私立東海大學化學工程暨材料工程研究所

碩士論文(草稿)

Master Thesis

指導教授：顏宏偉博士

Advisor：Hong-Wei Yen, Ph.D.

利用氣舉式發酵槽培養基因重組大腸桿菌生產 colicin 1B 之研究

The Study of Recombinant Protein-Colicin 1B Production in *Escherichia coli* by Using Airlift Bioreactor

研究生：狄雷薩

Graduate student：Resza Diwansyah Putra

中華民國 108 年 7 月

July, 2019

碩士學位論文指導教授推薦書

化學工程與材料工程研究所 狄雷薩 君所提供之論文

The Study of Recombinant Protein-Colicin 1B Production in Escherichia coli by Using Airlift Bioreactor

係由本人指導撰述，同意提付審查

此致

化學工程與材料工程研究所所長

指導教授：

日期：108年 7月 15日

（註：本頁可依個人意願是否貼附，可自行繕打後，列印出給指導老師、系主任簽名）

碩士學位論文口試委員會審定書

化學工程與材料工程研究所 狄雷薩 君所提供之論文

The Study of Recombinant Protein-Colicin 1B Production in Escherichia coli by Using Airlift Bioreactor

經本委員會審定通過，特此證明。

論文口試委員會

委　　員：

指導教授：

中華民國 108 年 7 月 15 日

Acknowledgement

Thanks to.

Allah Subhanallahu wa Ta'ala the Almighty, the greatest of all, on whom ultimately we depend for sustenance and guidance. I would like to thank Almighty Allah for giving me the opportunity, determination, and strength to do my research.

My parents, I owe everything to them for their struggle and sacrifice, supporting me so I can reach this point.

Professor Hong-Wei Yen, Professor in Chemical and Material Engineering Department, for being a great advisor and support my research. It such a great thing to work in your laboratory and having a lot of nice experience in these two years.

All of the professor and staff in Chemical and Material Engineering Department. Especially, Professor Shu-ming Chiao, Professor I-Kuan Yang, Professor Chi-Chang Lin, Professor Shueh-Hen Cheng, Professor Kuo-Tseng Li, and Professor Jia-Lin Liu who supported my academic activity in this department

All of the Indonesian friends who always give support beside of academic and research activity.

And all of those who give a contribution, idea, discussion to help me complete this research

Sincerely,

Resza Diwansyah Putra

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Escherichia coli (*E. coli*) is the most common host for recombinant protein production. A high cell density culture (HCDC) of *E. coli* is regarding be an efficient way to improve recombinant protein production, due to the low volume required, high efficiency, and relatively low cost of HCDC. To achieve HCDC, oxygen availability which is represented by dissolved oxygen (DO) is considered to be a crucial factor affects the production of recombinant protein in *E. coli*. In the other side, Induction step is crucial for the recombinant protein production. In the *E. coli* strain, there is a mechanism called SOS response.

This mechanism takes the role to repair the DNA when it is damaged or the replication of DNA is inhibited. As a result of the DNA repair, the certain protein is produced in the biomass. This experiment conducted to produce colicin 1B protein which is usually used as antibiotics. *E. coli* biomass was cultured in 4 liters Luria Bertani (LB) medium inside the airlift fermenter. After the desired Optical Density (OD) value of the biomass was achieved, the mitomycin C was added as an inducer to give damage to the biomass. So, the SOS response mechanism was able to do recombinant protein production. Some variation conducted in this experiment were a variation of aeration, a variation of temperature, and variation of pH. In the variation of aeration, it showed that keeping low aeration after induction can increase the protein expression. This optimal aeration is 2 vvm before induction and 0.5 vvm after induction which obtain 2^{14} protein expression. Besides that, it showed that the temperature did not give many changes to the protein expression which is stable between 2^{11} and 2^{12}. Meanwhile, the acid or base condition is not good for the recombinant protein since the resulted protein expression is stable between 2^{5} and 2^{6} which is not as good as the neutral condition.

Besides using the SOS response induction, the Isopropylthio-b-thiogalactopyranoside (IPTG) induction strain was also employed to evaluate the effect of the induction time point. The gene called T7, presenting in the pET vectors, is used for recombinant protein. When IPTG is present, the gene will activate the transcription of the desired protein. The evaluated induction time points are 0-hour, 5-hours and 24-hours. The result showed that the induction time point did not give effect to the protein activity. However, it gave effect to the result in cell concentration. The later induction time resulted in higher cell concentration since the metabolism resource was utilized in the growth phase.

Keywords: *Escherichia coli*, IPTG, SOS response, protein activity, Optical Density (OD) value

CHAPTER I

INTRODUCTION

1.1 Thesis Background

The recombinant proteins produced in the microbial system has revolutionized the biotechnology. Instead of requiring several days for either animal tissue, plant tissue or other large biological content to purify the protein, many researchers have developed a new project to obtain the purified protein immediately in recombinant protein researches. Furthermore, this protein recombinant is also allowed to be produced and industrialized in large quantity for obtaining commercial goods [1]. In the previous research, the topic of recombinant protein has been covered in detail to obtain the best result of the recombinant protein [2]–[4]. By obtaining the desired result of recombinant protein, it will rise to the advanced stage to the drug discovery process.

The recombinant protein requires the host cell to harbor the gene that producing the protein. *Escherichia coli* is a prokaryotic system that is popular as a host of recombinant protein. There are many advantages provided by the *E. coli* cells. For instance, it has unparalleled growth kinetics and the high cell density culture are easy to achieved [1], [5], [6]. Besides of its fast growth, *E. coli* also has the short-life cycle and easily manipulated [7]. Therefore, the manipulated gene may generate the desired protein after the induction step. To obtain the effective recombinant protein of *E. coli*, the fermentation process for the cell growth the induction gene are crucial.

Colicin is a kind of protein that is able to be produced in the *E. coli* cells recombinant protein. However, the colicin is the protein which lethal to the *E. coli* so that it is able to be employed as a vaccine that usually applied to the animal. The gene that can generate this

colicin is harbored in the *E. coli* cells. This thesis takes the observation about the recombinant protein of colicin 1B in *Escherichia coli* cells

1.2 Problem Statements

Based on that background, this thesis has the concern to obtain the optimum condition for the recombinant protein of colicin 1B. The optimum condition is observed by using these constraint points below which is divided by the two kinds of induction method.:

- Recombinant protein in the SOS response induction method
    1. What is the effect of aeration rate on the recombinant protein of colicin 1B?
    2. What is the effect of temperature on the recombinant protein of colicin 1B?
    3. What is the effect of pH on the recombinant protein of colicin 1B?
- Recombinant protein in the IPTG induction method
    1. What is the effect of induction time point to the recombinant protein of colicin 1B?

1.3 Aim of Investigation

Based on the background and the problem statements, obtaining the optimum condition to produce the recombinant protein is the main concern of this thesis. This optimum condition involving both the environmental factor such as temperature, oxygen supply, pH and the induction technique such as the SOS response gene and the IPTG induction system. The experiment will be conducted in the 5L-airlift bioreactor equipped with 4-holes sparger. As a comparison, this experiment was also scaled-up inside the 100L-airlift bioreactor. The working volume in the 5L-ALB was 4 liters and the working volume for 100L-ALB was 80 liters.

The produced protein in this experiment is colicin 1B produced by the special strain of *Escherichia coli*. Colicin is usually used as the antibiotics since its characteristic which is lethal to the *Escherichia coli* cells. By employing this characteristic, the obtained protein can be analyzed by the protein activity method. Furthermore, the optical density (OD) value of the *E. coli* cells will be also analyzed to know the profile of biomass growth.

CHAPTER II

LITERATURE REVIEW

2.1 Fermentation of *Escherichia coli* cells

Escherichia coli is a prokaryotic system which is mostly used for recombinant protein. Among all of the host for recombinant protein purpose, *Escherichia coli* is the most preferred choice due to its short life cycle and easily manipulated [7]. The good fermentation design is required to obtain the good *E. coli* cultivation for recombinant protein production. The most preferred *E. coli* expression system for recombinant protein is High Cell Density Culture (HCDC) of *E. coli*. HCDC gives the advantage of high efficiency, reduce culture volume, reduced wastewater, enhanced down-stream processing, low-cost culture [8].

Oxygen consideration, medium consideration, and acetate inhibition are the factors which are important to affect the *E. coli* cells growth [5]. The oxygen availability is crucial since growing the *E. coli* is an aerobic process. It was reported that the cell concentration greater than 100 grams of dry cell weight per liter can be obtained by fed-batch culture operation combined with the provided pure oxygen [6]. Medium is also an important factor because the medium gives nutrition for cell growth. A balance of nutrients provided is also important for cell growth. By utilizing the glucose control feeding strategy with a rate between 10 and 20 g/L, the final *E. coli* cells obtained after 50 hours cultivation can be as high as 164 g/L (source). By using pH-stat fed-batch cultivation, a high cell concentration of 183 g/L was obtained after 30 hours cultivation and plasmid stability was maintained at high levels for the production of recombinant insulin-like growth factor-2 in *E. coli* (source).

In the other side, the formed acetate content during the fermentation process may hinder the cell growth when the acetate concentration over the inhibition level. Acetate is formed not only when the *E. coli* is grown under oxygen-limiting condition but also when there is a glucose excess condition. The reason for the acetate accumulation is the cells take up more glucose than they can fully oxidize. Consequently, the cells eliminate the excess of carbon source by forming the overflow product which is mostly acetate [9]. Based on that explanation, there are several methods to keep the acetate level as low as possible. First, by limiting the carbon source feeding which can be performed by developing several fed-batch methods [5] [10]. That fed-batch method should be able to regulate the substrate feed rate in order to avoid overfeeding. Beside the fed-batch method, the dialysis combined with fermentation process can be also utilized to limit the carbon source feeding. This method is possible to grow *E. coli* to high cell density up to 190g/l dry weight and avoiding the acetate accumulation [5]. Besides the limiting carbon source method, the simpler way to control the acetate acid content is controlling the pH by using the high concentration of the base solution which is usually NaOH. Since the *E. coli* normally grow over internal pH 7.4-7.9 [11].

2.2 Bioreactor for *Escherichia coli* Fermentation

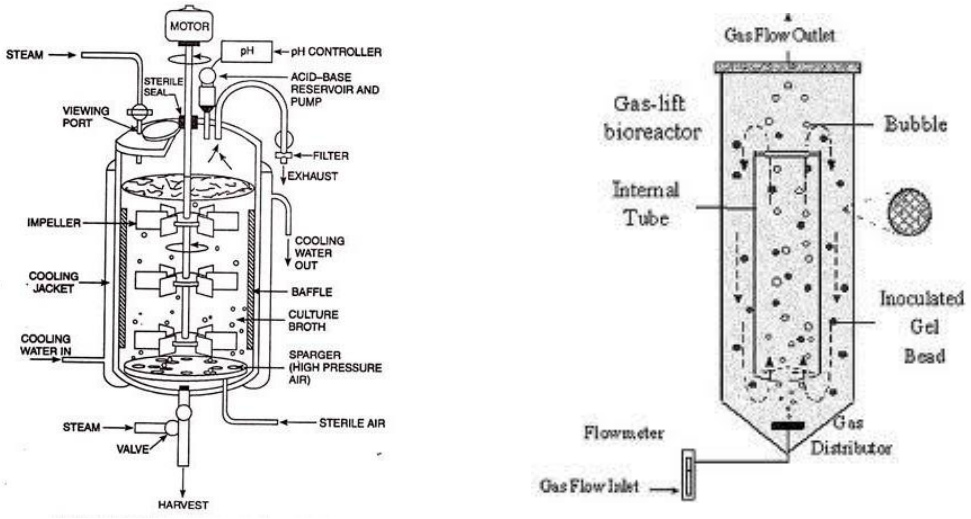
Microbial growth is affected by many environmental factors such as temperature, pH, and dissolved oxygen (DO). Dissolved oxygen is one of the important factors in the aerobic microbial process, because of the molecular oxygen role that is involved as the final electron acceptor in respiratory [12]. To fulfill the oxygen demand of aerobic microbial process, oxygen supply to the fermenter is crucial. There are several types of bioreactor i.e. Stirred Tank Bioreactor (STB), Airlift Bioreactor (ALB), Fluidized Bed Bioreactor and Packed Bed Bioreactor.

Some reactor might need the packed bed, this packed bed is usually made from polymer, ceramic, glass, other materials and available in the various sizes and shapes that allows the fluids to flow through the packed bed. In packed bed bioreactor, the cells are immobilized in the packed bed in the bioreactor column and fed with the nutrients flows through that packet bed. The disadvantages of this reactor are the poor temperature control, and unwanted side reaction [13]. In the fluidized bed bioreactor, the packed bed has the smaller size than the usual packed bed reactor. This reactor involves the biomass in solid phase carrier that is suspended using high liquid or gas flow rates. Thus, the bed behave as though it was a fluid [14]. The disadvantages of using fluidized bed reactor are increased reactor vessel size, pumping requirements and pressure drop, and erosion of internal components [13]. Growing *E. coli* biomass requires well pressure, temperature control and also avoiding the erosion. Since its disadvantages, either packed bed reactor or fluidized bed reactor is not suitable for growing *E. coli* biomass.

Oxygen transfer rate (OTR) is influenced by some variables that depend on temperatures such as viscosity, density, and oxygen diffusivity and solubility [15]. Since the influence of temperature on OTR is depends on reactor configuration, the presence of agitation impeller in STB may provide a better oxygen transfer rate (OTR) inside the bioreactor. Furthermore, the agitation inside the STB is not only able to distribute the heat from the jacket evenly, but also able to distribute the gas molecule from the sparger. However, the STB also has some disadvantages than ALB i.e. consuming more power, having more risk of contamination, and more complicated construction.

In contrast, ALB consumes lesser power since the absence of agitation impeller, having simpler construction so that it is easier to be scaled up. In contrast, because of the absence of the agitation impeller, this bioreactor type has the lower OTR [16].

Besides, it only depends on the sparger to distribute the gas molecule and the heat inside the bioreactor.



(a) (b)

Figure 2.1 Example of (a) Stirred Tank Bioreactor (STB) and (b) Airlift Bioreactor (ALB)

Airlift bioreactor is employed in this thesis since it has the advantage in the lesser power consumption and its simplicity design so that it easier to cleaned and sterilized. In industrialization those advantages are important to obtain the economic benefit. Although airlift bioreactor has the lower oxygen transfer rate than the stirred tank bioreactor, some studies develop many project to increase the oxygen transfer rate in airlift bioreactor such as adding membrane to increase the volumetric gas-liquid mass transfer coefficient [17][18].

2.3 Cell engineering of *Escherichia coli* for the recombinant protein-colicin

There are many types of researches developed about using *E. coli* strain for recombinant protein. Those researches involved some strategies to conduct the well-designed fermentation and developing *E. coli* strain to produce some protein [2]. The DNA of *E. coli* is well known and able to be manipulated thus the protein expressions are able to be formed [7]. The DNA of *E. coli* can be manipulated by altering genes which contain coded information that generate the protein production. The mutation of the genes such as

amino acid substitution and altered DNA sequence is useful to form the gene that produces the desired protein [19]. However, the main problem is to evaluate the steps that are transcription, RNA processing, translation, and protein processing that lead to final protein production [20].

Colicin is a one of kind protein that is able to use *E. coli* as the host strain for recombinant protein. One colicinogenic plasmid, pCol, is harbored in *E. coli* strain [21]. This pCol are usually contained in strains which are called colicinogenic strain. There are two types of pCol: Type I and Type II. Type I plasmids are tiny and contain 6 to 10 kb in about 20 copies cell. This type mostly encodes colicin group A. Type II plasmids are plasmids about 40 kb which are monocopy plasmids. This type usually used for encode group B. Although colicins are produced by *E. coli* strain, it is lethal for the related strain of *E. coli* [21]. Colicins contained in group A are translocated by the Tol system, for instance, colicins A, E1 to E9, K, L, N S4, U, and Y. On the other hand, Colicins contained in group B utilize the TonB system, for instance, colicin B, D, H, 1a, 1b, M, 5 and 10 [22], [23]. For developing numerous genetic and the biochemical mechanism purpose, colicin has been developed. The plasmid reproduction mechanisms of colicin have been broadly studied to the utilization of the ColE1 plasmid to build the original pBR322 vector and its many derivatives.

Besides colicin, there are many kinds of protein that can be expressed by *E. coli* strain. Activin A is also a kind of protein that can be expressed by recombinant protein in *E. coli* strain. Activin A is expressed using the pET 21a expression vector which is harbored in T7 promoter [24]. The same vector mechanism is also applied to express other proteins such as nitrilases [25].

There are two inner membrane dividing *E. coli* cells become three compartments. The cytoplasm (inner), the periplasm (middle), and extracellular space (outer). Each compartment can be the target to perform the recombinant protein. Each expression system in those compartments has specific biological activity, process, cost, and results. Therefore, targeting recombinant proteins to the cytoplasmic space, periplasmic space or culture medium has each own characteristic including their advantages and disadvantages.

Cytoplasm takes the role as the place to characterize the cell division which requires the precise regulation of cell envelope growth and orientation in concert with another process. Thus, this process regulates envelope rearrangements during the cell cycle, for instance, in signal transduction pathways or septum initiation and formation [26]. Recombinant protein in cytoplasm compartment or cytosolic production has some advantages such as it is able to result in the highest protein yield and simple plasmid construct. In contrast, it also has some disadvantages such as the purification is more complex and the misfolding formation in the inclusion body [8]

In the other side, production in the periplasm space or secretory production and production in extracellular space or excretory production have simple purification, soluble protein production, improved folding and prevention from protease degradation. However, secretory production and excretory production also have disadvantages. Both of it can occur the cell lysis and obtain low protein yield, especially for excretory production [8], [27]. The examples of recombinant protein in the different compartment are shown in Table 2.1.

Table 2.1: The production of recombinant protein at different compartments of *Escherichia coli*

Compartment	Protein resulted	Protein Expression	Reference
Cytosolic production	FtsL protein	13.6 kDa	[26]
	Bovine phosphate carrier (m)	5[1] Expression level	[28]
	Huwentoxin-IV	18 kDa	[29]
	Anti-VEGF	25 kDa	[30]
	EhCP1	0.65 mg (100% pure)	[31]
Secretory production	Alkaline phosphatase	5.2 g/l	[32]
	Mouse endostatin	40 mg/l	[33]
	Hirudin III	60 mg/l	[34]
	Bile salt hydrolase (BSH)	1.18 ± 0.08 U/mL	[35]
	Human interleukin-10	130 ± 40 ml/mL	[36]
	β-fructofuranosidase	66.4 kDa	[37]
	Pseudomonas fluorescens BJ-10 thermostable lipase	26 kDa	[38]
Excretory production	Winter flounder antifreeze	16 mg/l	[39]
	Exoglucanase	143 U/ml	[40]
	Pectate lyase	2200 U/ml	[41]
	Polyhydroxybutyrate depolymerase	3.47 U/l	[42]
	Green fluorescent protein (GFP)	-	[43]
	Xylanase	43 kDa	[44]
	Trx-hPTH	1440 mg/l	[45]

2.4 Induction System as The Key of Recombinant Protein

2.4.1 The SOS Response

E. coli shows a complex response to several conditions that damage DNA or inhibit the DNA replication this response called SOS response [46]. This SOS response also includes some phenomena such as the enhanced capacity for mutagenesis and DNA repair, inhibition of cell division and prophage induction. This SOS response is controlled by the gene regulation system which involves the LexA protein and RecA protein [47]. LexA protein represses the SOS genes via transcriptional repression while RecA protein can form the RecA* that enhance the autocatalytic cleavage of LexA protein. When the host of SOS response is triggered by some agents such as chemical drugs (mitomycin C, methyl methanesulfonate, etc) and ultraviolet (UV) exposure that leads to DNA damage, the expression of RecA is stimulated [48]. In colicin synthesis example, The SOS response involves the binding sites to promote the colicin-encoding operons [49]. After LexA is triggered by the inducer such as physical agents (UV light and chemical drugs) and stress conditions. It will perform the autocatalytic cleavage that leads to colicin production [21]. Mitomycin C is a most used chemical drug to induce the colicin formation. Furthermore, the stress condition can be achieved by setting up the parameters in the fermenter such as temperature, pH and oxygen availability. Previous research reported that the SOS response induction is able to construct the heterologous gene expression. The previous record was once studied via checking out the increase in the SOS-induced genes expression either from the natural genes or by using the use of a reporter gene construct, which used to be fused a putative SOS-induced gene promoter with promoter-less lacZ gene encoding β-galactosidase [50]. Based on that explanation, induction by SOS gene response have some advantages and disadvantages. The advantage is the time of induction is not strictly important, as long as the cell concentration is sufficient the protein expression is still able

to be induced. In contrast, since SOS gene response employs the antibiotic, toxin or UV light, it becomes more expensive than the IPTG inducer. Furthermore, the UV exposure time and operation are hard to be controlled for industrialization.

2.4.2 The Isopropylthio-b-thiogalactopyranoside (IPTG)

Besides the SOS gene, Small amounts of T7 RNA polymerase and *lac* operon can lead the *E. coli* cell to the expression of the specific gene for protein production purpose [51]. Inside the *E. coli* cell, there is a *lac* promoter which is the key component of *lac* operon as the key to protein production. This *lac* promoter is an array of three structural genes encoding the protein involved in lactose metabolism. For instance, *lacZ* encodes the enzyme β-galactosidase (which splits lactose into glucose and galactose); *lacY* encodes lactose permease, and *lacA* encodes a lactose transacetylase [52]. This promoter system can be induced by lactose adding and this sugar can be employed for protein production. However, carbon source (such as glucose) which already present in the media may hinder the induction by the lactose [53]. Because, lactose cannot be transferred into the cell which contains the glucose since the lactose permease, LacY, is inactive in the presence of glucose [54]. Isopropyl β-D-1-thiogalactopyranoside (IPTG, also known as lad-y) is a molecular biology reagent. This compound is a molecular mimic of allolactose, a lactose metabolite that triggers transcription of the *lac* operon, and it is therefore used to induce *E. coli* protein expression [55].

The *lac* operon is controlled by molecules, which can turn on the gene for protein expression and off to response to the nutrient concentration. However, some organisms require regulation of genes with time as the key consideration such as the production of phages (bacteriophages or bacterial viruses) in an infected cell [56]. The T7 promoter, present in the pET vectors, is used for recombinant protein. The T7 promoter is classified

from Phage T7 for the transcription of T7 DNA, which can be identified by *E. coli* RNA polymerase. The gene coding for T7 RNA polymerase itself has been altered into many commercial products which available *E. coli* strains under a modified *lac* operon system such as BL21 (DE3) cells together with the construction of the T7 promoter on the commercial *E. coli* pET vectors for recombinant protein production. The gene induction has two stages as described following: First, there is a *lac* promoter sequence that native *E. coli* RNA polymerase is able to bind. When IPTG is present, the *lac* repressor protein (*Lacl*) is released from the *lac* promoter region in the host chromosome and the T7 RNA polymerase is transcribed and translated. After that, the T7 RNA polymerase is able to activate the transcription of the gene of interest, which is inserted after the T7 promoter and the *lac* operator in pET expression plasmid. Thus, although the T7 promoter is weakly activated on the *E. coli* RNA polymerase, the gene induction can also strongly dependent upon IPTG [57].

Some protein such as Insulin-like growth factor-2 (IGF-2), Insulin-like growth factor-2 (IGF-2), Therapeutic proteins and Activin A are induced by IPTG. There are some advantages in producing protein by IPTG induction such as it can use the lactose or another carbon source so it will be cheaper than using toxic content like mitomycin C. Furthermore, The T7 promoter is most used in recombinant protein expression, since its target protein is able to show half of the total cell protein in successful cases [2]. In contrast, IPTG induction method also has some disadvantages such as induced to the right time is crucial. Otherwise, the desired protein expression might not be produced.

2.5 Factors affecting recombinant protein production in *E. coli*

Recently, there are many types of research and studies conducted to develop recombinant protein by *E. coli* growing method [2, 53, 54]. To have the optimal protein production in *E. coli*, a well-developed fermentation process is required. As shown in the previous section, HCDC and a suitable induction system might be crucial to maximizing recombinant protein production in *E. coli*. The effects of the induction strategy will be introduced in the following section. Besides, the effects of dissolved oxygen, temperature and pH in the recombinant protein production will also be discussed.

2.5.1 Induction Strategy

Induction method is the crucial thing to produce the protein expression. As described in the beginning, the SOS response method and IPTG method can provide different result. Besides the gene induction method, there are some strategies which can result in the desired protein expression such as induction time point and concentration of inducer. Based on the protein type, induction can be conducted in several ways.

First of all, induction in high biomass enables dissolution between cells growth and recombinant protein production (RPP) phases. After induction, the growth becomes slow because all metabolic resources are going into protein production. Then, the induction at low biomass that only can be conducted when the growth of biomass and the RPP are evenly obtained the same amount of metabolic resources. Otherwise, the growth of biomass and/or recombinant protein can be hindered. The last is when the toxic protein is produced, the growth of biomass will be inhibited and/or occur the cell death after induction [57].

As for the inducer of IPTG system, lactose is the common carbon source that is also used as the inducer in IPTG system of recombinant *E. coli*. Since lactose is also playing the role of carbon source, the best condition is when the main carbon source inside the

fermenter, usually is glucose, is already completely consumed. The existence of glucose will inhibit the uptake of lactose due to *lac* permease becomes inactive under that condition of glucose existing [60]. Besides the use of lactose as the inducer, the direct addition of IPTG as an inducer is another choice [53]. The inducer concentration also takes a role to affect the resulted protein expression. The comprehensive experiment should be conducted to determine the best IPTG concentration for obtaining the desired protein expression. In other words, the higher or lower IPTG concentration does not mean the higher protein expression can be expressed. Since there are the *lac* permease and permease independent pathways which are the ways that IPTG can enter the cell [61]. Because the expression *lac* permease is heterogeneous and the number of active permeases in each cell is highly variable, protein expression cannot be predicted by the IPTG concentration [1]. on the other hand, there are other supporting factors as shown in Table 2.2, such as the ethanol and the compound of IPTG and lactose that could affect the recombinant protein expression. In MEX67 and RPB5 production by using *E. coli* strain, the presence of ethanol may enhance the protein expression. Meanwhile, the compound of 1.5 mM IPTG without lactose content can enhance the expression of activin A by using *E. coli* BL21 (DE3) host strain.

Table 2.2 shows the results of recombinant proteins production by using different conditions of IPTG induction strategy. Based on Table 2.2, IPTG induction system is more popular than the SOS response gene due to its advantage to express more protein and easy to be employed. Related to that, the proper induction strategy can be determined to produce the desired protein expression. The induction strategy involves some factors such as the induction timing, inducer concentrations, and other supporting factors. It was observed that the optimum IPTG induction conditions will strongly depend on the recombinant protein produced in *E. coli*.

Table 2.2. Results of the recombinant protein production by using different conditions of IPTG induction strategy

Products	Host	Induction characteristic	Productivity and characteristic	Reference
Protective antigen protein	*E. coli* DH5α	IPTG	125 mg/L	[62]
Animolevulinate synthease	*E. coli* MG1655	IPTG 0.005 - 5 mM	5.2 g/L	[63]
Insulin-like growth factor-2 (IGF-2)	*E. coli* BL21(DE3)	IPTG 0.06 mM	9.69 g/L, inclusion body 1.2 g/L	[64]
Annexin-V-hirudin chimeric protein	*E. coli* BL21(DE3)	IPTG 0.7 mM	10 mg/L/DO_{600}	[65]
MEX67	*E. coli* SG13009	Adding ethanol in IPTG 1.0 mM induction	Protein expression is 66 kda (presence 3% ethanol) and 50 kda (absence of ethanol)	[66]
RPB5	*E. coli* pET-28a(+)	Adding ethanol in IPTG 1.0 mM induction	Protein expression is 25 kda (presence 3% ethanol and absence of ethanol)	[66]
Therapeutic proteins	*E. coli* BL21	Adding galactose and IPTG 1 mM inducer	37 ± 2 arbitrary titers per OD_{600} unit	[51]
Activin A	*E. coli* BL21(DE3) and BL21(DE3) plysS	1.5 mM IPTG + 0% w/v lactose 2.25 mM IPTG + 1% w/v lactose 0.75 mM IPTG + 1% w/v lactose 1.5 mM IPTG + 2% w/v lactose 2.25 mM IPTG + 3% w/v lactose	6.59 mg/ml 3.76 mg/ml 1.14 mg/ml 0.65 mg/ml 0.32 mg/ml	[24]

2.5.2 Dissolved Oxygen

Since *E. coli* culture is an aerobic culture, the availability of DO will be crucial to achieving HCDC condition for recombinant production. In most case, the air is normally supplied in the cultivation. However, pure oxygen needs to be provided to fulfill the demanding of high cell density required. In contrast, since the utilization of oxygen-enriched air is a common strategy to support high-density growth of *E. coli*, it was important to investigate the effect of oxidative stress resulting from the high dissolved oxygen concentrations on the physiology and growth of *E. coli*. The previous study reported that the monoamine oxidase (MAO) enzyme generated in *E. coli* could be damaged by the oxidative stress during the highly aerobic processes by supplying oxygen-enriched air. Therefore, the optimal condition for MAO production is aerated, instead of oxygenated, and induced at moderate cell density, and obviously serves a compromise between oxygen supply effects on specific growth rate/induction cell density, acetate accumulation, and high specific MAO activity [67]. The transcriptional analysis and enzyme activity results indicated that when *E. coli* is exposed to O_2 shift, the superoxide stress regulator SoxRS is activated and causes the stimulation of the superoxide dismutase system. This enables *E. coli* to protect itself from the poisoning effects of oxygen [68]. Molecular oxygen is producing the reactive oxygen species (ROS) for instance, superoxide anion ($O2^{-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (HO*) [69]. High levels of ROS are recognized to be stress conditions for *E. coli* and causing irreversible damages to cellular components. At normal growth conditions, SoxR is generated in an inactivated form, but when it exposed to superoxide or redox-cycling drugs, SoxR is activated together with simultaneous activation of the following series of SoxS genes [70]. In addition to providing high-cost oxygen-enriched air, reactor pressurization is an alternative approach to improve oxygen mass transfer. By pressurizing the bioreactor up to 0.41 MPa, without pure oxygen

supply, an 8.7-fold increase in economic efficiency is estimated, what shows the potential of this innovative strategy for aerobic cultures [16].

The effects of aeration rate and cultivation conditions on the growth of recombinant *E. coli* are shown in Table 2.3. *E. coli* culture theoretically may grow up to a cell density of 350 g/L with 0.2 L/h grow rate if the average fermentation system is able to supply oxygen at 1.5 M O_2 L/h [5]. Since oxygen availability is important for *E. coli* growth, oxygen limitation is able to trigger more than 200 genes expression for adjusting the cell's metabolic capacities to the availability of oxygen that the metabolic change might affect the production of recombinant protein [1]. In the cultivation of *Escherichia coli* K-12 strain W3110 under varied oxygen-enriched conditions, exponential growth could proceed for a longer time and higher growth rates could be maintained with oxygen enriched air supply. However, a higher specific oxygen consumption rate per glucose was measured after the start of the oxygen enrichment, indicating higher maintenance and consequently, the growth rate and yield coefficient decreased drastically at the end of the process. The chance for improving the performance of a process by enrichment of the air supply with oxygen is better when the product formation is growth associated [71].

Table 2.3. Results of the recombinant protein at the different condition of aeration

Products	Host	Air supply condition	Productivity and characteristic	Notes	Reference
TrpLE-hybrid	*E. coli* W3110	20%	0.3 g/l protein	Air supply condition is in DOT (Dissolved Oxygen Tension)	[72]
Protein Titer for vaccine	*E. coli* BL21	Start at 100% DO, set up k_La at 0.8 s^1	0.8 g/L	The working volume is 2 l	[73]
AviPure	*E. coli* BL21 (DE3): pAV01	Start at 80% DO	2.10 ± 0.12 g/L protein	Using LB media	[74]
PsPA	*E. coli*	0.4 MPa	4 g/l	Using pressured airlift bioreactor	[75]
P64k	*E. coli* K12 GC366	1 vvm	546 ± 51 g/l		[76]
rPDT fusion protein	*E. coli* pET28a-pdt	DO maintained in 10% and 50%	9104.195 when DO is 10% 9437.5 when DO is 50%	The result is in rPDT band intensity	[77]

2.5.3 Temperature

Temperature is also a crucial environmental factor not only for the *E. coli* culture but also for its recombinant protein. The solubility of recombinant protein can be increased by continued induction at low temperature and a lower amount of IPTG [7]. The previous study reviewed the temperature around 28^0C to 37^0C are commonly set up for culturing the high-cell-density culture of *E. coli* [6] since the set up below 20^0C and above 40^0C result the slower growth [78]. In the recombinant protein strategy, the temperature setup also gives a significant effect. The preceding research about producing lycopene in *E. coli* BL21 show result that lower temperature increases the higher lycopene content in ppm. As the comparison, At the same inducer, 0.1 mM IPTG inducer, produced lycopene content at 28^0C is 4800 ppm while at 37^0C is 1000 ppm [79]. Another study about producing

progesterone 5β-reductase (P5βR) reported that the induction is conducted at a low temperature. Since the induced cultures at 37^0C and 25^0C did not produce the active protein at any density, the induction is conducted at 15^0C and 4^0C [80].

Beside the resulted protein, inclusion body (IB) can also be affected by culture temperature. Inclusion body described as an insoluble protein aggregate that resulted by overproduction of recombinant protein [81]. Inclusion body is usually formed in high-level expression recombinant protein. This inclusion body can be avoided or decreased by reducing the cultivation temperature. However, protein expression such as chaperonine may also reduce drastically. The strain of *E. coli* called ArcticExpress conquered this issue since these genetically engineered strain co-express cold-adapted chaperonins *Cpn10* and *Cpn60* from the psychrophilic bacterium called *Oleispira antartica* [7]. Based on that explanation, although the low temperature is optimum for some cases of recombinant protein, the other cases show that it also can reduce the protein expression. Therefore, the optimum temperature needs to set up for obtaining best protein expression.

2.5.4 pH

Since formed acetic acid in *E. coli* culture can hinder the cell growth, pH is an important indicator of the culture process. The optimum pH condition needs to be settled to obtain the desired cell mass. However, pH also affects the protein expression in the recombinant system, As the stress condition can trigger the protein production in the recombinant protein. Although the acetate excess is a common issue in this recombinant protein, the improvements in acetate tolerance have been developed through genetic strategies and medium supplementation. By employing those strategies, increasing the protein expression under the acetate stress can be conducted. [82]

The engineered gene of *E. coli* BL21 (DE3) is employed to produce the protein expression of glutathione S-transferase (GST), green fluorescent protein (GFP) and cytochrome P450 monooxygenase (CYP) in the recombinant protein system. The recombinant protein in pH 6.5 and 7.5 were evaluated. It was reported that under the stress of 300 mM acetate, the intracellular acetate accumulation was reduced at pH 7.5. Besides that, the pH 7.5 provided the improved expression of GST, GFP and CYP protein. [82].

The inclusion body (IB) is not also be affected by temperature but also affected by culture pH. Mendoza and coworkers investigated the effect of pH-setting to the inclusion body formation in the recombinant protein in the *E. coli* BL21-Gold (DE3). The manipulation of pH gives an impact to IB formation. By using the controlled pH at 7.5, the IB of sphingomyelinase-D are produced and become smaller (<500 nm) after 24 hours, while at uncontrolled pH the IB is produced higher (>500). [83]

CHAPTER III

MATERIALS AND METHODS

3.1 Growing *Escherichia coli* cells in Luria Bertani (LB) Medium

To provide the proper environment for growing the biomass, the proper medium should be employed. The Luria-Bertani broth is the popular medium due to offer fast biomass growth and the good growth yield for many spices [84]. Thus, many microbial kinds of research used LB medium to grow the biomass, especially for *E. coli*. This thesis also employed the LB medium to grow the *E. coli* biomass. This medium consists of 10 g of tryptone, 10 g of NaCl, 5 g of yeast and 1 liter of distilled water; adjusted pH at 7.0 and sterilized in the autoclave at 120^0C during 20 minutes.

The growth of *E. coli* in LB broth medium is carried out by the steady-state period which is called an exponential phase or log phase. The advantage of the period in this state is extreme reproducibility of the physiological state of the bacteria. During the steady-state growth in the medium, the same achieved state will happen in the given strain. This state is usually achieved after the lag phase, the phase at low density, that the removed nutrient from medium does not affect the growth rate [84].

This thesis involved several steps to produce the recombinant colicin 1B protein in *Escherichia coli* cell which is described in figure 3.1

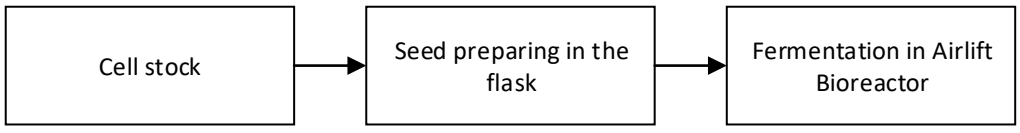
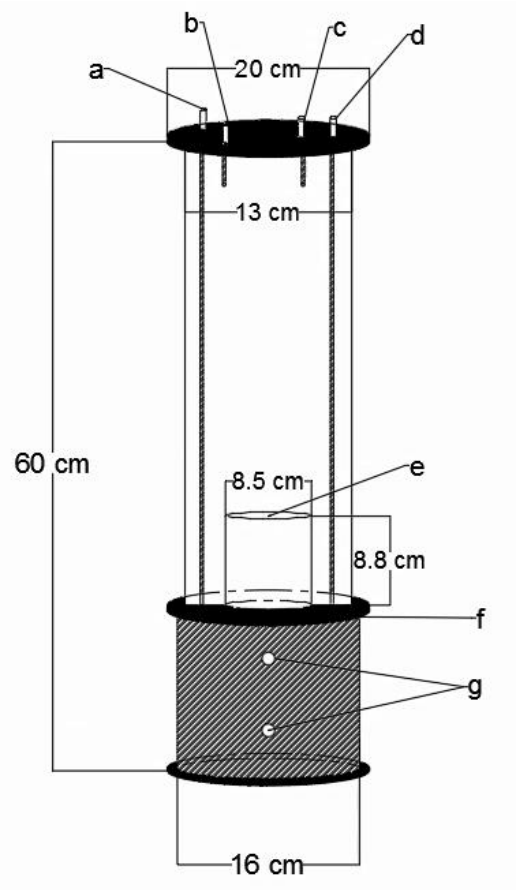


Figure 3.1 Steps to grow *Escherichia coli* cells in LB broth medium

(a) (b) (c)

Figure 3.2. *Escherichia coli* in the different volume (a) the cell stock (b) 100 ml LB medium in the flask (c) LB medium in the 5-liters airlift fermenter

Cell stocks of *Escherichia coli* are stored in the small vials at -80^{o}C temperature. Each vial contains 1 ml *E. coli* Cell. To culture these cells, each vial is prepared in the flask which contained 100 ml LB medium to obtain the seed for the fermentation in the airlift bioreactor. After the seed preparation, the next step is inoculation to the airlift bioreactor. The total working volume in this thesis is 4 liters. Therefore, by using 10% inoculation rate, 400 ml seed are prepared for the 3.6 liters LB medium prepared in the airlift bioreactor.



Legend:

a. Air inlet
b. Liquid inlet
c. Air outlet
d. Liquid outlet
e. Buffle
f. Heating insulation tube
g. Heating/cooling liquid circulation

Figure 3.3 Sketch of airlift bioreactor used in this thesis

Figure 3.4 The real picture of airlift bioreactor

3.2 Carbon Source Consumption

Besides the medium, the growth of *E. coli* is also carried out by carbon sources such as sucrose, glucose, fructose and etc. However, glucose is the most preferred carbon source since its rapid utilization which depends on the phosphoenolpyruvate: carbohydrate phosphotransferase system (PTS) [85]. This thesis utilized glucose at 40 g/l LB broth supplied to the 5-liters airlift bioreactor while for the 100-liters airlift bioreactor, utilized glucose is 20 g/l LB broth.

3.3 Material

3.3.1 *Escherichia coli* cells strain

This thesis employed two kind different strains of *E. coli*. These two strains are developed by Chung-Hsing University, Taiwan. The first is the *E. coli* strain that can be induced by the SOS response. By using either the Mitomycin C or UV exposure, the SOS gene response can encode the desired colicin protein. This strain is utilized to evaluate the effect of the aeration, temperature and pH to the recombinant protein-colicin 1B of *E. coli*.

The second strain is the *E. coli* strain that can be induced by Isopropylthio-b-thiogalactopyranoside (IPTG). The lactose or IPTG can be employed as the inducer to produce the desired colicin protein. This strain is utilized to evaluate the effect of the induction time point to the recombinant protein-colicin 1B of *E. coli*.

3.3.2 Chemical and organic materials

Chemical and organic materials are employed in many necessities such as medium, carbon source, analysis and other utilities. This following Table 3.1 shows the list of materials used in this thesis.

Table 3.1 The list of utilized material

Material name	Company/Brand
Tryptone	Bacto BD
Sodium Cloride (NaCl)	SHOWA
Yeast Extract	Bacto BD
Sodium Hydroxide (NaOH)	SHOWA
Glucose ($C_6H_{12}O_6$)	ROQUETTE
Mitomycin C	KYOWA
Isopropylthio-b-thiogalactopyranoside (IPTG)	CYRUSBIOSCIENCE
Granulated Agar	Difco BD

3.4 The SOS Response Induction System Strategy

When the DNA receives the damage, The SOS response takes a role to repair the damaged DNA. As a consequence, the proteins are also produced after the DNA recovery. The damage that can be repaired by the SOS response is usually caused by two factors i.e. drugs or UV exposure. This thesis applies the mitomycin C as the inducer to give the stress damage to the *E. coli* cell.

Mitomycin C solution 1 g/ml were utilized to induce the SOS gene in *E. coli* cells so that it can produce the desired colicin. The induction is conducted at 16 hours point fermentation time. Because after 16 hours, the biomass generally achieves the high concentration that is enough to conduct the induction. The amount of used mitomycin C is 0.4 ml/1 liter working volume.

These experiments were conducted by varying the environmental condition which are aeration rate, pH and temperature. Each condition is varied in two types which are one stage and two stages. In one stage, the set-up is not changed during the fermentation while in the two stages, the condition is changed after the induction.

3.4.1 Aeration Rate Variation

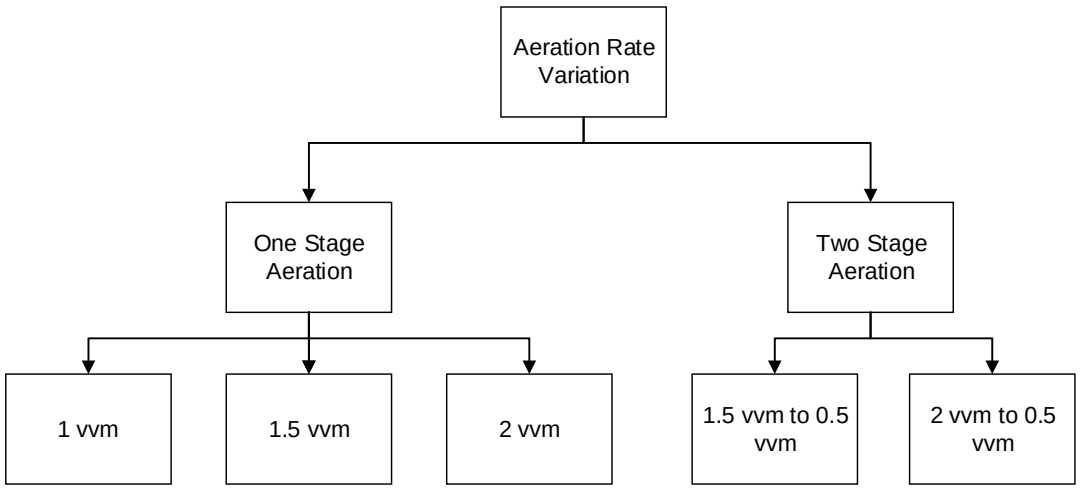


Figure 3.5 Scheme of experiment for aeration rate variation

The fermentation was conducted in settled pH and temperature which are 7 and 37^0C respectively. This experiment varying aeration rate which is divided into two sections which are one stage aeration and two-stage aeration. In one stage aeration, the aeration is varied at 1 vvm, 1,5 vvm, and 2 vvm. In two-stage aeration, they are set at 1.5 vvm before induction then 0.5 vvm after induction; and 2 vvm before induction then 0,5 vvm after induction.

3.4.2 Temperature Setting Variation

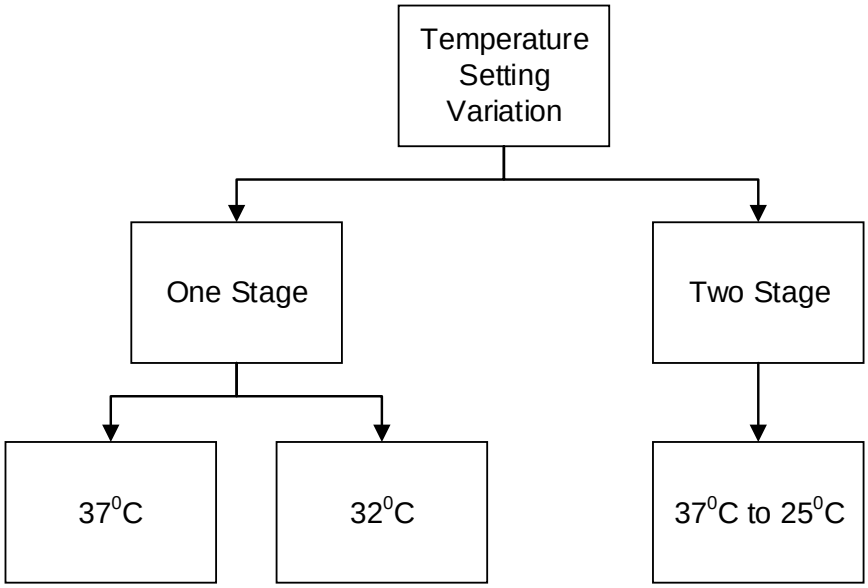


Figure 3.6 Scheme of the experiment for temperature setting variation

The aeration rate and culture pH are settled at 1.5 and 7 respectively. The temperature settings varied in one stage are 37^0C and 32^0C. in two-stage, the temperature is varied in 37^0C before the induction then 25^0C after the induction.

3.4.3 pH Setting variation

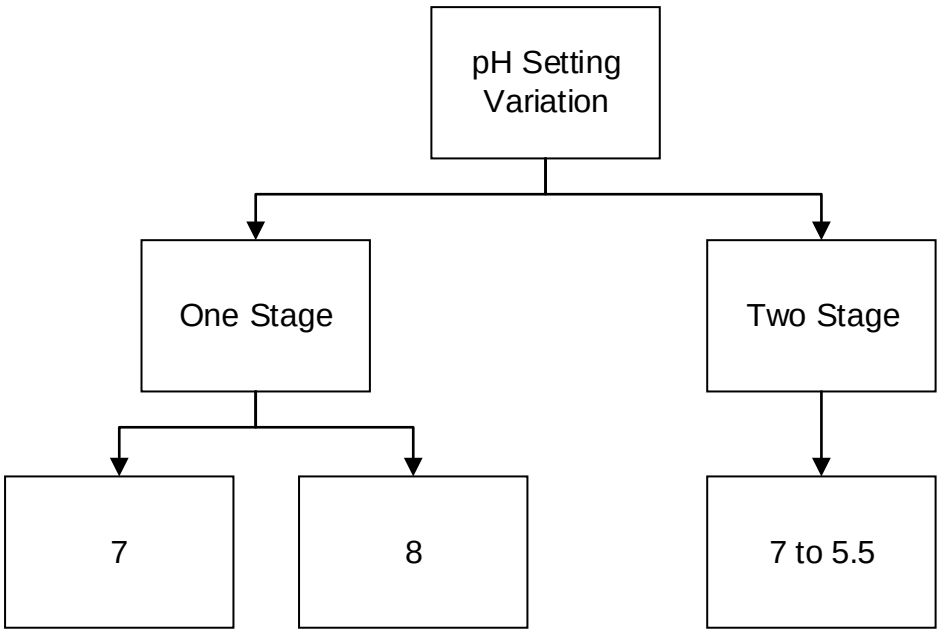


Figure 3.7 Scheme of the experiment for pH setting variation

The fixed condition in this variation is temperature and aeration rate which are 37^0C and 1.5 vvm respectively. In one stage, this section varies the culture pH at 7 and 8 since the pH in the neutral condition and base condition would be compared regarding to its effect to the recombinant protein. In two-stage, the pH is settled at 7 before induction then settled at 5.5 after induction to evaluate the effect of acid condition to the recombinant protein. The acid condition is set up after the induction since the high biomass before induction is required and the *E. coli* normally grow at neutral condition [11].

3.5 The IPTG Induction System Strategy

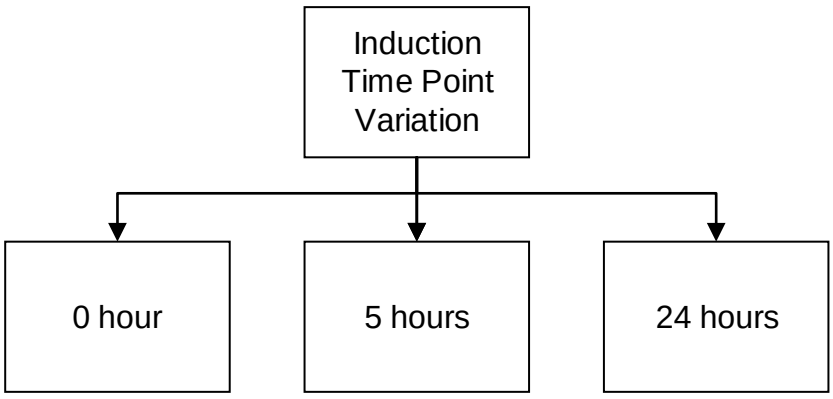


Figure 3.8 Scheme of the experiment for induction time point variation

To induce colicin production, 1 M IPTG solution were employed. The amount of used IPTG is 0.1 ml/1 liter working volume. This experiment varied the induction time point to observe its effect on protein expression. The time induction point variation used in this experiment is 0, 4, and 24 hours. Those points represent the different states of *E. coli* culture which are the lowest biomass, biomass starts growing, the high biomass. Besides the induction time point, the 100 liters fermenter and 5 liters fermenter performance are compared to investigate the *E. coli* growth and resulted in protein expression.

Figure 3.9 Airlift fermenter 100 liters capacity

3.6 Recombinant Protein in *E. coli* by Using the 100-Liters Airlift Bioreactor

The same procedure of recombinant protein by using the IPTG induction method is also applied to the 100-liters capacity of airlift bioreactor. The working volume is settled at 80 liters of LB broth contains 20 g/l glucose. The settled operating conditions are the temperature at 37^0C, aeration rate at 1.5 vvm and pH at 7. The inducer is 1 M IPTG solution which used at 0.1 ml/1 liter working volume. This inducer is added when the fermentation at 24 hours point.

3.7 Protein Expression Analysis by Using Colicin Activity Method

Colicin is a kind of protein produced by the gene hosted in *E. coli.* In the other side, this colicin is also lethal to the *E. coli* cell. By using this characteristic, the produced colicin

activity can be investigated. This thesis applied the sequences step to investigate the colicin activity obtained from the LB broth which contains the *E. coli* cell.

Step 1 Separating cells from its broth. As mentioned in the 2.1 points, LB medium broth, which is employed in this thesis, contain many contents that can provide the nutrition and appropriate environment to the microbial growth of *E. coli*. To have the proper protein activity analysis, this broth needs to be removed from the cell. 5 ml broth is centrifugated to separate the cell from the broth, then the cell is diluted in 5 ml sterile water.

Step 2 Diluting the biomass solution. After the broth removal, the obtained cells are diluted by the sterile water. Since the biomass has to be settled for the protein activity analysis, the biomass is settled by value 1.

Step 3 Sonication of *E. coli* cells. Since the recombinant protein of colicin is produced intracellularly, the sonication process is needed to break down the cell and obtain the colicin. Sonication process is conducted at amplitude 10 for 1 minute 30 seconds. Sonication process will release some amount of heat which is able to give damage to the protein, so that, some ice cubes were added to keep the protein from the heat damage.

Step 4 Diluting the protein in various concentration. To investigate the protein activity, the obtained protein after sonication process is diluted in sterile water to various concentration. The 0.5 ml protein, which called 2^0 dilution, was taken and diluted in 0.5 ml sterile water to have the protein with 2^1 dilution. Then it was conducted to have protein with concentration from 2^0 until 2^{20}.

Step 5 *E. coli* cells in the LB agar media preparation and protein activity analysis. After preparing those proteins, the next step is preparing the area to test those proteins. The mentioned area is the thin LB agar broth media which contains the *E. coli* cells at the small biomass (OD_{600} at 0.4 - 0.7). This analysis method employs the characteristic of the colicin

that is lethal to the *E. coli* cells. Therefore, after the LB agar medium preparation, the colicins with various dilutions are dropped on the agar. Then, it stored in the incubator at 37^0C for 6 hours.

Step 6 Determining the colicin activity. After stored in the incubator, the colicin activity can be determined by counting the area which has no *E. coli* cells. Figure 3.7 below is the example of how the colicin activity is observed.

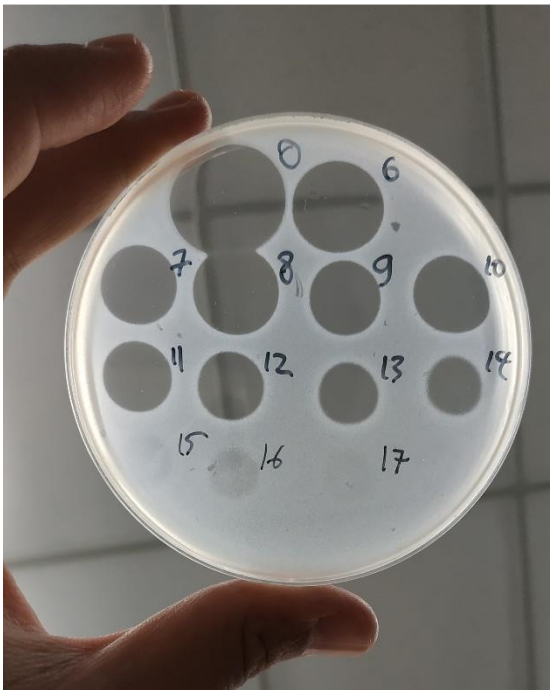


Figure 3.10 The observation of colicin activity; there is no activity after 2^{17} which means the activity is 2^{17}

3.8 The Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) for Protein Analysis

The SDS-PAGE is a method of protein separation by using electrophoresis principle. In general, electrophoresis is a separation of macromolecule in an electric field. In order to perform a protein separation, SDS-PAGE utilize sodium dodecyl sulfate (SDS) to denature the proteins and a discontinuous polyacrylamide gel as a medium. Since SDS is an anionic detergent, its molecule has a net negative charge within a wide pH range when it is dissolved.

Polyacrylamide gels hinder larger molecule from moving as quick as smaller molecules. Since the charge-to-mass ratio is almost the same among SDS-denatured polypeptides, the separation of proteins depends almost entirely on the differences of molecular mass of polypeptide. There are some steps protocol of experiment needed to perform an SDS-PAGE.

Step 1 Preparing the gel. The gel consists of two kind of gel solution which are the resolving gel and stacking gel. The resolving gel is first loaded to the casting gel frame. After the resolving gel get firmed, the stacking gel is stacked over the resolving gel into the casting frame and place the comb to cast the well onto the upper part of the gel. After the entire gel get firmed, the gel is ready to be used. This following Table 3.2 shows the ingredients of the resolving gel and stacking gel.

Table 3.2 The ingredients of resolving gel and stacking gel

Materials	Resolving gel	Stacking gel
Distilled water	22.6 ml	12.3 ml
1.5 M Tris	10 ml	-
0.5 M Tris	-	5 ml
10% SDS	40 µl	20 µl
10% Ammonium Persulphate	120 µl	60 µl
Acrylamide-bis	7 ml	2.5 ml
TEMED	60 µl	30 µl

Step 2 Sample preparation. The protein sample is prepared by denaturing it into the denatured solution which consists of Tris-HCl pH 6.8, SDS, β-mercaptoethanol, bromophenol blue, glycerol and distilled water. The sample mixed with the buffer solution is boiled for 10 minutes to denature the protein.

Step 3 Placing the gel to the electrophoresis unit. The gel cassette needs to be placed in the electrophoresis unit and removing the comb to have the well. After that, the gel is submerged into the buffer solution. After the sample and electrophoresis unit are ready, each well on the gel is loaded by 20 µl sample by consecutive order.

Step 4 Performing the electrophoresis. The safety cover is placed to the electrophoresis unit and the lead is connected to the power supply. The voltage is set on 180 volts and the electrophoresis is performed for one hour.

Step 5 Removing the gel. The gel cassette is taken out from the chamber and the cassette plate is removed to take out the gel. After that, the polyacrylamide gel is stained by using the page blue for 10-20 minutes. After the destain process by using water, the gel will show the protein band. The scale of protein molecular weight can be referred to the protein ladder as shown by Figure 3.9 below.

Figure 3.11 The protein ladder for determining the protein molecular weight

3.9 Glucose Concentration Analysis by High-Performance Liquid Chromatography

High-Performance Liquid Chromatography (HPLC) is a specific form of column chromatography used in analysis to separate, identify, and quantify the active compounds [86]. HPLC generally employs a column as the stationary phase, a pump to flow the mobile phase through the column, and the detector for showing the retention time. The retention time is depending on the interaction between the stationary phase and the mobile phase, the molecules being analyzed and the solvent [87]. HPLC method can be used to analyze the glucose concentration. This thesis applied this sequence of steps to utilize the HPLC method in analyzing glucose concentration in broth sample. The glucose was used as the standard. Peak identification was based on HPLC retention times compared with the selected standard. The calibration curve was determined by plotting the peak area with the concentration of glucose solution injected (range covered 10-0.625 g/l)

The samples are taken and centrifuged to remove the cell from its broth and the broth was filtered through 0.2 μm model nylon013N020I filter (Pure Tech® Syringe Filter). Then the filtrate was stored in the vial bottle 1 ml/bottle to be injected into the HPLC. To prepare the carrier solution, 0.008 N H_2SO_4 solution is utilized as a carrier in the liquid chromatography method.

When the sample was injected to the HPLC, the carrier solution flew to carry the sample as the mobile phase by the 0.6 ml/minute flowrate. The flow of mobile phase passed through the R1 column operated at 60^0C to conduct the separation to the mobile phase compound. Then, it entered to the detector to analyze the retention time. the analysis took 30 minutes/sample to show the peak area. The data of the peak area was plotted to the calibration curve to determine the glucose concentration.

3.10 Biomass Measurement

Spectrophotometer UV-Visible Genesys 150 was employed to measure the biomass concentration. The principle of this spectrophotometer is using beam with the specific wavelength through the sample cell and determine the concentration of the sample by using the beam passed the sample and proceed to the detector. The 600 nm wavelength beam is utilized to measure the *E. coli* biomass. This biomass measurement result is represented by OD_{600} value, which equals to cells/ml.10^8.

Figure 3.12 Spectrophotometer UV-Visible Genesys 150

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Recombinant Colicin 1B Protein by SOS Response Induction

As mentioned in the previous chapter, this section observes the effect of aeration rate, temperature, and pH in recombinant protein using SOS response induction. Besides biomass and glucose concentration, the most concerned aspect is protein activity.

4.1.1 Effect of Aeration Rate

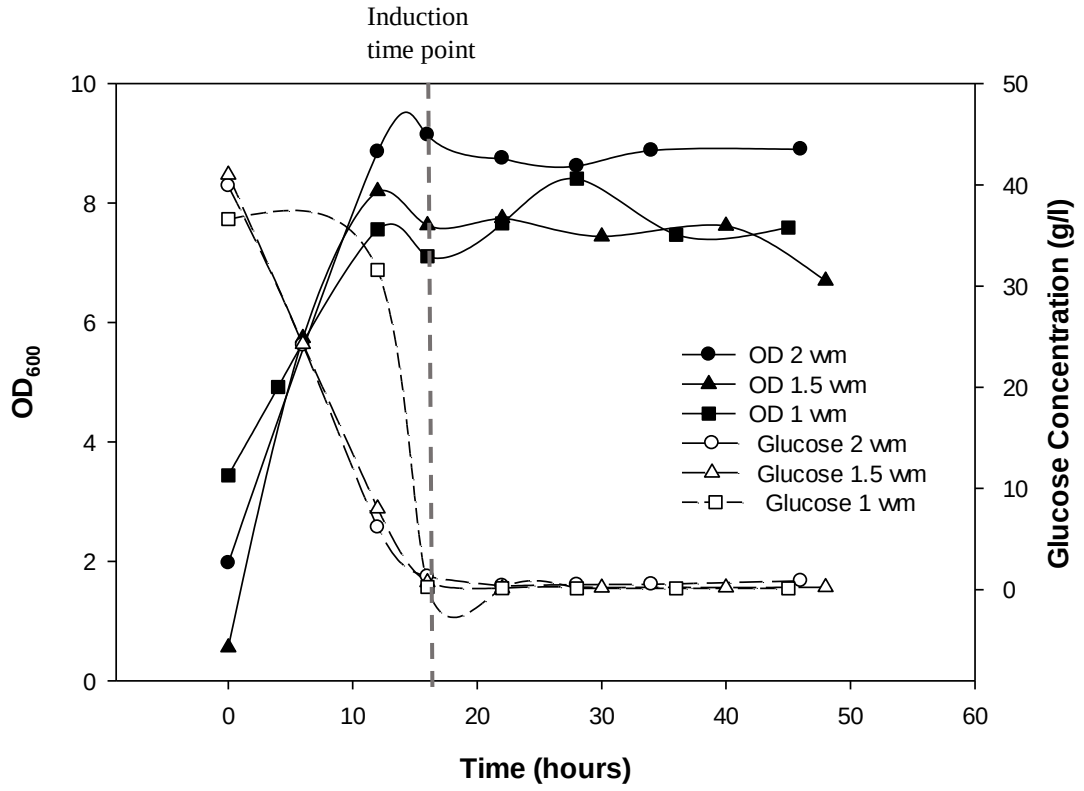


Figure 4.1 Biomass and glucose concentration respect to the time in different one stage aeration rate in 5-liters fermenter by using SOS response induction

Table 4.1 Biomass growth by time in different one stage aeration rate (a) 1 vvm, (b) 1.5 vvm, and (c) 2 vvm

(a)

Time (hour)	0	4	12	16	22	28	36	45
OD_{600}	3.44	4.92	7.56	7.11	7.66	8.41	7.47	7.59

(b)

Time (hour)	0	6	12	16	22	30	40	48
OD_{600}	0.56	5.74	8.2	7.63	7.75	7.44	7.62	6.7

(c)

Time (hour)	0	12	16	22	28	34	46
OD_{600}	1.97	8.86	9.14	8.75	8.62	8.88	8.9

Figure 4.1 shows the effect of one-stage aeration rate 1 vvm, 1.5 vvm and 2 vvm to the biomass and glucose concentration. It is a crystal clear that the higher aeration rate, the higher the obtained biomass. These data show there is no lag phase and proceed to the growth phase at the 6 hours data. Generally, the growth phase stops after 12 hours fermentation when the achieved biomasses are around 7-9. After 12 hours, there is no significant growth and the biomass tend to decreased since the produced colicin killed the cells.

On the other side, the glucose concentrations are rapidly decreased in the growth phase and almost completely consumed. The consumed glucose does not mean that it is oxidized and support cell growth. Some of that glucose is turned to the acetate content. Since the acid condition can hinder the cell growth, the base solution of NaOH was prepared to maintain the pH at 7.

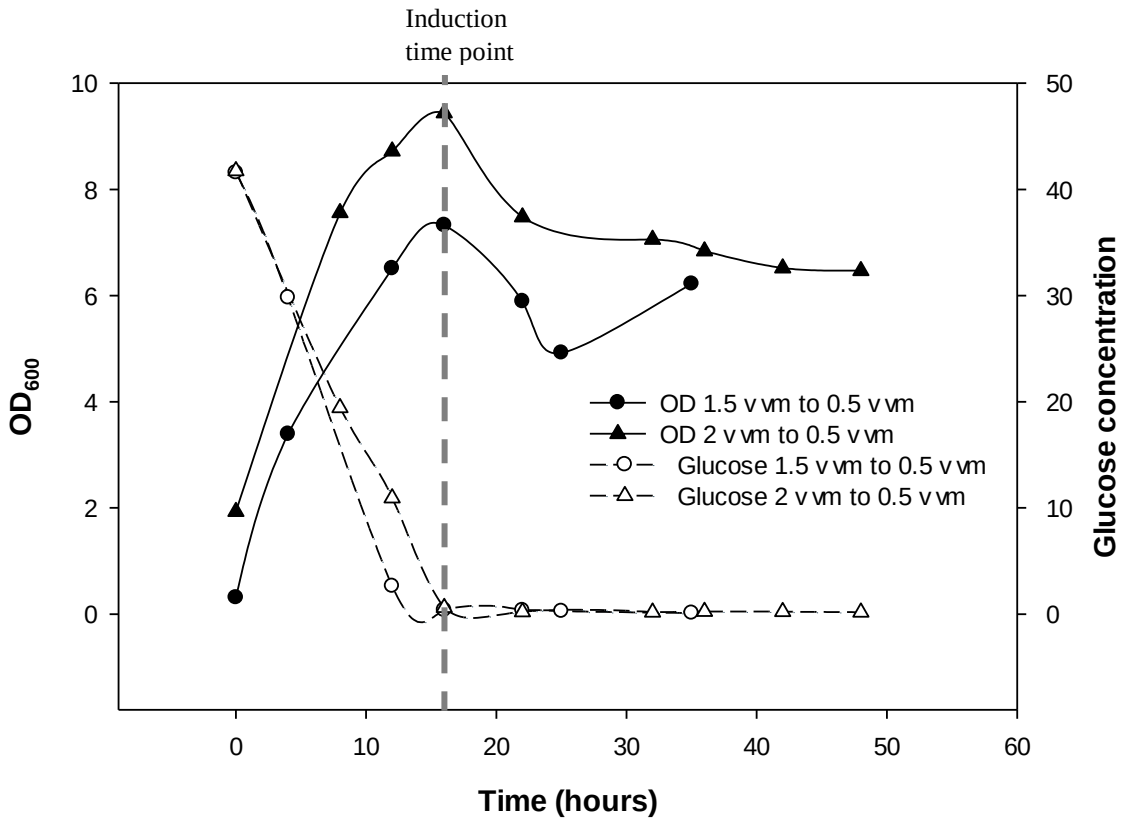


Figure 4.2 Biomass and glucose concentration respect to the time in different two-stages aeration rate in 5-liters fermenter by using SOS response induction

Table 4.2 Biomass growth by time in different two stage aeration rate (a) 1.5 vvm (before induction) to 0.5 (after induction) (b) 2 vvm (before induction) to 0.5 vvm (after induction)

(a)

Time (hour)	0	4	12	16	22	25	35
OD_{600}	0.3	3.39	6.51	7.32	5.89	4.92	6.22

(b)

Time (hour)	0	8	12	16	22	32	36	42	48
OD_{600}	1.93	7.56	8.72	9.44	7.48	7.06	6.84	6.52	6.47

The same phenomenon is also occurring in the two-stage aeration setting. The higher aeration set-up, the higher biomass that can be obtained. However, since the aeration rates are decreased after the induction at 16 hours point, the biomass is also decreased faster compared to the one-stage aeration setting.

Table 4.3 The resulted activity in different aeration rate

Stage	One-stage aeration			Two-stages aeration	
Aeration rate (vvm)	1	1.5	2	1.5 to 0.5	2 to 0.5
Activity	2^{12}	2^{13}	2^{13}	2^{11}	2^{14}

Table 4.1 reveals that the effect aeration condition to the protein expression. In one stage aeration, the changes are not too significant, since there is only a slight change between 1 vvm and the others. Moreover, there is no change between the 1.5 vvm and 2 vvm. However, the two-stage aeration gives the more significant change compared to the one stage. The change from 2 vvm to 0.5 vvm after induction has more protein activity. In contrast, the change from 1.5 vvm to 0.5 vvm after induction have lesser protein activity. In this point, it can be said that setting the higher aeration before the induction then setting the lower aeration after the induction can result in the higher protein activity.

4.1.2 Effect of Temperature

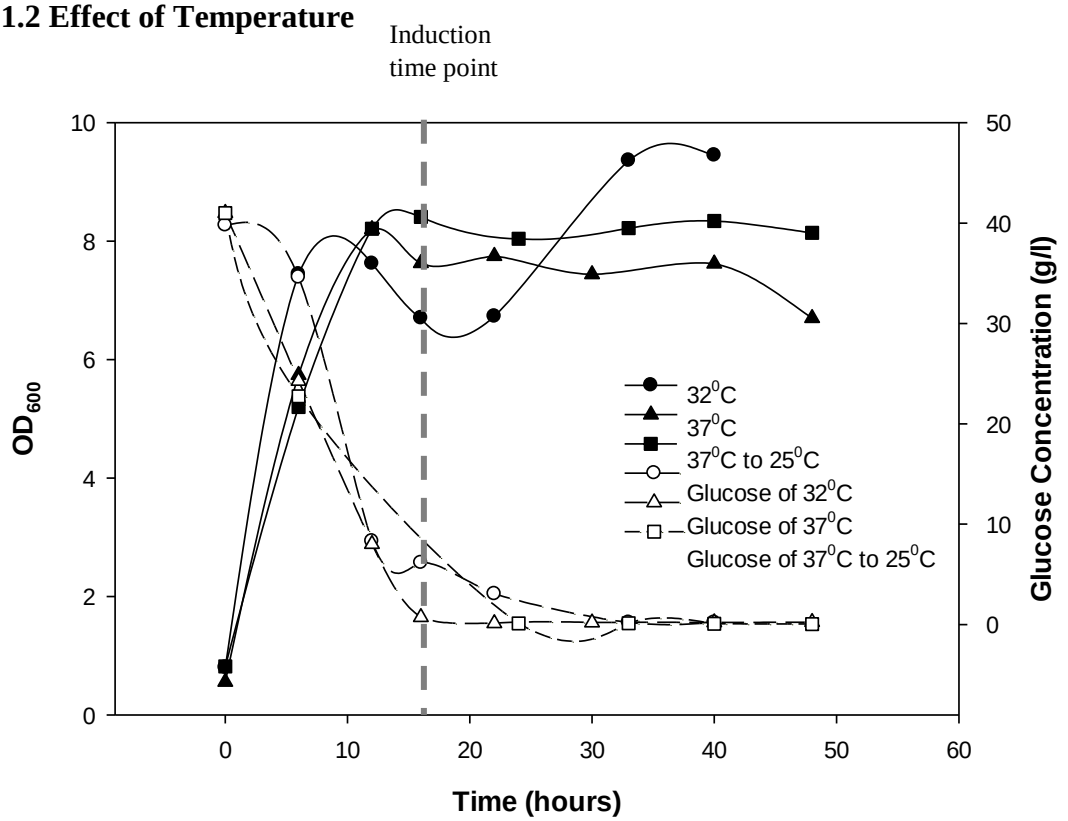


Figure 4.3 Biomass and glucose concentration respect to the time in different temperature in 5-liters fermenter by using SOS response induction

Table 4.4 Biomass growth by time in different temperature (a) 32^0C, (b) 37^0C, and (c) 37^0C (before induction) to 25^0C (after induction)

(a)

Time (hour)	0	6	12	16	22	33	40
OD_{600}	0.8	7.44	7.62	6.7	6.73	9.36	9.45

(b)

Time (hour)	0	6	12	16	22	30	40	48
OD_{600}	0.56	5.74	8.2	7.63	7.75	7.44	7.62	6.7

(c)

Time (hour)	0	6	12	16	24	33	40	48
OD_{600}	0.82	5.2	8.21	8.41	8.04	8.22	8.34	8.14

The effect of temperature to cell growth is observed in the data shown in Figure 4.3. in the one-stage temperature, there is a slight change between the 32^0C and 37^0C. The fermentation at 32^0C gave higher cell growth than the fermentation at the 37^0C. In the other side, the two-stages temperature setting, from 37^0C to 25^0C after induction, did not give many changes compared to the one-stages temperature setting at 37^0C.

These three batches also show their glucose consumption characteristic. The one-stage temperature 37^0C and two-stages temperature show similar behavior. Both the cell growth rate and glucose consumption stop their growth and consumption after 12-hours point. Then the cell growth proceeds to the stationary phase and remains the slight amount of glucose content. Besides, the glucose consumption in 32^0C stops slower. it remains a slight amount of glucose after 24 hours fermentation. Consequently, cell growth can be higher since the glucose is consumed gradually.

Table 4.5 The resulted activity in different temperature

Stage	One-stage temperature		Two-stages temperature
Temperature (^{0}C)	32	37	37 to 25
Activity	2^{11}	2^{12}	2^{11}

Based on Table 4.2, the variation of temperature did not give a significant effect on the resulted protein activity. Even though the stress was given by decreasing the temperature after the induction, the resulted protein activity almost similar to the one-stage temperature.

4.1.3 Effect of pH

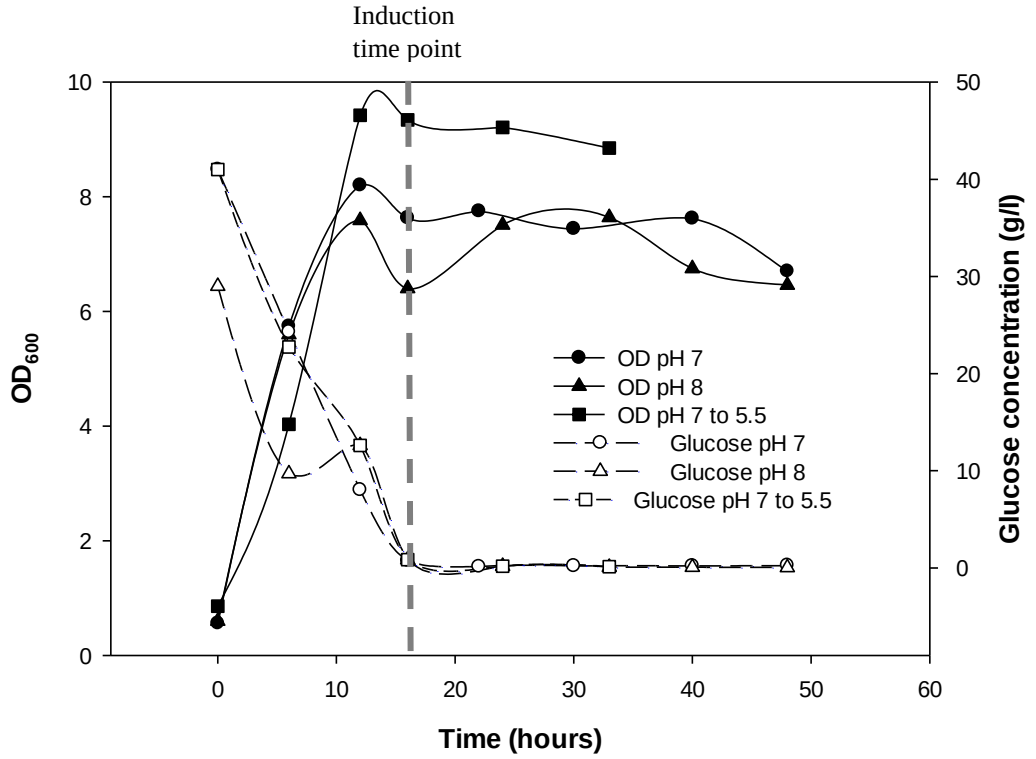


Figure 4.4 Biomass and glucose concentration respect to the time in different pH in 5-liters fermenter by using SOS response induction

Table 4.6 Biomass growth by time in different pH (a) 7, (b) 8, and (c) 7 (before induction) to 5.5 (after induction)

(a)

Time (hour)	0	6	12	16	22	30	40	48
OD_{600}	0.56	5.74	8.2	7.63	7.75	7.44	7.62	6.7

(b)

Time (hour)	0	6	12	16	24	30	40	48
OD_{600}	0.6	5.6	7.59	6.4	7.51	7.64	6.75	6.46

(c)

Time (hour)	0	6	12	16	24	33
OD_{600}	0.86	4.03	9.42	9.34	9.21	8.85

As mentioned in the previous chapter that the acetate content, causing the acid environment, can hinder the *E. coli cell* growth. In contrast, the little bit base condition did not give the obstacle to the *E. coli* cell growth. It is shown in Figure 4.4 that fermentation at pH 8 has a similar growth compared to the fermentation at pH 7. The acetate formation related to the glucose consumption to the cell. In the growth phase, since the glucose is consumed and oxidized by the cell, the glucose concentration is decreased significantly. However, not all consumed glucose content is oxidized by the cell. Consequently, the cells form the acetate content as the by-product. Therefore, in the growth phase, the pH tends to decrease. To maintain the pH-7, the NaOH solution is added periodically.

Table 4.7 The resulted activity in different pH

Stage	One-stage pH		Two-stages pH
pH	7	8	7 to 5.5
Activity	2^{12}	2^{5}	2^{5}

Based on the data shown in Table 4.3, either base or acid condition can decrease protein activity. In the one-stage pH, the base condition, pH-8, resulted in the much lower protein activity than the neutral pH condition. In the two-stages pH, the decreased pH after induction also resulted in the lower protein activity than one-stage at pH-7.

4.2 Recombinant Colicin 1B Protein by IPTG Induction

4.2.1 Effect of Induction Time Point

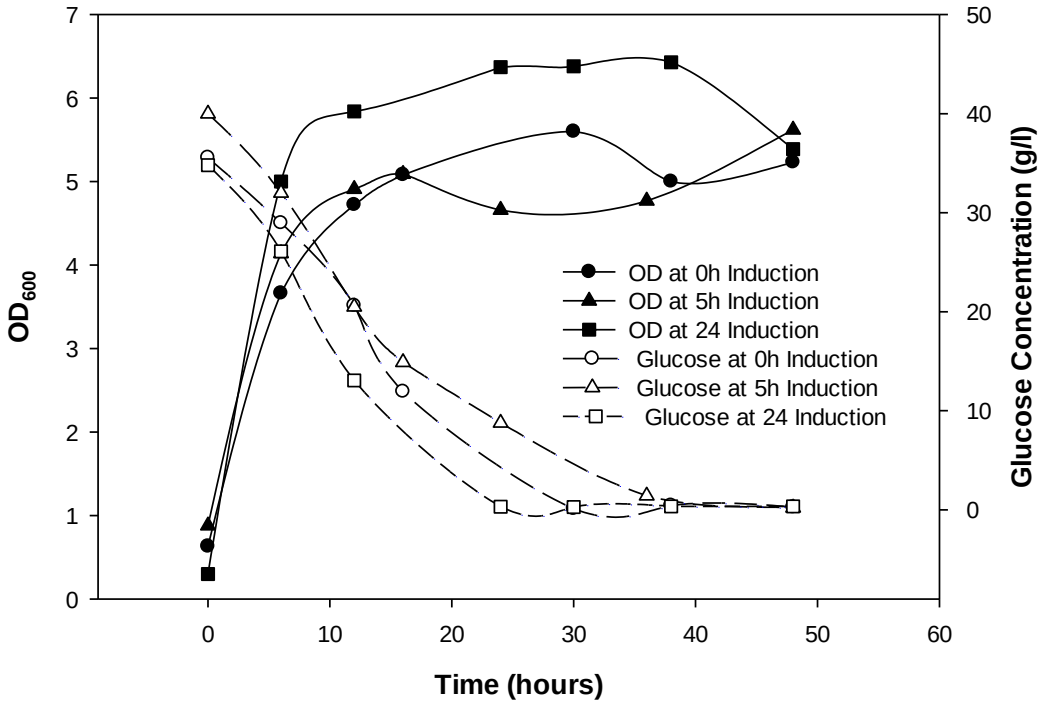


Figure 4.5 Biomass and glucose concentration respect to the time in different induction time point in 5-liters fermenter by using IPTG induction

Table 4.8 Biomass growth by time in different induction time point (a) 0-hour, (b) 5-hours, and (c) 24-hours

(a)

Time (hour)	0	6	12	16	30	38	48
OD_{600}	0.63	3.66	4.72	5.08	5.6	5	5.23

(b)

Time (hour)	0	6	12	16	24	36	48
OD_{600}	0.88	4.14	4.91	5.09	4.66	4.77	5.62

(c)

Time (hour)	0	6	12	16	24	36	48
OD_{600}	0.3	5	5.84	6.37	6.38	6.43	5.39

When induction is conducted, the *lac repressor* protein will be released and trigger the production protein. Since the production protein requires the metabolic resources, the induction time point gives the effect to the *E. coli* cell growth. The utilized inducer is 1 M IPTG solution. the amount of the inducer is 0.1 ml/1 liter working volume. When induction is conducted at 0-hour, the metabolic resources are split to the cell growth and recombinant protein at 0-hour point. Moreover, the produced colicins are lethal to the *E. coli* cells. Therefore, the obtained biomass is lower than the induction time point at 5-hour and 24-hour.

Table 4.9 The resulted activity in different induction IPTG time point by using 1 M IPTG solution in 5-liters fermenter

Induction time point	0-hour	5-hour	24-hour
Activity	2^{17}	2^{17}	2^{17}

Based on Table 4.4, the induction time point did not give the effect to the protein expression. Since the time induction point only affects the cell growth based on the split metabolic resources. Therefore, whenever the induction point is, the gene inside the cell produces the protein in the same way. However, to obtain the mass product of colicin protein, the high cell density culture of *E. coli* needs to be produced.

4.2.2 Comparison Between 5-liter and 100-liter Airlift Fermenter

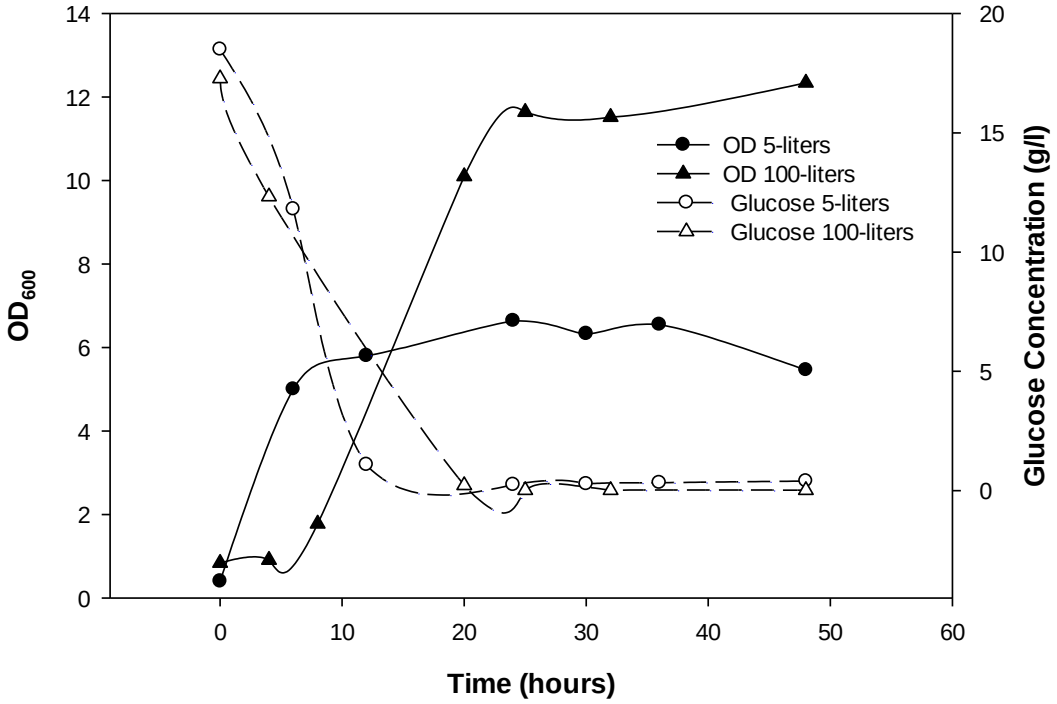


Figure 4.6 Biomass and glucose concentration respect to the time in different volume of the fermenter by using IPTG induction

Table 4.10 Biomass growth by time in different volume of airlift fermenter (a) 5-liters, and (b) 100-liters

(a)

Time (hour)	0	6	12	24	30	36	48
OD_{600}	0.4	5	5.8	6.64	6.33	6.55	5.46

(b)

Time (hourS)	0	4	8	20	25	32	48
OD_{600}	0.84	0.91	1.78	10.1	11.64	11.52	12.34

There is a difference inoculation rate between fermentation in 100-liters fermenter and 5-liters fermenter. In the 5-liters fermenter, the inoculation rate is 10% in the 4 liters total working volume while in the 100-liters fermenter, the inoculation rate is 2% in the 80 liters total working volume. In Figure 4.6, There is a lag phase in 100-liters fermenter. After the lag phase, the growth phase occurred until 24 hours point and achieve the biomass above 10. Then the stationary phase occurred. In the other side, there is no lag phase in 5-liters fermenter and proceed to the growth phase until the 12-hours point and achieve the lower biomass than the 100-liters fermenter.

This both 5-liters and 100-liters fermenter experiments employ 20g/l medium glucose for supplying the carbon source. In Figure 4.6, it can be observed that in the 5-liter fermenter, the glucose is consumed faster than in the 100-liters fermenter. In the same way, the growth phase in the 5-liters fermenter finished faster than the growth phase in the 100-liters fermenter.

Table 4.11 The resulted protein activity in different volume of the airlift fermenter by using IPTG inducer

Volume of fermenter	5-liters	100-liters
Activity	2^{16}	2^{19}

Regarding the protein activity, fermentation in 100-liters fermenter gave more protein activity than the fermentation in 5-liters fermenter. Even though using the same strain, the volume difference may give different resulted in protein activity.

4.3 The Summary of Recombinant Protein in *Escherichia coli*

Fermentation of *Escherichia coli* in 5-liters fermenter is employed to conduct the recombinant protein-colicin 1B. The SOS gene response, induced by mitomycin C, is used to generate the protein product. This following Table 4.6 shows the result of recombinant protein by using the SOS gene response as an induction system.

Table 4.12 Result of recombinant protein in *Escherichia coli* inside 5-liters fermenter by using the SOS response induction

Set up Variables				
Aeration rate (vvm)	Temperature (^{0}C)	pH	Final OD_{600}	Activity Result
1	37	7	7.59	2^{12}
1.5	37	7	6.7	2^{13}
2	37	7	8.9	2^{13}
Before induction: 1.5; after induction: 0.5	37	7	6.22	2^{11}
Before induction: 2; after induction: 0.5	37	7	6.47	2^{14}
1.5	32	7	9.45	2^{11}
1.5	37	7	6.7	2^{12}
1.5	Before induction: 37; after induction: 25	7	8.14	2^{11}
1.5	37	7	6.7	2^{12}
1.5	37	8	6.46	2^5
1.5	37	Before induction: 7; after induction: 5.5	8.85	2^5

Beside of using the SOS response gene as the induction system, the IPTG induction method was also observed. By using the IPTG induction, the induction time point effect to the resulted recombinant protein was investigated. The resulted recombinant protein in different volume of airlift bioreactor was also compared. This following Table 4.7 shows the result of recombinant protein by using the IPTG induction system in the default operating condition aeration rate, temperature and pH were 1.5 vvm, 37^0C, and 7 respectively.

Table 4.13 Result of recombinant protein in *Escherichia coli* by using the IPTG induction

Variables				
Induction time point (hours)	Volume of airlift bioreactor (liter)	Initial glucose concentration (g/l)	Final OD_{600}	Activity Result
0	5	40	5.23	2^{17}
5	5	40	5.62	2^{17}
24	5	40	5.39	2^{17}
24	5	20	5.46	2^{16}
24	100	20	12.34	2^{19}

4.4 The SDS-PAGE Result for Protein Analysis

Beside the protein activity analysis method, SDS-PAGE was also employed to observed the protein existence in the sample. Figure 4.7 shows the SDS-PAGE result to show the protein band in the recombinant protein production (RPP). Lane 1 and 2 shows the marker band which represent the scale in the left column in kilodalton (kDa). The lane 3-6 show the protein band at RPP in the 32^0C temperature. It is shown that the protein bands are not so visible. Probably, the protein macromolecules in these lanes are very small. The lane 7-10 show the protein band at RPP in the 37^0C temperature. The bands on the lane 7-10 are more visible than the bands on the lane 3-6. Therefore, in this point, the RPP in the 37^0C temperature has the better protein expression than the RPP in the 32^0C temperature

Figure 4.7 SDS page result; lane 1-2 marking band; lane 3-6 colicin 1B from Recombinant Protein Production at 32^0C; lane 3-6 colicin 1B from Recombinant Protein Production at 37^0C

CHAPTER V

CONCLUSION

Dissolved oxygen enrichment can be beneficial for *E. coli* cell growth. The higher aeration rate may increase the resulted biomass. On the other hand, the lower aeration rate can give the stress to the SOS response gene. Consequently, it may trigger more recombinant protein production. Therefore, the higher aeration rate before induction then the lower aeration rate after induction can be conducted to obtain a high amount of protein colicin 1B product.

The culture temperature probably cannot give a significant effect on protein activity. However, it still can give effect to cell growth. Thus, this is important to keep the temperature in a range that the cell growth keeps high. In the other side, the pH setting gave a significant effect to the recombinant protein. The neutral condition, pH 7, is an ideal condition for the recombinant protein of colicin 1B. The acid and base environment did not give the satisfying resulted in protein activity. Thus, pH control is crucial to keep the culturing pH at 7.

In an experiment that using the IPTG induction gene to produce the colicin, the induction time point did not affect the protein activity. However, it affected cell growth since the metabolic resources are divided to produce the protein and grow the cell. Therefore, the induction time point after the growth phase is a good choice to obtain higher cell growth. The comparison between fermentation in 100-liters and 5-liters shows the trend that the bigger volume can grow more cells even the inoculation rate is lower. Besides the cell growth, the obtained protein activity in the 100-liters fermenter is higher the 5-liters fermenter. Therefore, to obtain the optimum recombinant protein result in IPTG strain, the scaled-up volume and the induction time point after the growth phase is the good choices.

Based on this thesis experiment, the gene inducted by IPTG has the better protein expression result. Since the resulted protein activities are 10^{19} in the 100-liters fermenter and 10^{16} and 10^{17}. On the other side, the resulted protein activity in the SOS response induction are around 10^{11} -10^{14}. Beside its protein activity result, the reason why the IPTG induction is more preferred than the SOS response is the IPTG induction is easier and has the lower cost. Induction by using the SOS response need the UV exposure and drugs to give the cell a stress. Since those inducers are costly, instead of using the SOS response induction, the IPTG is an alternative to produce the recombinant protein.

CHAPTER VI

FUTURE WORK

This thesis experiment shows that the recombinant protein colicin 1B hosted by *E. coli* strain can be influenced by several factors. The significant differences are shown by the protein expression resulted in these both induction methods. In the same operating conditions, the IPTG induction method shows the better result than the SOS response method. However, by using this experiment result, there are some further works that can be conducted to develop this research.

First of all is investigating the environmental factor effect to the recombinant protein in *E. coli* by using IPTG induction method. Regarding the environmental factor such as aeration rate, temperature, and pH that give effect to the recombinant protein using the SOS response induction method, the same way should be conducted by using the IPTG induction since it is more preferred. Thus, the comprehensive data can be collected to decide the best operating procedure in order to obtain the desired product.

Since the aeration rate, that is also related to dissolved oxygen, is the factor which gave the significant effect to the resulted protein expression, the design of airlift fermenter can be modified to increase the oxygen transfer rate. Some research developed the project to increase the oxygen transfer rate that can be applied to the airlift bioreactor. For instance, adding membrane to increase the volumetric gas-liquid mass transfer coefficient [17][18].

Beside of the modification of fermenter design, scaling up the fermenter to the pilot scale also need to be conducted. In order to lead this research to the industrial scale. Since this thesis research provide the data of recombinant protein production by using 100-liters airlift fermenter, this data can be a reference to consider the experiment model in pilot scale.

APPENDIX

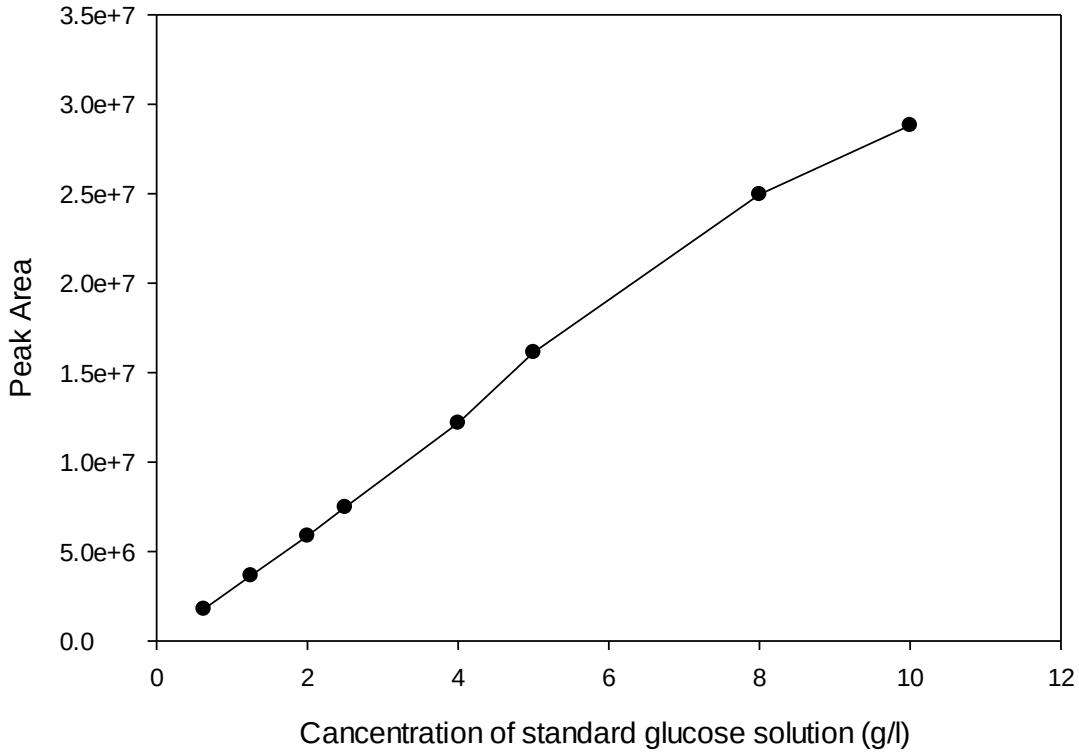


Figure A.1 Calibration curve of glucose concentration vs peak area in HPLC by using 0.008 N H_2SO_4 as a carrier solution; flowrate: 0.6 ml/minute; max pressure: 408 kgf/cm^2; injected sample volume: 30 µl; RI column temperature: 60^0C; RI detector stop time: 30 minutes.

Reference

[1] G. L. Rosano and E. A. Ceccarelli, "Recombinant protein expression in *Escherichia coli*: Advances and challenges," *Front. Microbiol.*, vol. 5, no. APR, pp. 1–17, 2014.

[2] F. Baneyx, "Recombinant Protein Expression in *Escherichia coli*," *Curr. Opin. Biotechnol.*, vol. 10, pp. 411–421, 1999.

[3] F. Hoffmann and U. Rinas, "Stress Induced by Recombinant Protein Production in *Escherichia coli*," pp. 73–92, 2012.

[4] H. P. Sørensen and K. K. Mortensen, "Advanced genetic strategies for recombinant protein expression in *Escherichia coli*," *J. Biotechnol.*, vol. 115, no. 2, pp. 113–128, 2005.

[5] J. Shiloach and R. Fass, "Growing *E. coli* to high cell density - A historical perspective on method development," *Biotechnol. Adv.*, vol. 23, no. 5, pp. 345–357, 2005.

[6] S. Y. Lee, "High cell-density culture of *Escherichia coli*," *Trends Biotechnol.*, vol. 14, no. 3, pp. 98–105, 1996.

[7] G. J. Gopal and A. Kumar, "Strategies for the production of recombinant protein in *Escherichia coli*," *Protein J.*, vol. 32, no. 6, pp. 419–425, 2013.

[8] J. H. Choi, K. C. Keum, and S. Y. Lee, "Production of recombinant proteins by high cell density culture of *Escherichia coli*," *Chem. Eng. Sci.*, vol. 61, no. 3, pp. 876–885, 2006.

[9] S. Schaepe, A. Kuprijanov, R. Simutis, and A. Lübbert, "Avoiding overfeeding in high cell density fed-batch cultures of *E. coli* during the production of heterologous proteins," *J. Biotechnol.*, vol. 192, no. Part A, pp. 146–153, 2014.

[10] W. Xue *et al.*, "Effects of acetic acid and its assimilation in fed-batch cultures of recombinant *Escherichia coli* containing human-like collagen cDNA," *J. Biosci. Bioeng.*, vol. 109, no. 3, pp. 257–261, 2010.

[11] P. Small, D. Blankenhorn, D. Welty, E. Zinser, and J. L. Slonczewski, "Acid and base resistance in *Escherichia coli* and Shigella flexneri: Role of rpoS and growth pH," *J. Bacteriol.*, vol. 176, no. 6, pp. 1729–1737, 1994.

[12] U. Onken and E. Liefke, "Effect of total and partial pressure (oxygen and carbon dioxide) on aerobic microbial processes," *Bioprocesses Eng.*, vol. 40, pp. 137–169, 2005.

[13] J. Singh, N. Kaushik, and S. Biswas, "Bioreactors – Technology & Design Analysis," *SCITECH J.*, vol. 01, no. 06, pp. 28–36, 2016.

[14] B. Özkaya, A. H. Kaksonen, E. Sahinkaya, and J. A. Puhakka, "Fluidized bed bioreactor for multiple environmental engineering solutions," *Water Res.*, no. November, pp. 452–465, 2019.

[15] E. Daniil, "Temperature dependence of liquid film coefficient for gas transfer," *J Env. Eng*, vol. 114, no. 5, pp. 1224–1229, 1989.

[16] G. Campani *et al.*, "Recombinant protein production by engineered *Escherichia coli* in a pressurized airlift bioreactor: A techno-economic analysis," *Chem. Eng. Process. Process Intensif.*, vol. 103, pp. 63–69, 2016.

[17] S. Popovic, N. Lukic, P. Kojic, D. Jovicevic, D. Petrovic, and I. Sijacki, "Volumetric gas-liquid mass transfer coefficient in an external-loop airlift reactor with inserted membrane," *Chem. Ind. Chem. Eng. Q.*, vol. 22, no. 3, pp. 275–284, 2015.

[18] C. Wei, B. Wu, G. Li, K. Chen, M. Jiang, and P. Ouyang, "Comparison of the hydrodynamics and mass transfer characteristics in internal-loop airlift bioreactors utilizing either a novel membrane-tube sparger or perforated plate sparger,"

Bioprocess Biosyst. Eng., vol. 37, no. 11, pp. 2289–2304, 2014.
[19] L. Balabanova, V. Golotin, A. Podvolotskaya, and V. Rasskazov, "Genetically modified proteins: Functional improvement and chimeragenesis," *Bioengineered*, vol. 6, no. 5, pp. 262–274, 2015.
[20] P. T. Lomedico, "Use of recombinant DNA technology to program eukaryotic cells to synthesize rat proinsulin: a rapid expression assay for cloned genes.," *Proc. Natl. Acad. Sci.*, vol. 79, no. 19, pp. 5798–5802, 2006.
[21] S. Slatin *et al.*, "Colicin Biology," *Microbiol. Mol. Biol. Rev.*, vol. 71, no. 1, pp. 158–229, 2007.
[22] H. Pilsl and V. Braun, "-dependent uptake via to TonB- Tsx receptor and to," vol. 16, pp. 57–67, 1995.
[23] Pilsl H and Braun V, "Strong function related homology between the pore forming colicins K and 5," *J. Bacteriol.*, vol. 177, no. 23, pp. 6973–6977, 1995.
[24] Z. Hajihassan and N. Biroonro, "Enhanced expression of recombinant activin a in *Escherichia coli* by optimization of induction parameters," *J. Sci. Islam. Repub. Iran*, vol. 29, no. 2, pp. 105–111, 2018.
[25] S. Sathe, P. P. Wangikar, D. Nelapati, V. Javadekar-Subhedar, S. V. Sohoni, and R. P. Gaikaiwari, "Optimization of high cell density fermentation process for recombinant nitrilase production in *E. coli*," *Bioresour. Technol.*, vol. 188, pp. 202–208, 2015.
[26] L. M. Guzman, J. J. Barondess, and J. Beckwith, "FtsL, an essential cytoplasmic membrane protein involved in cell division in *Escherichia coli*," *J. Bacteriol.*, vol. 174, no. 23, pp. 7717–7728, 1992.
[27] J. H. Choi and S. Y. Lee, "Secretory and extracellular production of recombinant proteins using *Escherichia coli*," *Appl. Microbiol. Biotechnol.*, vol. 64, no. 5, pp. 625–635, 2004.
[28] B. Miroux and J. E. Walker, "#044_Miroux et al_Over-production of proteins in *Escherichia coli*_ mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels J_ Mol_ Biol_ 260_ 289-298 (1996)_.pdf," pp. 289–298, 1996.
[29] I. Sermadiras, J. Revell, J. E. Linley, A. Sandercock, and P. Ravn, "Recombinant expression and in Vitro characterisation of active huwentoxin-IV," *PLoS One*, vol. 8, no. 12, pp. 1–11, 2013.
[30] F. Rezaie *et al.*, "Cytosolic expression of functional Fab fragments in *Escherichia coli* using a novel combination of dual SUMO expression cassette and EnBase® cultivation mode," *J. Appl. Microbiol.*, vol. 123, no. 1, pp. 134–144, 2017.
[31] E. Jalomo-Khayrova, R. E. Mares, P. L. A. Muñoz, S. G. Meléndez-López, I. A. Rivero, and M. A. Ramos, "Soluble expression of an amebic cysteine protease in the cytoplasm of *Escherichia coli* SHuffle Express cells and purification of active enzyme," *BMC Biotechnol.*, vol. 18, no. 1, pp. 1–7, 2018.
[32] J. H. Choi, K. J. Jeong, S. C. Kim, and S. Y. Lee, "Efficient secretory production of alkaline phosphatase by high cell density culture of recombinant *Escherichia coli* using the Bacillus sp. endoxylanase signal sequence," *Appl. Microbiol. Biotechnol.*, vol. 53, no. 6, pp. 640–645, 2000.
[33] Q. Zhang, T.-P. Li, R.-B. Gan, R. Xu, P. Du, and J.-J. Fan, "High-Level Expression and Secretion of Recombinant Mouse Endostatin by *Escherichia coli*," *Protein Expr. Purif.*, vol. 24, no. 3, pp. 453–459, 2002.
[34] S. Tan, W. Wu, J. Liu, Y. Kong, Y. Pu, and R. Yuan, "Efficient expression and secretion of recombinant hirudin III in *E. coli* using the L-asparaginase II signal sequence," *Protein Expr. Purif.*, vol. 25, no. 3, pp. 430–436, 2002.

[35] Z. Dong, J. Zhang, G. Du, J. Chen, H. Li, and B. Lee, "Periplasmic Export of Bile Salt Hydrolase in *Escherichia coli* by the Twin-Arginine Signal Peptides," *Appl. Biochem. Biotechnol.*, vol. 177, no. 2, pp. 458–471, 2015.
[36] F. Guggenbichler, C. Büttner, W. Rudolph, K. Zimmermann, F. Gunzer, and C. Pöhlmann, "Design of a covalently linked human interleukin-10 fusion protein and its secretory expression in *Escherichia coli*," *Appl. Microbiol. Biotechnol.*, vol. 100, no. 24, pp. 10479–10493, 2016.
[37] C. Cheng, S. Wu, L. Cui, Y. Wu, T. Jiang, and B. He, "A novel Ffu fusion system for secretory expression of heterologous proteins in *Escherichia coli*," *Microb. Cell Fact.*, vol. 16, no. 1, pp. 1–12, 2017.
[38] W. Zhang, J. Lu, S. Zhang, L. Liu, X. Pang, and J. Lv, "Development an effective system to expression recombinant protein in *E. coli* via comparison and optimization of signal peptides: Expression of Pseudomonas fluorescens BJ-10 thermostable lipase as case study," *Microb. Cell Fact.*, vol. 17, no. 1, pp. 1–12, 2018.
[39] Q. Lin *et al.*, "Extracellular Expression, Purification, and Characterization of a Winter Flounder Antifreeze Polypeptide from *Escherichia coli*," *Protein Expr. Purif.*, vol. 18, no. 2, pp. 175–181, 2002.
[40] T. L. Lam, R. S. C. Wong, and W. K. R. Wong, "Enhancement of extracellular production of a Cellulomonas fimi exoglucanase in *Escherichia coli* by the reduction of promoter strength," *Enzyme Microb. Technol.*, vol. 20, no. 7, pp. 482–488, 1997.
[41] T. Matsumoto, D. Katsura, A. Kondo, and H. Fukuda, "Efficient secretory overexpression of Bacillus subtilis pectate lyase in *Escherichia coli* and single-step purification," *Biochem. Eng. J.*, vol. 12, no. 3, pp. 175–179, 2002.
[42] S. W. Park, M. G. Chung, H. Y. Lee, J. Y. Kim, and Y. H. Rhee, "Stable expression and secretion of polyhydroxybutyrate depolymerase of Paucimonas lemoignei in *Escherichia coli*," *J. Microbiol.*, vol. 46, no. 6, pp. 662–669, 2008.
[43] C. W. Chung, J. You, K. Kim, Y. Moon, H. Kim, and J. H. Ahn, "Export of recombinant proteins in *Escherichia coli* using ABC transporter with an attached lipase ABC transporter recognition domain (LARD)," *Microb. Cell Fact.*, vol. 8, pp. 1–12, 2009.
[44] L. Su, C. Xu, R. W. Woodard, J. Chen, and J. Wu, "A novel strategy for enhancing extracellular secretion of recombinant proteins in *Escherichia coli*," *Appl. Microbiol. Biotechnol.*, vol. 97, no. 15, pp. 6705–6713, 2013.
[45] Z. Y. Chen, J. Cao, L. Xie, X. F. Li, Z. H. Yu, and W. Y. Tong, "Construction of leaky strains and extracellular production of exogenous proteins in recombinant *Escherichia coli*," *Microb. Biotechnol.*, vol. 7, no. 4, pp. 360–370, 2014.
[46] A. N. Bugay, E. A. Krasavin, A. Y. Parkhomenko, and M. A. Vasilyeva, "Modeling nucleotide excision repair and its impact on UV-induced mutagenesis during SOS-response in bacterial cells," *J. Theor. Biol.*, vol. 364, no. March, pp. 7–20, 2015.
[47] J. W. Little and D. W. Mount, "The SOS Regulatory *Escherichia coli* System of Review," *Cell*, vol. 29, no. May, pp. 11–22, 1982.
[48] V. E. Galkin, C. E. Bell, D. Ndjonka, X. Yu, E. H. Egelman, and J. Bielnicki, "Cleavage of Bacteriophage λ cI Repressor Involves the RecA C-Terminal Domain," *J. Mol. Biol.*, vol. 385, no. 3, pp. 779–787, 2008.
[49] Z. Podlesek, A. Ihan, D. Zgur-Bertok, P. Mrak, J. Mulec, and A. Kopitar, "A cka-gfp Transcriptional Fusion Reveals that the Colicin K Activity Gene Is Induced in Only 3 Percent of the Population," *J. Bacteriol.*, vol. 185, no. 2, pp. 654–659, 2003.

[50] C. Janion, “Inducible SOS response system of DNA repair and mutagenesis in *Escherichia coli*,” *Int. J. Biol. Sci.*, vol. 4, no. 6, pp. 338–344, 2008.
[51] J. Xu, A. Banerjee, S. H. Pan, and Z. J. Li, “Galactose can be an inducer for production of therapeutic proteins by auto-induction using *E. coli* BL21 strains,” *Protein Expr. Purif.*, vol. 83, no. 1, pp. 30–36, 2012.
[52] A. Marbach and K. Bettenbrock, “Lac operon induction in *Escherichia coli*: Systematic comparison of IPTG and TMG induction and influence of the transacetylase LacA,” *J. Biotechnol.*, vol. 157, no. 1, pp. 82–88, 2012.
[53] B. L. Wanner, R. Kodaira, and F. C. Neidhardt, “Regulation of lac operon expression: Reappraisal of the theory of catabolite repression,” *J. Bacteriol.*, vol. 136, no. 3, pp. 947–954, 1978.
[54] Y. Matsuoka and K. Shimizu, “Metabolic regulation in *Escherichia coli* in response to culture environments via global regulators,” *Biotechnol. J.*, vol. 6, no. 11, pp. 1330–1341, 2011.
[55] W. S. Reznikoff, “The lactose operon-controlling elements: a complex paradigm,” *Mol. Microbiol.*, vol. 6, no. 17, pp. 2419–2422, 1992.
[56] N. Bhagavan, *Regulation of Gene Expression. Essential of Medical Biochemistry (Second Edition) with Clinical Cases*. 2015.
[57] T. W. Overton, “Recombinant protein production in bacterial hosts,” *Drug Discov. Today*, vol. 19, no. 5, pp. 590–601, 2014.
[58] J. W. Dubendorfft, W. Studier, and R. August, “Controlling Basal Expression in an Inducible T7 Expression System by Blocking the Target T7 Promoter with Zac Repressor,” pp. 45–59, 1991.
[59] F. W. Studier, “Protein production by auto-induction in high density shaking cultures.,” *Protein Expr. Purif.*, vol. 41, no. 1, pp. 207–234, 2005.
[60] H. H. Winkler and T. H. Wilson, “Inhibition of β-galactoside transport by substrates of the glucose transport system in *Escherichia coli*,” *BBA - Biomembr.*, vol. 135, no. 5, pp. 1030–1051, 1967.
[61] A. Fernández-Castané, C. E. Vine, G. Caminal, and J. López-Santín, “Evidencing the role of lactose permease in IPTG uptake by *Escherichia coli* in fed-batch high cell density cultures,” *J. Biotechnol.*, vol. 157, no. 3, pp. 391–398, 2012.
[62] V. Chauhan, A. Singh, S. M. Waheed, S. Singh, and R. Bhatnagar, “Constitutive expression of protective antigen gene of Bacillus anthracis in Escherichia coil,” *Biochem. Biophys. Res. Commun.*, vol. 283, no. 2, pp. 308–315, 2001.
[63] L. Xie, D. Hall, M. A. Eiteman, and E. Altman, “Optimization of recombinant aminolevulinate synthase production in *Escherichia coli* using factorial design,” *Appl. Microbiol. Biotechnol.*, vol. 63, no. 3, pp. 267–273, 2003.
[64] S. Y. Hu, J. L. Wu, and J. H. Huang, “Production of tilapia insulin-like growth factor-2 in high cell density cultures of recombinant *Escherichia coli*,” *J. Biotechnol.*, vol. 107, no. 2, pp. 161–171, 2004.
[65] H. Yuan, X. Yang, and Z. C. Hua, “Optimization of expression of an Annexin V-Hirudin chimeric protein in *Escherichia coli*,” *Microbiol. Res.*, vol. 159, no. 2, pp. 147–156, 2004.
[66] G. Chhetri, P. Kalita, and T. Tripathi, “An efficient protocol to enhance recombinant protein expression using ethanol in *Escherichia coli*,” *MethodsX*, vol. 2, pp. 385–391, 2015.
[67] I. Voulgaris, S. A. Arnold, R. Speight, L. M. Harvey, and B. McNeil, “Effects of dissolved oxygen availability and culture biomass at induction upon the intracellular expression of monoamine oxidase by recombinant *E. coli* in fed batch bioprocesses,” *Process Biochem.*, vol. 46, no. 3, pp. 721–729, 2011.

[68] A. Baez and J. Shiloach, "*Escherichia coli* avoids high dissolved oxygen stress by activation of SoxRS and manganese-superoxide dismutase," *Microb. Cell Fact.*, vol. 12, no. 1, pp. 1–9, 2013.
[69] S. B. Farr and T. Kogoma, "Oxidative stress responses in *Escherichia coli* and Salmonella typhimurium.," *Microbiol. Rev.*, vol. 55, no. 4, pp. 561–85, 1991.
[70] M. Gu and J. A. Imlay, "The SoxRS response of *Escherichia coli* is directly activated by redox-cycling drugs rather than by superoxide," *Mol. Microbiol.*, vol. 79, no. 5, pp. 1136–1150, 2011.
[71] A. Castan, A. Näsman, and S. O. Enfors, "Oxygen enriched air supply in *Escherichia coli* processes: Production of biomass and recombinant human growth hormone," *Enzyme Microb. Technol.*, vol. 30, no. 7, pp. 847–854, 2002.
[72] E. A. Sandoval-Basurto, G. Gosset, F. Bolivar, and O. T. Ramírez, "Culture of *Escherichia coli* under dissolved oxygen gradients simulated in a two-compartment scale-down system: Metabolic response and production of recombinant protein," *Biotechnol. Bioeng.*, vol. 89, no. 4, pp. 453–463, 2005.
[73] P.-P. Champagne *et al.*, "The use of dissolved oxygen-controlled, fed-batch aerobic cultivation for recombinant protein subunit vaccine manufacturing," *Vaccine*, vol. 33, no. 48, pp. 6752–6756, 2015.
[74] V. Yelemane, M. Grasselli, K. D. D. Gorrepati, M. Fernández-Lahore, A. N. Polat, and M. Kangwa, "High-level fed-batch fermentative expression of an engineered Staphylococcal protein A based ligand in *E. coli*: purification and characterization," *AMB Express*, vol. 5, no. 1, 2015.
[75] G. Campani, G. Gonçalves da Silva, T. C. Zangirolami, and M. Perencin de Arruda Ribeiro, "Recombinant *Escherichia coli* cultivation in a pressurized airlift bioreactor: assessment of the influence of temperature on oxygen transfer and uptake rates," *Bioprocess Biosyst. Eng.*, vol. 40, no. 11, pp. 1621–1633, 2017.
[76] R. Espinosa Pérez *et al.*, "Scaling-up fermentation of *Escherichia coli* for production of recombinant P64k protein from Neisseria meningitidis," *Electron. J. Biotechnol.*, vol. 33, pp. 29–35, 2018.
[77] N. N. Koopaei *et al.*, "Optimization of rPDT fusion protein expression by *Escherichia coli* in pilot scale fermentation: a statistical experimental design approach," *AMB Express*, vol. 8, no. 1, 2018.
[78] P. Jones, R. Van Bogelen, and F. Neidhardt, "Induction of proteins in response to low temperature in," *Escherichia C. Bacteriol 1987; 169^ ...*, vol. 169, no. 5, pp. 2092–2095, 1987.
[79] K. Zhou, L. Zhou, Q. Lim, R. Zou, G. Stephanopoulos, and H. P. Too, "Novel reference genes for quantifying transcriptional responses of *Escherichia coli* to protein overexpression by quantitative PCR," *BMC Mol. Biol.*, vol. 12, 2011.
[80] T. San-Miguel, P. Pérez-Bermúdez, and I. Gavidia, "Production of soluble eukaryotic recombinant proteins in *E. coli* is favoured in early log-phase cultures induced at low temperature," *Springerplus*, vol. 2, no. 1, pp. 1–4, 2013.
[81] W. Hale, V. A. Saunders, and J. Margham, *Collins Dictionary of Biology, 3rd ed.* 2005.
[82] H. Wang *et al.*, "Improving the expression of recombinant proteins in *E. coli* BL21 (DE3) under acetate stress: An alkaline ph shift approach," *PLoS One*, vol. 9, no. 11, pp. 1–11, 2014.
[83] A. Olvera *et al.*, "Influence of pH control in the formation of inclusion bodies during production of recombinant sphingomyelinase-D in *Escherichia coli*," *Microb. Cell Fact.*, vol. 13, no. 1, pp. 1–14, 2014.
[84] G. Sezonov, D. Joseleau-Petit, and R. D'Ari, "*Escherichia coli* physiology in

Luria-Bertani broth," *J. Bacteriol.*, vol. 189, no. 23, pp. 8746–8749, 2007.
[85] K. Martínez-Gómez *et al.*, "New insights into *Escherichia coli* metabolism: Carbon scavenging, acetate metabolism and carbon recycling responses during growth on glycerol," *Microb. Cell Fact.*, vol. 11, pp. 1–21, 2012.
[86] M. Martin and G. Guiochon, "Effects of high pressure in liquid chromatography," *J. Chromatogr. A*, vol. 1090, no. 1–2, pp. 16–38, 2005.
[87] Y. Xiang, Y. Liu, and M. L. Lee, "Ultrahigh pressure liquid chromatography using elevated temperature," *J. Chromatogr. A*, vol. 1104, no. 1–2, pp. 198–202, 2006.