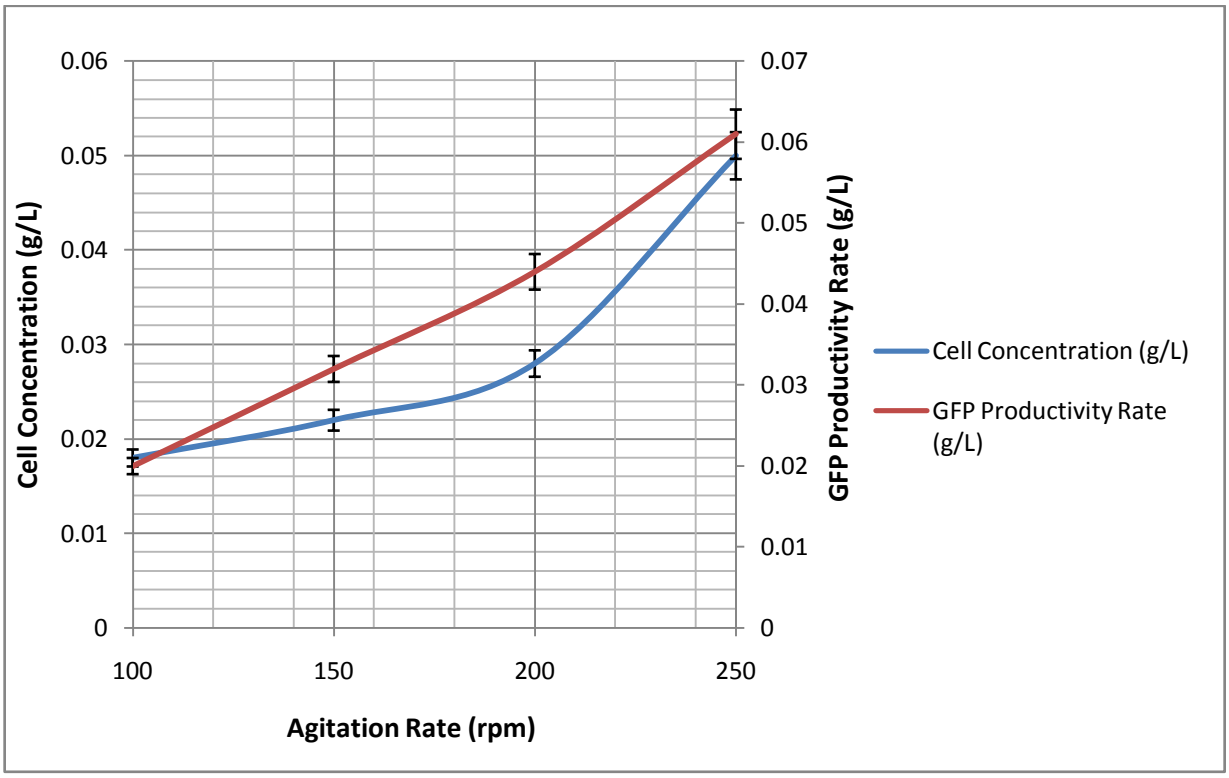

Figure 4.2: Cell biomass concentration and EGFP production rate (g/L) as a function of agitation rate. The duplicate runs for fermentation were performed at 20% working volume and 30°C.

An optimum agitation rate is required to provide thorough mixing of the microbial cells with the nutrients available in the medium. At the same time, it also helps in homogenous availability of oxygen to the cells. The EGFP and cell concentration were increased as the agitation rate increase from 100 to 250 rpm (Figure 4.2). The highest cell concentration and EGFP production were produced at 250 rpm with values of 0.05g/L and 0.061g/L respectively. The lowest cell concentration and EGFP production were produced at 100 rpm with values of 0.018g/L and 0.02g/L respectively (The raw data is shown in Appendix D).

The expression of EGFP was largely growth associated. Castilow (1981) had reported that growth rate and metabolism of aerobic bacteria depends upon the availability of dissolved oxygen. Naturally, oxygen does not dissolve completely in liquid. When propagating bacteria in liquid culture, agitation is required to increase the dissolved oxygen (Brock and Madigan, 1988). At low agitation rate, low functional EGFP production and

cell biomass concentration were obtained. This may due to incomplete mixing and insufficient oxygen transfer in culture (Chew et al., 2012).

Agitation rate not only provides good mixing efficiency in culture but also plays a major role in improving mass and heat transfer rate. Feng et al. (2002) has reported that higher agitation rate increased the amount of dissolved oxygen and dispersion of macromolecules in the medium. Thus, it might have contributed to the higher growth of *E.coli* and better EGFP productivity. This result is further supported by Heim et al. (1994) who reported that oxygen is crucial for the formation of EGFP chromophore.

Other than that, these research fermentations were conducted using lab scale stackable incubator shaker with a maximum speed of 550 rpm. By observing the increasing trend of EGFP production and cell biomass concentration in Figure 4.2, further experiments by using higher agitation rates can be conducted for higher EGFP production. However, if agitation rate higher than 250 rpm is used, it may induce shearing effect on the cells and product degradation as reported by Manolov (1992) and Shioya et al. (1999). This will probably lead to decrease in EGFP production and cell biomass concentration. Hence, optimization of the agitation rate is important to provide optimal EGFP production.

4.4 TEMPERATURE

The fermentation process was conducted at varied temperature of 25, 30, 35 and 40°C. Based on the previous results, 20% of working volume and agitation rate of 250 rpm was maintained. Figure 4.3 shows the EGFP production rate (g/L) and cell biomass concentration (g/L) at different temperatures. The raw data is shown in Appendix E.

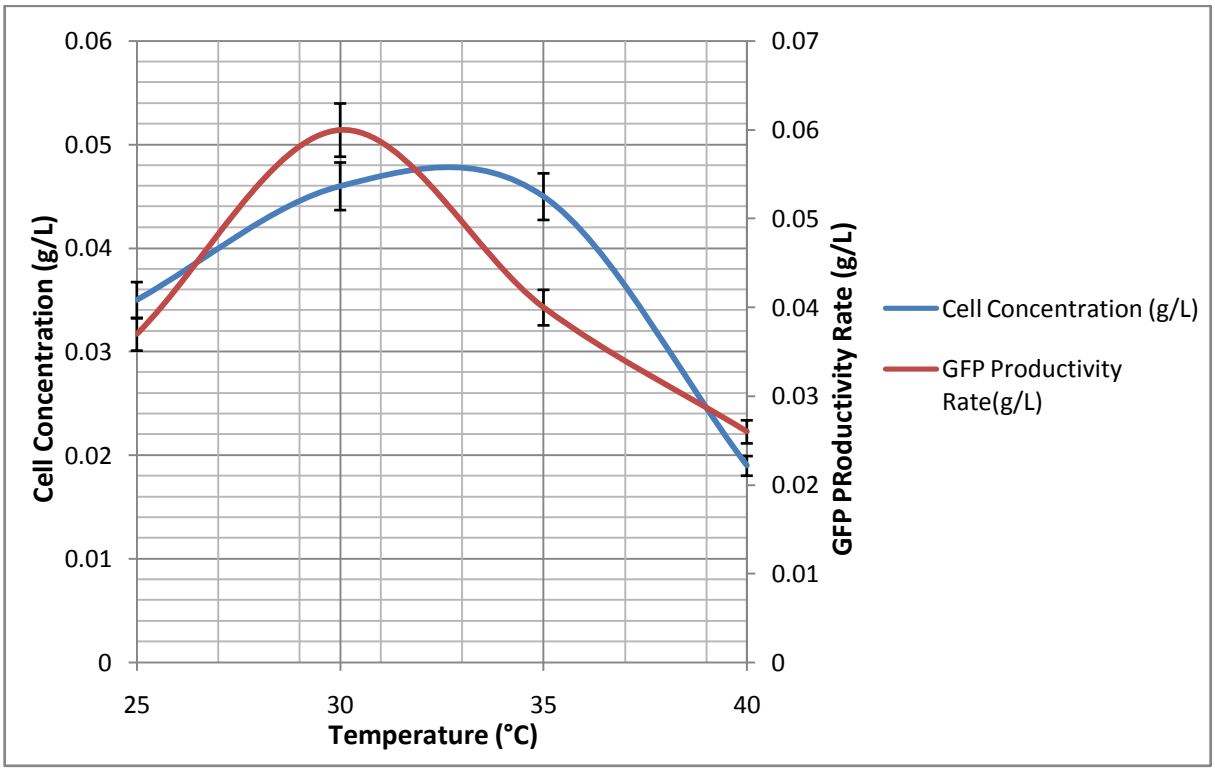

Figure 4.3: Cell biomass concentration and EGFP production rate (g/L) as a function of temperature. The duplicate runs for fermentation were performed at 20% working volume and 250 rpm.

Microorganisms have its favorable growth temperature for their metabolism process. An optimum temperature is important to maximize the growth of *E.coli* and production of EGFP. As demonstrated in Figure 4.3, EGFP production and cell biomass concentration were increased from 25 to 30°C. At low temperature (< 30 °C), low EGFP production and cell biomass concentration were obtained (Figure 4.3). The result may due to slow growth rate of *E.coli* at temperature 25°C (Herendeen et al., 1979). Nedwell (1999) has discussed that growth of microorganism will slow down due to its decreased affinity towards substrate in the medium at temperature below its optimum point.

A fermentation temperature of 30°C gave the optimum fermentation process, at which the EGFP production and cell biomass concentration were 0.06 g/L and 0.046 g/L respectively. According to Arellano and Martinez (2003), temperature at 30°C had increased the amount of functional EGFP yield in *Lactobacillus casei*. Oddone et al. (2007) had reported the similar results as this research by using *Lactococcus lactis* to produce

EGFP. It has demonstrated by Waldo et al. (1999) that emission of EGFP occurs when the protein has the correct tertiary structure. Therefore, the cell has optimum growth and maximum expression of fluoresced EGFP at 30°C. Previous researches done by Webb et al. (1995) and Lim et al. (1995) has reported similar results in which a lower fermentation temperature of bacteria and yeast have enhanced EGFP fluorescence.

A further increase in temperature resulted in a decrease of EGFP and biomass production. This might be due to improper folding of proteins which results in protein aggregation as inclusion bodies at high temperatures in *E.coli* (Siemering et al., 1996). Luo et al. (2006) have reported that EGFP formed inclusion bodies at 37°C when induced with IPTG. Most of the EGFP that been expressed at high temperature was non-fluorescent protein.

4.5 TIME- COURSE STUDY OF CELL CULTIVATION

From this study, the best condition was obtained from each parameter (working volume, agitation rate and temperature) by conducting the experiments using one factor at a time method. Hence, the yield of EGFP and cell biomass concentration was studied in shake flask fermentation for 12 hours under the best condition identified from each parameter (20% working volume, 250 rpm, 30°C). Figure 4.4 shows the time profile of cell biomass concentration and functional EGFP production. At 60 min of incubation time, IPTG was induced into the fermentation medium at OD_{600nm} (0.08-0.1) for expression of functional EGFP. Samples were collected every 2 hour starting after the induction time and analyzed using gel based imaging method in order to determine EGFP production rate. At the same time, the optical density readings of samples were recorded (Refer to Appendix G).

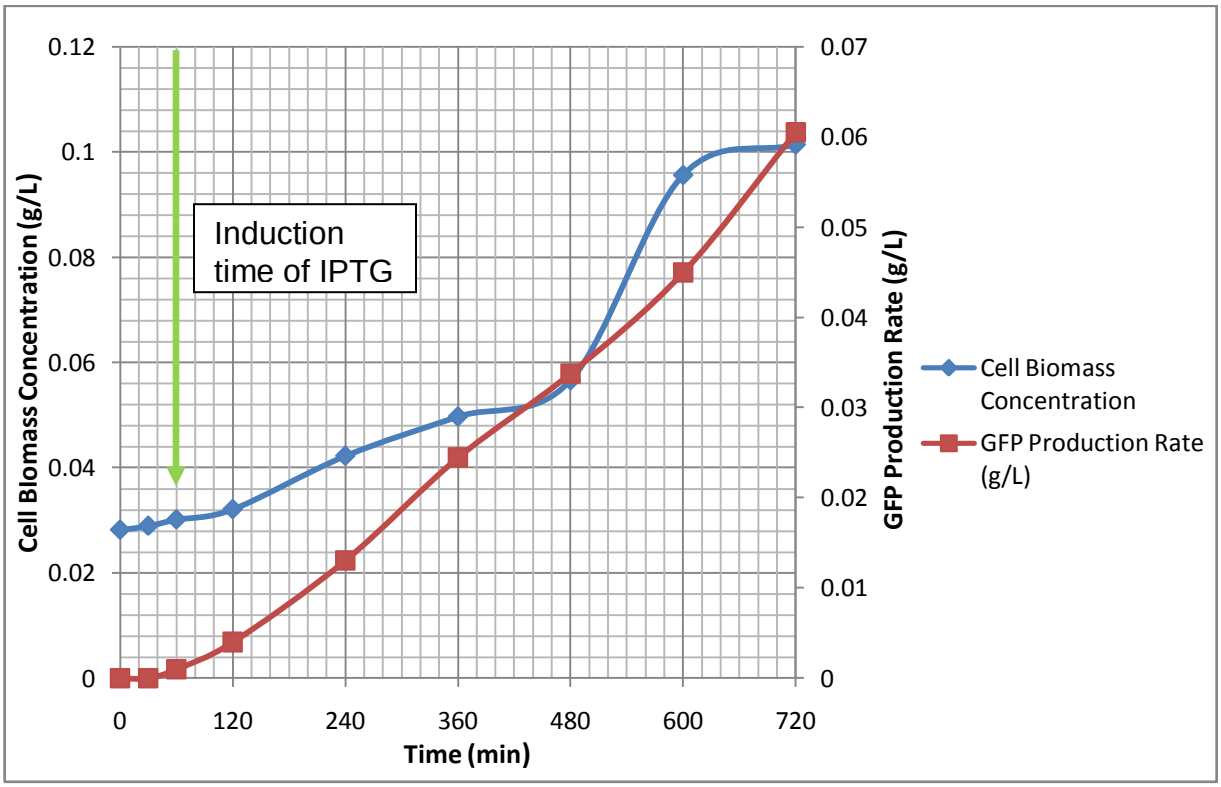

Figure 4.4: Time-course study of cell cultivation at 20% working volume, agitation rate of 200 rpm and temperature of 30 °C using shake flask for 12 hours.

From Figure 4.4, there was low detectable amount of EGFP until 2 hours of post induction period of IPTG. However, the functional EGFP production increases abruptly after 4 hrs of induction period. EGFP fluorescence can only be emitted when the protein has correct tertiary structure. Several hours are required for EGFP to undergo autocatalytic cyclization and proper folding of protein structure (Ferreira et al, 2007). Moreover, increasing trend of functional EGFP production was observed in Figure 4.4. Thus, there is a possibility of obtaining higher production of EGFP at longer fermentation time.

4.6 CONCLUSION

The effects of different cultivation condition are interrelated, and they are important to maximize the EGFP production. The experimental results showed that at 20% of working volume, 250 rpm and 30°C, the production of functional EGFP is the highest with 0.04, 0.061 and 0.06 g/L respectively. Besides that, there is also possibility of increasing EGFP yield at a longer cultivation period.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

Working volume affects the dissolved oxygen level of the fermentation broth in a shaking flask. Therefore the smaller the medium volume in a fermentation flask, the higher the dissolved oxygen which leads to better EGFP production. Hence, EGFP production by *E.coli* decreases as the working volumes was increased from 20 to 50%.

Agitation provides good mixing efficiency and sufficient oxygen transfer in culture. At low agitation rates, low functional EGFP production and cell biomass concentration was obtained. 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. Growth of *E.coli* will slow down due to its decreased affinity towards substrate in the medium at low temperature (25°C). Consequently, functional EGFP production decreased at low temperature. A high temperature (> 30°C) resulted in a decrease of EGFP and biomass production due to improper folding of proteins and formation of inclusion bodies.

In conclusion, the yield of functional EGFP was highest in shake flask fermentation under condition, working volume of 20%, agitation rate of 250 rpm and temperature of 30°C with 0.04, 0.061 and 0.06 g/L respectively.

5.2 RECOMMENDATIONS

This research done is a potential tool to identify best growth conditions to maximize production of functional EGFP. A wide range of studies can be done in the future, which include:-

1.0 Cultivation time can be increased to increase the functional EGFP production.

2.0 Effect of other growth conditions such as time of induction on production of EGFP can be studied.

3.0 Optimized temperature and agitation rate can be scaled up in bioreactor for better EGFP yield.

4.0 Statistical optimization can be done to study the interaction between parameter used and to develop a regression equation.

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APPENDICES

APPENDIX A: RAW DATA OF EGFP STANDARD CURVE

Table A-1: EGFP intensity for duplicate runs

Run / Dilutions	1	2	Average Intensity	Standard Deviation	CV(%)
2x	18606	18605	18605.5	0.7071	0.0038
4x	8297	10303	9300	1418.4562	15.2522
6x	7688	6288	6988	989.9495	14.1664
8x	7371	4160	5765.5	2270.5199	39.3811
10x	4792	3733	4262.5	748.8261	17.5678

Table A-2: Dilution for the standard curve

Dilutions	Concentrations (µg/ µL)	2x dilution (µg/ µL)	Amount (µg) (Times with 10µL)	Average Intensity
2x	0.5	0.25	2.5	18605.5
4x	0.25	0.125	1.25	9300
6x	0.17	0.085	0.85	6988
8x	0.125	0.063	0.63	5765.5
10x	0.10	0.05	0.50	4262.5

APPENDIX B: RAW DATA OF CELL BIOMASS CONCENTRATION STANDARD CURVE

Table B-1: Data obtained before and after drying the cell pellet

	Tube (g)		Tube + Cell (g)		Cell (g)	
Run / Dilutions	**1**	**2**	**1**	**2**	**1**	**2**
1x	5.6079	5.6471	5.6253	5.664	0.0174	0.0169
2x	5.6032	5.718	5.6136	5.7265	0.0104	0.0085
5x	5.7045	5.6474	5.7101	5.6543	0.0056	0.0069
10x	5.6165	5.6675	5.619	5.6712	0.0025	0.0037
15x	5.7248	5.5992	5.7291	5.6017	0.0043	0.0025
20x	5.5553	5.5015	5.5574	5.5046	0.0021	0.0031

Table B-2: Data used to plot standard curve of cell biomass concentration

	Average Cell Dry Weight (g)	Average Cell Biomass Concentration (g/L)	Optical Density (OD)		Average OD	Standard deviation	CV (%)
			1	2			
1x	0.01715	0.0034	0.904	0.941	0.9225	0.0262	2.8361
2x	0.00945	0.0019	0.509	0.534	0.5215	0.0177	3.3898
5x	0.00625	0.0013	0.24	0.251	0.2455	0.0078	3.1683
10x	0.0031	0.0006	0.129	0.149	0.139	0.0141	10.1742
15x	0.0034	0.0007	0.085	0.092	0.0885	0.0050	5.5929
2x	0.0026	0.0005	0.065	0.079	0.072	0.0099	**13.7493**

APPENDIX C- EGFP PRODUCTION AND CELL BIOMASS CONCENTRATION DATA FOR WORKING VOLUME

Table C-1: Raw data of average EGFP intensity

Working Volume (%)	First Run			Second Run			Average Intensity
	1st reading	**2nd reading**	**3rd reading**	**1st reading**	**2nd reading**	**3rd reading**	
20	11 395	11 454	11 861	11 529	11 615	11 467	11554
30	10 080	9759	9666	10776	10 955	10950	10 365
40	7849	7230	7790	6799	7043	7155	7311
50	6257	6095	6385	7074	6957	6426	6233

Table C-2: EGFP production data

Working Volume (%)	Average Intensity	Amount (µg)	÷ by 10µL	Times 2x dilution	Amount µg in 1.5ml (times 200microL)	Production Rate (g/L)
20	11 554	1.512	0.1512	0.3024	60.4806	0.04
30	10365	1.3427	0.1343	0.2685	53.7066	0.036
40	7311	0.9077	0.0908	0.1815	36.3071	0.024
50	6233	0.7541	0.0754	0.1508	30.1654	0.02

Table C-3: Raw data of average optical density (OD) of cell biomass

Working Volume (%) / Runs	20	30	40	50
1	0.452	0.315	0.505	0.584
2	0.444	0.309	0.490	0.578
Average OD	**0.448**	**0.312**	**0.498**	**0.581**

Table C-4: Cell biomass concentration data

Working volume (%)	Average OD	Cell concentration(g/l)	Times 10
20	0.448	0.0018	0.018
30	0.312	0.0013	0.013
40	0.498	0.0020	0.020
50	0.581	0.0022	0.022

APPENDIX D- EGFP PRODUCTION AND CELL BIOMASS CONCENTRATION DATA FOR AGITATION RATE

Table D-1: Raw data of average EGFP intensity

Agitation Rate (rpm)	First Run			Second Run			Average Intensity
	1st reading	**2nd reading**	**3rd reading**	**1st reading**	**2nd reading**	**3rd reading**	
100	6490	5857	6626	5907	6523	6541	6324
150	9655	9082	9691	8845	9851	9732	9476
200	12 964	11876	12 547	12 634	11 955	12 797	12 462
250	17 320	17 047	16 254	17 577	16 026	17 019	16 874

Table D-2: EGFP production data

Agitation Rate (rpm)	Average Intensity	Amount (µg)	÷ by 10µL	Times 2x dilution	Amount µg in 1.5ml (times 200microL)	Production Rate (g/L)
100	6324	0.7671	0.0767	0.1534	306839	0.020
150	9476	1.2160	0.1216	0.2432	48.6417	0.032
200	12 462	1.6413	0.1641	0.3283	65.6537	0.044
250	16 874	2.2700	0.2270	0.4540	90.7901	0.061

Table D-3: Raw data of average optical density (OD) of cell biomass

Agitation Rate (rpm) / Runs	100	150	200	250
1	0.468	0.564	0.745	0.934
2	0.461	0.579	0.763	0.874
Average OD	**0.465**	**0.572**	**0.754**	**0.581**

Table D-4: Cell biomass concentration calculations

Agitation rate (rpm)	OD	Cell concentration(g/l)	Times 10
100	0.465	0.0019	0.018
150	0.572	0.0022	0.022
200	0.754	0.0028	0.028
250	0.904	0.0033	0.050

APPENDIX E- EGFP PRODUCTION AND CELL BIOMASS CONCENTRATION DATA FOR TEMPERATURE

Table E-1: Raw data of average EGFP intensity

Temperature (°C)	First Run			Second Run			Average Intensity
	1st reading	**2nd reading**	**3rd reading**	**1st reading**	**2nd reading**	**3rd reading**	
25	10 502	10 821	11 005	11 263	10 269	10 796	10 776
30	16 605	16 832	16 863	17 028	16 704	16 569	16 767
35	12 733	12 694	12 911	12 843	12 956	12 538	12 779
40	7663	7839	7841	7412	7986	7945	7781

Table E-2: EGFP production data

Temperature (°C)	Average Intensity	Amount (µg)	÷ by 10µL	Times 2x dilution	Amount µg in 1.5ml (times 200microL)	Production Rate (g/L)
25	10 776	1.4012	0.1401	0.2802	56.0481	0.037
30	16 767	2.2545	0.2255	0.4509	90.1805	0.060
35	12 779	1.6865	0.1687	0.3373	67.4598	0.045
40	7781	0.9746	0.0975	0.1949	38.9848	0.026

Table E-3: Raw data of average optical density (OD) of cell biomass

Runs \ Temperature (°C)	25	30	35	40
1	0.452	0.634	0.567	0.197
2	0.444	0.556	0.590	0.210
Average OD	**0.448**	**0.595**	**0.579**	**0.204**

Table E-4: Cell biomass concentration data

Temperature (°C)	OD	Cell concentration(g/l)	Times 20
25	0.448	0.0018	0.036
30	0.595	0.0023	0.046
35	0.579	0.0022	0.045
40	0.204	0.0010	0.019

APPENDIX F- EGFP PRODUCTION AND CELL BIOMASS CONCENTRATION DATA AT BEST CULTIVATION

Table F-1: EGFP production data

Time (min)	Average Intensity	Amount (µg)	÷ by 10µL	Times 2x dilution	Amount µg in 1.5ml (times 200microL)	Production Rate (g/L)
60	1202	0.0376	0.0038	0.0075	1.5024	0.001
120	1991	0.1499	0.0150	0.0300	5.9976	0.004
240	4375	0.4895	0.0490	0.0980	19.5799	0.013
360	7376	0.9169	0.0917	0.1834	36.6774	0.024
480	9835	1.2672	0.1267	0.2534	50.6870	0.034
600	12 779	1.6865	0.1687	0.3373	67.4598	0.045
720	16 874	2.2698	0.2270	0.4540	90.7901	0.061

Table F-2: Cell biomass concentration data

Time (min)	OD	Cell concentration (g/L)	Times (600& 720 min x 20)
0	0.012	0.0028	0.028
30	0.034	0.0029	0.029
60	0.070	0.0032	0.030
120	0.128	0.0032	0.032
240	0.428	0.0042	0.042
360	0.649	0.0050	0.050
480	0.853	0.0057	0.057
600	0.593	0.0048	0.096
720	0.679	0.0051	0.101

APPENDIX G- FLUORESCENT IMAGE OF EGFP CAPTURED USING BIO-IMAGING SYSTEM

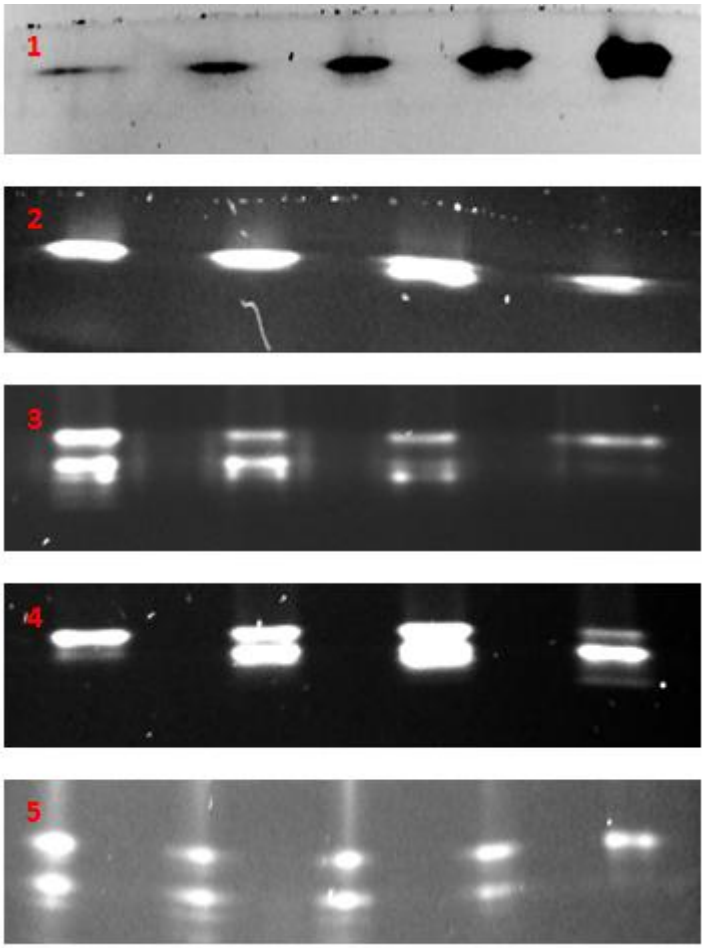

Figure G: Fluorescent image of EGFP on gel (1-Pure EGFP intensity for standard curve,
2-EGFP intensity at different working volumes, 3-EGFP intensity at different agitation rates, 4-EGFP intensity at different temperatures and 5-EGFP intensity at best cultivation conditions)