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EFFECT OF IMIDAZOLE CONCENTRATION AND TYPE OF METAL ION ON THE PURIFICATION OF RECOMBINANT GREEN FLUORESCENT PROTEIN USING AN AFFINITY CHROMATOGRAPHY

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ABSTRACT

The purification of protein using IMAC can be influenced by imidazole concentration and type of metal ion. Different type of metal ion and different imidazole concentration will give different result of purity and yield for target protein. The strength of binding between histidine- tagged protein and metal ion is varies depending on the type of protein as well as the ion used. Imidazole is one of the competitive agents that are effective at displacing protein. Because of this, imidazole is one of the best agent to be used an eluent. Imidazole at low concentration is commonly use to minimise binding of host cell protein and at somewhat higher concentration, imidazole may also decrease the binding of histidine tagged- protein. This paper presents the effect of imidazole concentration and type of metal ion studies on the purification of recombinant green fluorescent protein (GFP) using an affinity chromatography. *Escherichia coli* (*E.coli*) BL21 (DE3) strain carrying the pRSETGFP plasmid encoding the GFP was grown in Luria-Bertani (LB) broth at 30^0C and 200 rpm for 16 hours. An induction of protein expression, Isopropyl β-D-1- thiogalactopyranoside when the cell density (OD_{600}) reaches 0.6-0.8 enhanced the yield of functional GFP production. After harvesting, the cell suspension was disrupted using freeze and thaw method. Clarified GFP was then filter using nylon filter before being purified using a HisTrap™ FF 1 ml column (GE healthcare). The column was charged with different metal ion (0.1 M $NiSO_4$ or $CuSO_4$) and the concentration of imidazole at elution buffer was varied (100, 200, 300, 400, 500, 600 mM). For protein analysis, the sample was analysed using Lowry method for total protein determination, while amount of GFP was quantified using gel-based imaging method. This method has resulted in 95% purity at 500 mM imidazole concentration and 93% recovery of GFP at 300 mM imidazole concentration when the column was charged with Ni(II) ion while 89% purity at 500 mM imidazole concentration and 95 % recovery at 400 mM at imidazole concentration of GFP when the column was charged with Cu(II) ion.

ABSTRAK

Penulinan protein mengunakan IMAC boleh dipengaruhi oleh kepekatan imidazole and jenis ion logam yang digunakan. Ion logam yang berlainan dan kepekatan imidazole yang bebeza- beza akan menghasilkan penulilan dan jumlah protein yang berbeze-beza. protein. Kekuatan pengikat diantara histidine-tag protein dan ion logam adalah berbeza bergantung kepada jenis protein dan ion logam yang digunakan. Imidazole merupakan salah satu agent yang sangat kompetitif dalam menyinkirkan protein Oleh kerana itu,imidazole dipilih sebagai agent yang paling sesuai untuk proses penyingkiran protein . Pada kepekatan yang rendah, imidazole boleh mengurankan kekuatan pengikat bagi protein yang tidak diperlukan semas proses penulin.Pada pekatan yang rendah juga, imidazole boleh megurangkan kekuatan pengikat diantara histidine tag dengan protein.Penulisan ini bertujuan untuk mengkaji kesan kepekatan imidazole and ion logam yang berbeza dalam penulinan GFP dengan menggunakan affinity chromatography. *Escherichia coli* (*E.coli*) BL21 (DE3) strain yang membawa pRSETGFP plasmid dengan kod GFP telah dibesarkan di dalam Luria-Bertani (LB) broth pada suhu 30^0C dan pada kelajuan 200 rpm. Penambahan Isopropyl β-D-1-thiogalactopyranoside (IPTG) pada kepadatan sell (OD_{600}) mencecah 0.6-0.8 telah membantu dalam penghasilan GFP. Sampel GFP kemudianya ditapis dengan menggunakan penapis nylon sebelum sample melalui proses penulinan dengan menggunakan HisTrap™ FF 1 ml column (GE healthcare). Column ini kemudiannya di charge dengan ion logam yang berbeza-beza (0.1 M NiSO4 atau CuSO4) dan kepekatan imidazole yang berlainan (100, 200, 300, 400, 500, 600 mM). Untuk tujuan protein analisis, sampel telah dianalisis dengan menggunakan kaedah Lowry bagi penentukan jumlah protein yang terhasil , manakala jumlah GFP telah diukur dengan menggunakan kaedah gel pengimejan. Kaedah ini telah menghasilkn 95 % GFP pada 500 mM kepekatan imdazole dan 93 % pehasilan GFP apabila ion logam Ni (II) digunakan sebagai cas . Apabila column ini di cas semula mengunakan ion logam Cu (II), 89% penulinan pada 500 mM kepekatan imidazole dan 95% pehasilan pada 400 mM kepekatan imidazole berlaku pada GFP .

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LIST OF ABBREVIATIONS

CV	Column volume
E.coli	*Escherichia coli*
GFP	Green Fluorescent Protein
IMAC	Immobilized metal affinity chromatography

CHAPTER 1: INTRODUCTION

1.1 Background of study

The Green fluorescent protein (GFP) is originally isolated from bioluminescent jellyfish *Aequorea Victoria*. Osamu Shimomura was the first person who recovers this GFP as he started doing his researched on the bioluminescent phenomena of glowing jellyfish in early 1960. Moreover, Osamu Shimomura shows that GFP contain a special chromophore in 1997. Chromophore is a group of chemicals which absorb and emit light. This special chromophore is formed naturally from a tri-peptide motif Ser65, Tyr66, and Gly67 in the primary structure of GFP where its enables GFP fluorescence automatically when expressed in every single organism (Shimomura, 1960). In other words, the tri-peptide-based chromophore in GFP only requires oxygen (O_2) without the needs of enzymes or other auxiliary factors or others protein for it to fluorescence spontaneously. Therefore, the miraculous property of chromophore that found in GFP is responsible for it fluorescence.

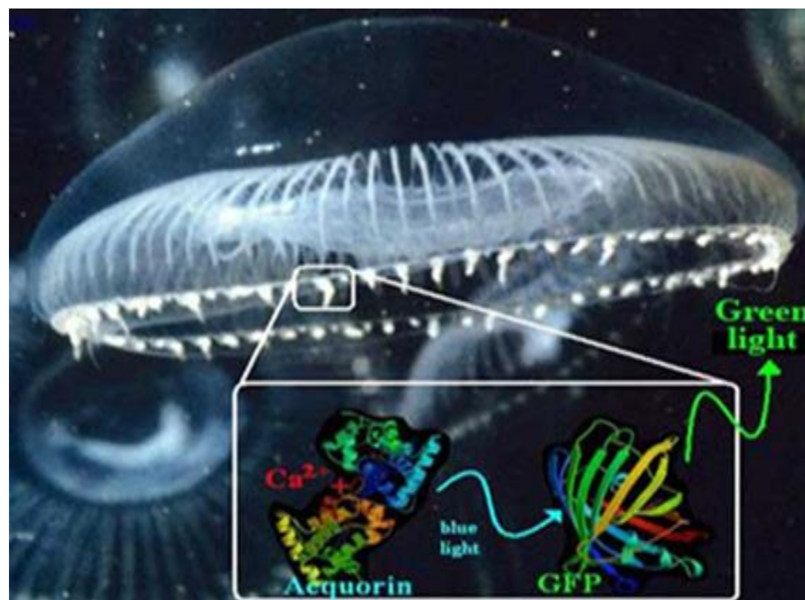

Figure 1-1: Bioluminescent jellyfish *Aequorea Victoria.*

(Osamu Shimamura, 1960)

This characteristic of GFP has been proved by Roger Tsien in his study on 1994. The effectiveness of GFP was first acknowledged by Chalfie *et al.* (1994), who cloned and expressed the GFP in *Escherichia coli* (*E.coli*) and *Caenorhabditis elegans* (*C elegans*). According to Wiedenmann *et al.* (2006), GFP has become one of the most broadly studied and exploited protein in biochemistry and cell biology. GFP has become a favourite marker of gene expression and protein translocation in mammalian, bacterial, and plant system because of its strong intrinsic visible fluorescence (Ha *et al*., 1996).

With its continued use, several reports on GFP recovery and purification methods have emerged, including organic extraction, size exclusion chromatography and ion exchange HPLC (Yakhnin *et al.*, 1998; Deschamps *et al*., 1995). Besides that, the recovery and purification of GFP can also be done using immobilized metal affinity chromatography (IMAC) method. Several advantages of IMAC in protein purification including its stability, high protein loading, mild elution condition, simple regeneration and low in cost (Arnold, 1991).

1.2 Problem statement and motivation

The purification of protein using IMAC is influenced by imidazole concentration and type of metal ion. Different type of metal ion and different imidazole concentration will give different result of purity and yield for target protein (Niebe *et al*., 1997; Chong *et al*., 2009). The strength of binding between histidine-tagged protein and metal ion is varies depending on the type of protein as well as the ion used. Thus, it is necessary for different metal ion to be test for their ability to bind the tagged protein (Niebe *et al*., 1997). Imidazole is one of the competitive agents that are effective at displacing protein. Because of this, imidazole is one of the best agents to be used as an eluent. Imidazole at low concentration is commonly used to minimize binding of host cell protein. However, at somewhat higher concentration, imidazole may also decrease the binding of histidine tagged-protein. Thus, imidazole concentration must be optimized to ensure the best balance of high purity and high yield of the GFP (Clemmit and Chase, 2000).

1.3 Objective

The aim of this study is to investigate the effect of imidazole concentration and types of metal ions on the purification GFP using IMAC.

1.4 Scope

The effect of imidazole concentration (100, 200, 300, 400, 500 and 600 mM) and the types of metal ion (1.0 M $NiSO_4$ and $CuSO_4$) used on the performance of IMAC purification method was studied. By varying the imidazole concentration and metal ion, the purity and yield of GFP were compared in order to identify the best condition for the purification of GFP. In order to calculate the purity and yield of GFP, two analytical methods were used including gel imaging method for GFP quantitation and Lowry method for total protein determination.

CHAPTER 2: LITERATURE REVIEW

2.1 GFP

GFP is a naturally fluorescent protein which can be found in a variety of bioluminescent organism such as the Pacific jellyfish, *Aequoria victoria* (Morin and Hasting, 1971). According to Prasher *et al*. (1992), GFP with 27 kDa is encompassed of total 238 amino acid residue. Three amino acid residues (Figure 2.1) which is Ser65, Tyr66, and Gly67 were the main factor that contributes to the ability of GFP in spontaneously exhibit strong intrinsic fluorescence (Figure 2.1). This amino acid residue will instinctively form fluorescent chromophore in the sequence of GFP (Tsien, 1998).

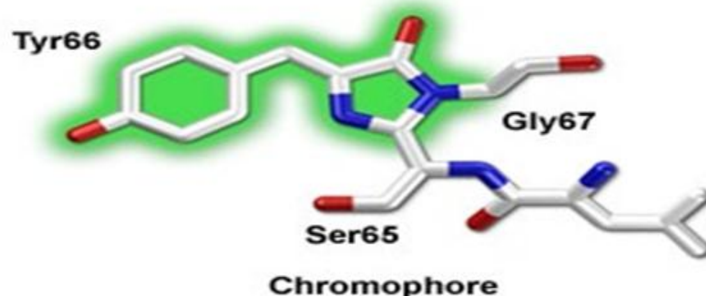

Figure 2- 1: the three amino acid residue (Ser65-Tyr66-Gly67)

(Ziess Campus)

In order for the chromophore to emit the fluorescence, GFP must fold into its native conformation. The attack of nucleophillic towards amide Gly67 on the carbonyl residue 65 has cause the formation of imidazolinone which later lead to the dehydration. The α-β bond of residue 66 is then dehydrogenated into conjugation with the

imidazolinone at the presence of oxygen molecule. This finally resulted in the fluorescence of GFP chromophore (Tsien, 1998).

E. coli and *C. elegans* is the two types of bacteria that have been used to demonstrate the fluorescent form of GFP (Chalfie *et al*., 1994). However, as more research and study has been done, it have been discovered that GFP can also be expressed in the yeast *Sacharomyces cerevisiae*, mammalian cell and Drosophila melanogaster (fruit fly) (Tsien, 1994; Epel *et al*., 1996; Wang and Hazelrigg, 1994).

According to Tsien (1998), in order to have high expression level and detectability of GFP, there are many factors that need to be considered such as promoter, codon usage, and splicing. The higher amount of protein per cell will be produced if the large number of gene is copied and the stronger promoter is used for its transcription. However, for plant and mammalian system, there is need for the codon to be altered so that cryptic splice site can be removed in the plant and the expression level in the mammalian system can be improved (Haseloff *et al*., 1997; Zolotukhin *et al*., 1996; Yang *et al*., 1996).

2.2 Application of GFP

GFP was first used as a reporter gene in 1994. Since that time, GFP has always been a favourite gene for the researcher. The most commonly used of GFP is for the entire organism visualization. Once the gene of GFP is expressed in the interest organism protein gene, the location, movement and others activities of the protein can be followed using microscopic by monitoring the GFP fluorescent (Wang and Hazelrigg, 1994). This fusion gene will not affect the activity of the interest protein as well as fluorescence of GFP (Chalfie *et al*., 1994).

Based on the research done by Elliot *et al*. (1998), using the sugarcane transformed with GFP-S65T, GFP have been used to replace antibiotic selection which later become a great used when the organogenesis or conversion segments of transformation procedures are inefficient under antibiotic or herbicide selection. Moreover, the development of GFP biosensors has become a great advantage for the small molecule detection in both environmental and bioprocess engineering (Kim *et al*.

2002; Stiner and Halverson, 2002; Eggeling *et al*., 2001). All this proved the GFP is important toward biotechnology industry.

2.3 Fundamental principle of affinity chromatography

Affinity chromatography was first introduced by Cuatrecasas *et al*. (1998). Since that day, affinity chromatography has been developed to become a powerful tool for the purification of substance in a complex biological mixture. This tool helps to separates a desired protein from the affinity ligand that coupled to chromatographic matrix. These biochemical separations depend on the reversible interaction between the protein to be purified and its cognate ligands. According to Dorsey and Cooper (1994), these reverse interaction is an interaction of hydrophobic protein (mobile phase) with an immobilized hydrophobic ligand (stationary phase) in a polar aqueous phase which is reversed from normal phase chromatography where usually, a hydrophilic polar ligand will bind to molecules in a hydrophobic nonpolar mobile phase. A basic purification step using affinity chromatography is shown in Figure 2.2.

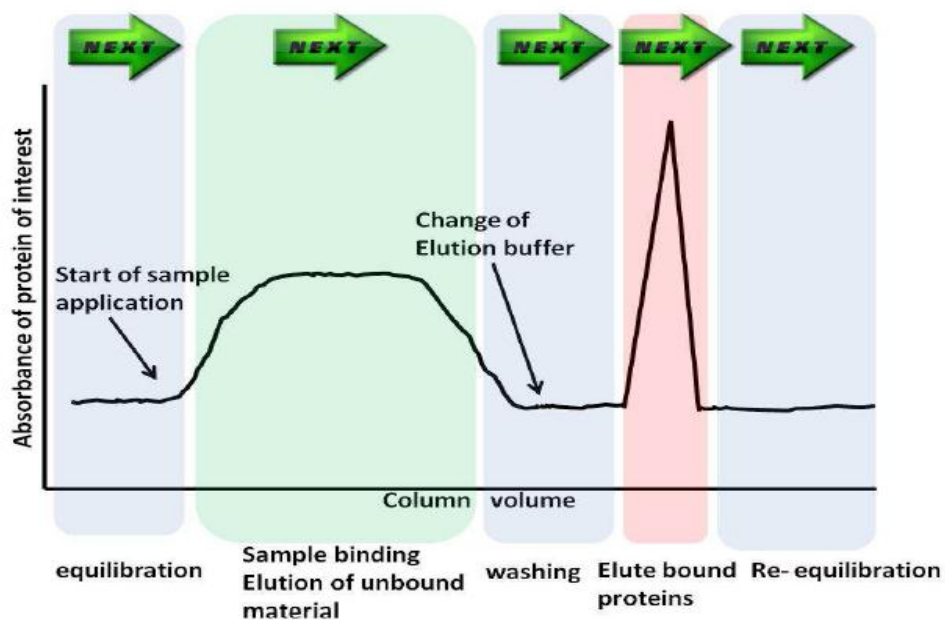

Figure 2- 2: Basic purification step using affinity chromatography

(Sameh and Annette, 2012)

The purification using affinity chromatography begins with the equilibration of its column by binding buffer. The purpose for this equilibration step is to ensure that the affinity interaction between the protein and immobilized molecules occurs in an optimum condition. After the sample is loading into the column, a washing step is applied to remove all unwanted substance and left out the desired protein who still attached to the affinity support. Then, desired protein is obtained by eluted it using high concentration of free ligand that will compete for the binding site of the protein (Luana *et al*., 2012).

2.4 Component of affinity medium

Common porous material such as agarose, polymethacrylate, polyacrylamide, cellulose, and silica are those support materials that have been used in affinity chromatography (Figure 2.3). Nevertheless, there are several factors that need to be taken into account when choosing a support material. The factors are chemical inertness, chemical stability, mechanical stability, pore size and particle size. Chemical inertness for the material support is required in order to ensure that the affinity support bind only to the interested molecule and no nonspecific binding (Sameh and Annette, 2012).

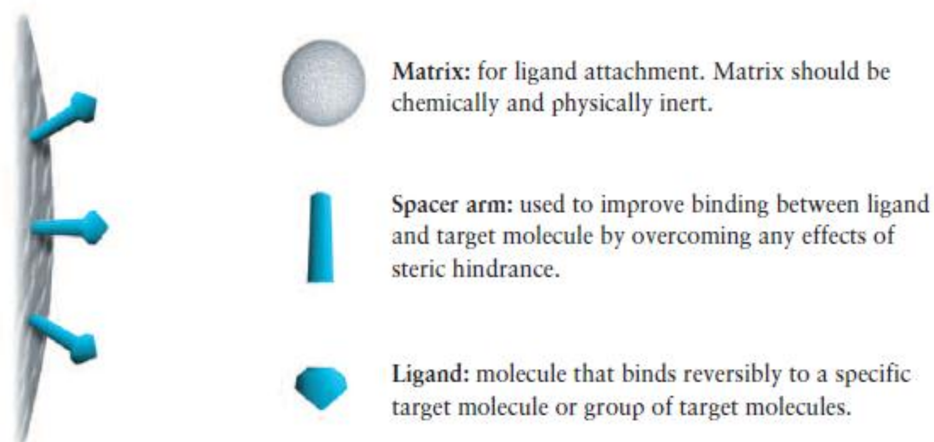

Figure 2- 3: Component of affinity medium

(GE healthcare, Affinity chromatography: Principle and method)

Next, the material support for affinity chromatography must be chemically stable so that it can resist to the degradation cause by all enzyme and microbes, elution buffers, regenerating solvent and cleaning agent that will be used in the affinity column. Besides that, the material support must also able to sustain the backpressure that occurs during the separation process without compressing. To ensure that a large amount of affinity ligands is allowed to immobilize on the surface of the support, small particles size of support materials is preferred compared to the large size (Sameh and Annette, 2012).

According to Renkin (1954), the diameter of the pore should at least 5 times larger than the diameter of biomolecule that being purified. This is must to ensure that biomolecules of the interest can be fully interacting with the affinity ligand. The spacer arms must not be too short or too long as it will affect the binding of biomolecule of interest which later result in the failure of its binding with the ligand. Last but not least, the ligand used must be able form reversible complexes with the protein to isolate.

2.5 IMAC

IMAC was first introduced by Porath and his coworkers in 1975 under the name of Metal chelate affinity chromatography. According to Chaga (2001), there is a lot of papers emerged, defining the use of this principle not only for group separations, but also can be used as a highly selective purification tool for target proteins from complex biological. Since from its first introduce until now, IMAC have become a technology with a very broad portfolio of applications. Some of the application of IMAC are immobilized metal ions in agarose and capillary electrophoresis, (Goubran *et al*, 1992), immobilized metal ions on soluble chelating polymers for selective precipitation and in two-phase systems, based on the introduction of the immobilized metal ion in either the PEG or dextran phase, (Van Dam *et al.,* 1989), and the purification of DNA and oligonucleotide derivatives tagged with histidine residues.

2.6 Principle of IMAC

IMAC is a separation principle that utilizes the differential affinity of proteins for immobilized metal ions to affect their separation. This differential affinity develops from the formation of coordination bonds between metal ions and certain amino acid side chains on the surface of the protein molecules. In addition, as the interaction of immobilized metal ion and amino acid side chain is already reversible. Thus, the utilization for adsorption and then denaturing can be done (Chaga, 2009). Most commonly used, transition-metal ions are Cu (II), Ni (II), Zn (II), Co (II), and Fe (III) which are electron-pair acceptor and can be considered as Lewis acid. Usually iminodiacetic acid (IDA) and nitrilotriacetic acid (NTA) are two chelating agent that is commonly used in the IMAC application (Westra *et al*., 2001). However, IDA is a commercially accessible from many producers since it forms a stronger protein binding (Valdka *et al*., 2001).

2.7 Metal ion

According to Pearson (1973), in IMAC field, metal ion can be classified based on the principle of hard and soft acid and base (HSAB). This principle state that, when two atom form a bond, one of them will act as Lewis acid and the other one act as Lewis base. Metal ion also can classify into three categories, hard, borderline or intermediate and soft based on their preferential reactivity toward nucleophile. At first it was reported that hard type of metal ion is used for the purification of protein. However, later on it is reported that immobilized Ca (II) ions is use for the purification of Lectin (Borrebaeck *et al*, 1981; Borrebaeck *et al*, 1984). Furthermore, the difference between affinity intermediate metal ions and hard metal ions is studied and the natural extension of this observation is used for the purification of calcium binding protein by using IMAC (Mantovaara *et al.*, 1991; Chaga *et al.*, 1996).

Table 2- 1: Types of metal ions and its preferential activity

Categories	**type of metal ion**	**preference**
hard	Fe (III), Al (III), Ca (II), Mg (II), and K (I)	oxygen, aliphatics nitrogen and phosphorous
borderline	Cu (II), Ni (II), Zn (II), and Co (II)	coordinate aromatic nitrogen, oxygen and sulphur
soft	Cu (I), Hg (I), and Ag (I)	sulphur

2.8 Imidazole concentration

Imidazole is a type of competitive agent together with histidine or amine group such as glycine. Imidazole is chose as an eluent because it is inexpensive, does not affect subsequent purification step and does not scavenge metals from the bed (Clemmit and Chase 2000). Tan *et al.* (2006) is using imidazole concentration as one of his parameter that need to be optimized in order to obtain the optimum condition for the yield and purity of Nucleocapsid protein. Chong *et al*. (2009) also used imidazole as one of her parameter in the purification of Nucleocapsid protein. However the result is different for both researchers as Tan *et al*. (2006) obtain optimum production of yield and purity at 350 mM while Chong *et al.* (2009) achieve optimum protein of yield and purity at 300 mM.

CHAPTER 3: MATERIALS AND METHODS

3.1 Overview

In this section, the experiment procedure will be explained in details. All chemicals used in this experiment were obtained from the faculty of engineering and natural resources (FKKSA) laboratory. However some of them were purchased from Sigma Aldrich (Malaysia). Experiment ware done in FKKSA laboratory for 14 weeks. Moreover, all the apparatus used for this experiment were borrowed form FKKSA store that were located inside the FKKSA laboratory. In order to ensure all the experiment were doing in appropriate manner by using the equipment properly, the training were taken under a guidance from lecture engineer (" Jurutera Pengajar" JP). Therefore, the experiment can be safely done.

3.2 Material

Table 3- 1: Chemicals for each process

Process	Chemical
Feedstock preparation	▪ Hydrochloric acid, HCl, ▪ Sodium hydroxide, NaOH, ▪ Luria-Bertani (broth) powder, ▪ Luria bertani (broth) agar, ▪ Inducer-isopropylthio-ß-D-galactoside (IPTG), ▪ Ampicilin
Native PAGE	▪ Acylamide, ▪ Lower buffer (1.5 M, Tris hydrochloride, pH 8.8) ▪ Upper buffer (0.5 M, Tris hydrochloride, pH 6.8) ▪ Tris-glycine buffer ▪ TEMED, ▪ Butanol, ▪ Ammonium persulfhate, ▪ Pure GFP
Lowry method	▪ Using Lowry Reagent ▪ BSA as standard protein
Purification	▪ Sodium chloride, NaCl ▪ Imidazole, $C_3H_4N_2$ ▪ Sodium phosphate dibasic, Na_2HPO_4. ▪ Sodium phosphate monobasic, NaH_2PO_4. ▪ Ethylenediaminetetraacetic acid, EDTA ▪ Isopropanol, C_3H_8O ▪ Sodium hydroxide, NaOH ▪ Copper sulphate, $CuSO_4$ ▪ Nickel sulphate, $NiSO_4$ ▪ Ethanol, C_2H_5OH

3.3 Block flow diagram for overall process

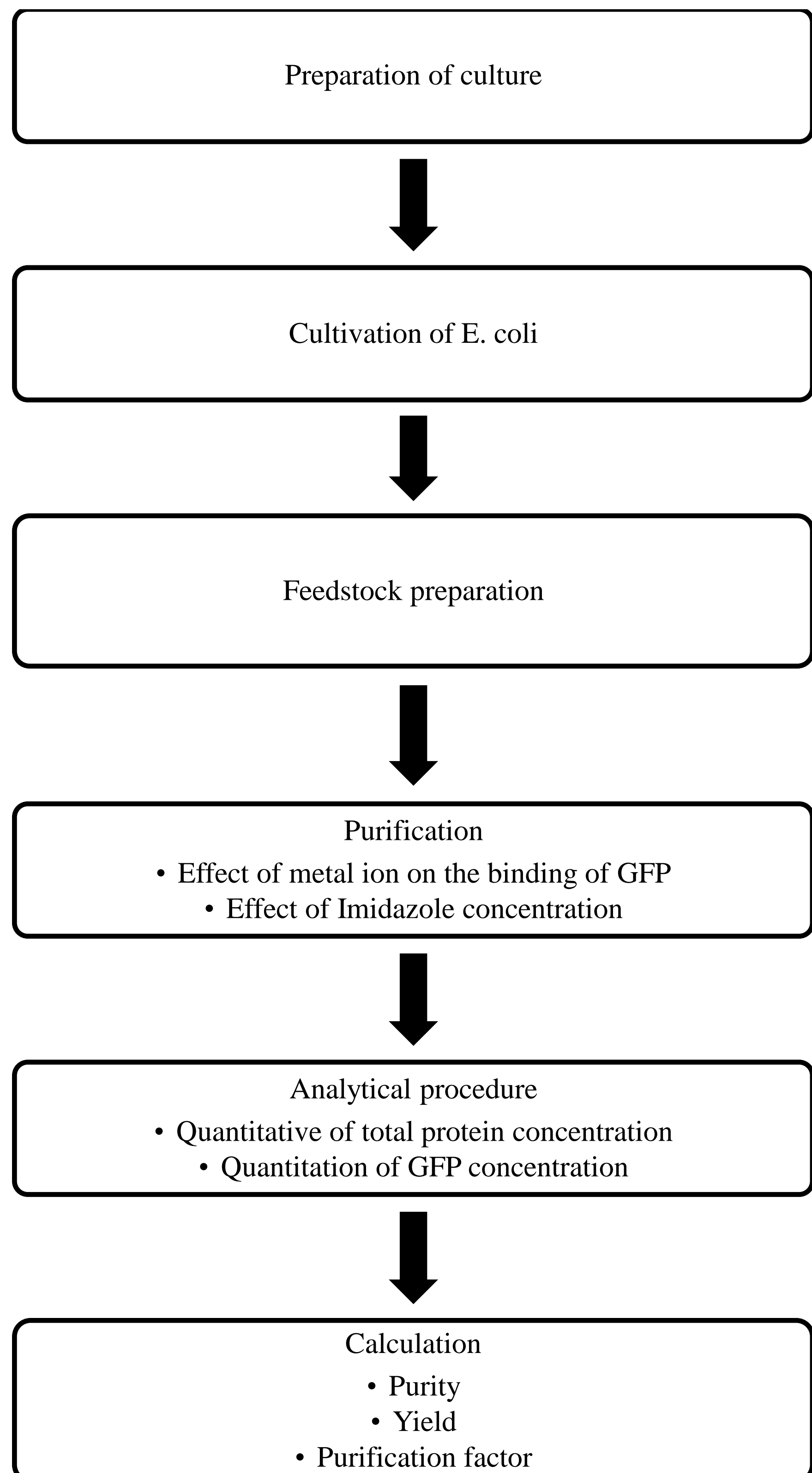

Figure 3- 1: Block flow diagram for overall process

3.4 Method

3.4.1 Preparation of culture

The nutrient agar was prepared by mixing 8 g of Luria-Bertani broth with 400 mL of distilled water. The powder was dissolved completely in water using magnetic stirrer before sent for autoclaving at 121°C for 15 min. After heat sterilisation process, the nutrient agar was allowed to cool for several minutes before pouring it into petri dish. The medium agar was allowed to solidify at room temperature. *E.coli* strain BL21 (DE3) carrying the pRSETGFP plasmid encoding enhanced EGFP was transferred to agar plate by streaking method, aseptically and seal with parafilm. Incubate at 37^{0}C for 24 h**.**

3.4.2 Cultivation of *E. coli*

Single colony of *E. coli* from agar plate was grown in 50 ml of Luria-Bertani broth with 100 µg/ml of ampicillin. The broths were then put into the incubator shaker (Ecotron, INFORS HT) at 30^{0}C and 200 rpm for 18 h. The pH of the culture medium was adjusting using hydrochloric acid (HCl) before inoculation and it was not controlled throughout the fermentation. The prepared inoculum was transfer to 1000 ml of Erlenmeyer flask (ratio of media/flask volume = 0.2) and grown for 16 h by shaking at 30^{0}C and 200 rpm. Protein expression was induced by adding Isopropyl ß-ᴅ-1-thiologalactopyranoside (IPTG) after optical density ($OD_{600\ nm}$) reached 0.6-0.8. After 16 h cultivation the cells were then harvested by centrifugation at 5000 x g for 30 min at 4^{0}C (Lee and Tan, 2008).

3.4.3 Feedstock preparation

The cell pellets were washing with sample buffer (20 mM sodium phosphate buffer at pH 7.4) and later were centrifuged at 5000 x g for 30 min at 4^{0}C. After that, the cell pellets were suspended in sample buffer (20 mM sodium phosphate buffer at pH 7.4) at 10% (w/v) biomass concentration. Later, the cells were then disrupted using

freezing and thawing method. The sample were frozen in the chiller at -80^{0}C for 30 min and then placed in the water bath for 30 min for thawing. The cycles were repeated for 3 times. The cell debris was then removed by centrifugation at 12,000 x g for 10 min at 4^{0}C. Finally the sample were filtered using 0.45 µl of nylon filter before proceed to the purification process.

3.4.4 EGFP Purification

3.4.4.1 Effect of metal ion on the binding of EGFP protein

HisTrap^{TM} FF 1 ml column (GE healthcare) were first stripped with 10 column volume (CV) stripping buffer (20 mM sodium phosphate, 0.5 M NaCl, 50 mM EDTA, pH 7.4) and wash with10 CV binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4) followed by 10 CV of distilled water before the column was recharged with different types of metal ion (0.1 M $NiSO_4$ or $CuSO_4$). The column was then washed and equilibrated with 5 CV of distilled water and 5 CV binding buffer before applying the sample. After the samples were loaded into the column, the samples were then washed with 15 CV of binding buffer. Eluted protein fractions were collected using 5 CV elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 100-600 mM imidazole, pH 7.4) and analysed for the amount of GFP and total protein. The purity and yield of GFP obtained in the purified fraction were then calculated as described by Chew *et al*. (2009).

3.4.4.2 Effect of imidazole concentration

Prior filling the feedstock into the HisTrap^{TM} FF 1 ml column, the affinity supports were first equilibrating with binding buffer. GFP lysate (5 ml) was then loaded into the column. The column was then washed 15 CV binding buffer to remove loosely bound protein. After washing step, the GFP was eluted from the column using 5 CV elution buffer with different concentration of imidazole (100, 200, 300, 400, 500, and 600 mM). Eluted protein fractions were collected and analysed for the amount of GFP and total protein.

3.4.5 Analytical procedure

3.4.5.1 Quantitative of total protein concentration

Protein determination was carried out using Lowry reagent with bovine serum albumin (BSA) as a standard protein (Lowry *et al.*, 1951). The concentration of BSA is prepared at 200, 500, 1000, 1500 and 2000 µg/ml. 1 ml of Lowry reagent was added into 0.2 ml of each concentration of BSA, mixes well and left at room temperature for 10 min. Then, 0.1 ml of 1.0 N Folin-Ciocalteu reagent was added into the solution, and left at room temperature again for 30 min. Optical density of the mixtures was measured at 750 nm using spectrophotometer (Hitachi, U 1800). A calibration curve (OD versus BSA concentration) was plotted and the amount of protein for various samples was obtained by comparing the OD with the standard curve (Figure 3.2).

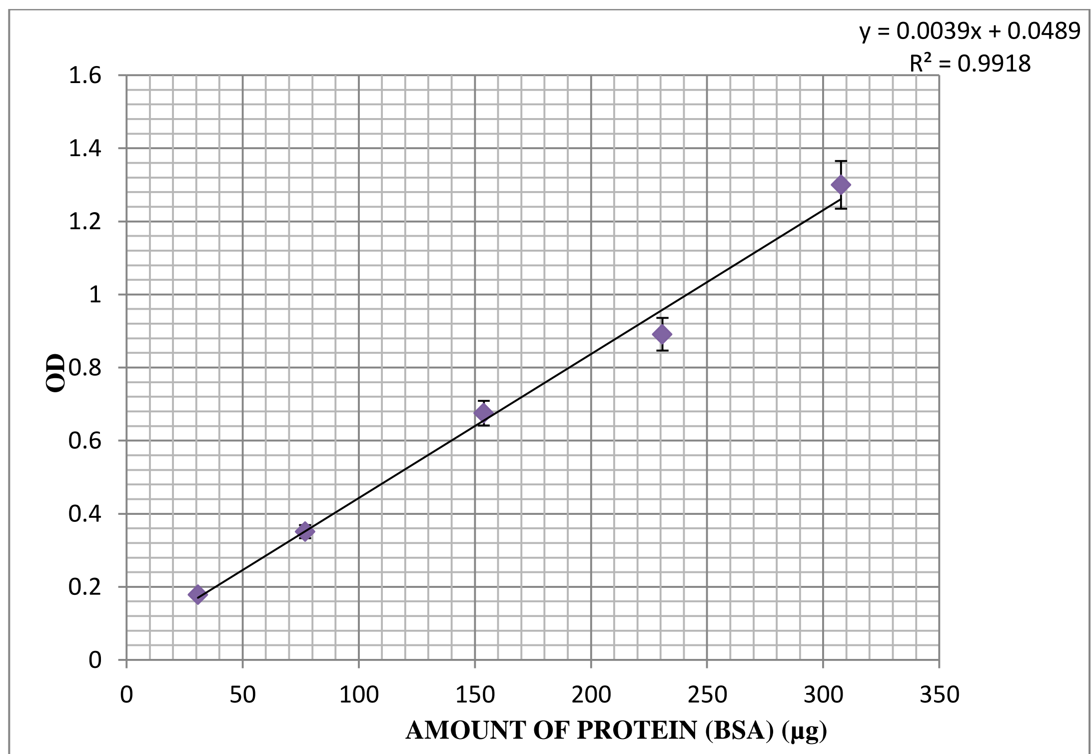

Figure 3- 2: Standard curves for protein analysis