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Correlation of fermentation redox potential and induction of recombinant *E. coli* expression system

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By

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ABSTRACT

Currently, *Escherichia coli* (*E. coli*) is widely adopted as a host for recombinant genes to overexpress. It is common to regard optical density (OD) reading as an indicator to guide IPTG induction of recombinant *E. coli* and harvest them at an empirical moment. However, it is found that OD reading cannot truly reflect the actively growing cells in the culture. With labor involvement, the frequent sampling for monitoring fermentation progress is prone to result in contamination. Besides, with an improperly designed sampling schedule, the optimal induction moment may be missed. Due to these reasons, a new measurement using redox potential (ORP) to replace OD as a more appropriate method to monitor the fermentation of recombinant *E. coli* was proposed, implemented and validated. ORP is known as an instantaneous reflection of organisms, either oxidized or reduced, which is related to the fermentation dissolved oxygen (DO) level and the intracellular metabolic reactions.

During the culture of recombinant *E. coli* at 16 ^{0}C, OD ORP and DO profiles were collected and compared. ORP profile was found as a portrait of a real-time growth stage of recombinant cells. And this relationship has been verified to be reproducible and reliable. With the cells rapid build up at the start of exponential phase, ORP declines promptly. Then ORP stayed at this minimal level for over sixty hours until *E. coli* grew to the end of stationary phase. When the culture was ready for harvest, ORP raised noticeably in a short period of time due to a living cell reduction. In addition, two special features of ORP profile were related to the crucial moments of *E. coli* growth. Specifically, the fluctuation during the decreasing phase of ORP was recognized as a certain timing for induction. Besides, harvesting cells when ORP increased rapidly was known as an optimal period for both enlarging the protein expression and improving the enzyme activity.

DO supply was then noticed insufficient during the fermentation which had a negative effect on cells' growth. Two methods increasing agitation rate and aeration rate were

implemented in order to improve and control DO level. The results demonstrated that DO level was able to be controlled at a sufficient amount by combining these two methods.

Comparing the fermentation technology developed in this research with the typical recombinant cell cultivation technique when OD is chosen to guide the IPTG addition, the expression level of prolinase of both techniques was compatible. Our fermentation technology (compared to the conventional cultivation technology) has various advantages, including less prone to culture contamination, less labor involvement, and low operating cost. This proposed technology can be further improved to become an automatic fermentation process towards the production of recombinant products.

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ABREVIATIONS

ACS	American Chemical Society
ALT	Alanine aminotransferase
AST	Alpartate aminotransferase
BSA	Bovine serum albumin
CA	Clavulanic acid
CSB	Colorless sulfur bacteria
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
E. coli:	*Escherichia coli*
EMP	Glycolytic pathway
HDVAg	Hepatitis D virus antigen
IPTG	Isopropyl-β-d-thiogalactoside
kDa	Kilodalton
lacA	Gene coding β-thiogalactoside transacetylase,
lacI	Gene coding lac repressor
LacI	Lac repressor
lacY	Gene coding β- galactoside permease
lacZ	Gene coding β-galactosidase
LB	Luria-Bertani broth
mGH	Mink growth hormone
OD	Optical density
ORP	Redox potential
pepR	Prolinase coding gene
PepR	Prolinase

pKK	Plasmid vector pKK223-3
PLD	Prolinase
PMLA	Polymalic acid
Pro	Proline
rH-PepR	Recombinant His-tagged prolinase
RNA	Ribonucleic acid
Rpm	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TB	Terrific broth
TCA	Tricarboxylic acid
TCW	Tender coconut water
TEMED	N-N-N'-N'-tetramethylethylenediamine
TMAO	Trimethylamine
UV	Ultraviolet

1. Introduction

Escherichia coli (*E. coli*) has been used extensively as a host for production of biopharmaceuticals, enzymes, and chemicals, due to its low culture cost and detailed knowledge about its genetic background and physiology [1]. In this research, *E. coli* TOP10F' strain introduced with pKK-PepR2 (pKK is a plasmid vector named pKK223-3; PepR2 is the DNA sequence of prolinase) is used for producing recombinant prolinase on a large scale. Prolinase is a strict iminodipeptidase which can contribute to hydrolyze dipeptides containing proline or hydroxyproline as N-terminal groups. These dipeptides are the main sources of the bitterness in cheese. Therefore, prolinase is significantly important to the dairy industry.

In general, the gene of target protein is introduced to *E. coli* with lactose-inducible system in order to avoid overexpression of target protein before organism accumulates to its optimum amount. Researchers usually use optical density (OD) as a signal to estimate the growth pattern of *E. coli* in order to switch the growth phase into protein production phase by adding inducer isopropyl-β-D-galactosidase (IPTG) [2-4]. However, the OD of fermentation broth cannot represent the real amount of living cells in the system. It is required to find out a certain time point for inducing directly. Fermentation oxidation-reduction potential (ORP) is known as an indicator showing the tendency of a chemical species to acquire electrons (to be reduced) or to donate electrons (to be oxidized). It is indicated that the fermentation ORP is related to the growth pattern of cultivated organism and the instantaneous amount of dissolved oxygen (DO) [5]. With a sufficient and constant DO supply, it is expected that the growth pattern of recombinant cells could be indicated by fermentation ORP directly. Therefore, control and increase the DO level of the system is an essential part to advance this project. Previous researches found that there was a shoulder-like stage in the dropping zone of ORP profile which was corresponding to the start of log phase of *E. coli* growth pattern [6]. In my M.Sc. thesis, I propose to illustrate

and establish correlation between fermentation ORP profile and the induction of *E. coli* expression system, to understand the metabolic meaning of the shoulder-like stage, and to prove that whether fermentation ORP profile could be used to replace culture OD to become a more accurate indicator to induce *E. coli* expressions system.

2. Literature review

2.1 Utilization of prolinase

2.1.1 Proline

Proline or Pro for short, is an α-amino acid used for synthesizing proteins. Its codons include CCU, CCC, CCA and CCG. As a non-essential amino acid, proline can be synthesized in human body. It is deliquescent, soluble and sweet. Proline can produce yellow component instead of purple, when heated with ninhydrin solution. Besides, it turns to 4-hydroxyproline after hydroxylation, which is a significant component in both collagens of animals and cell wall of plants. Proline is found in a hydrolysate of proteins like gelatin and casein. There are mainly two methods for proline producing: a fermentation of corynebacterium glutamicum with glucose, and chemical synthesis by using proline as a raw material. Proline is widely used in clinic, industry and biological materials. [7]

2.1.1.1 Utilization

As mentioned above, proline can be applied in various areas. Initially it is a material of amino acid treatments for curing malnutrition, protein deficiency and stomachache. As one of the twenty amino acids consisted in human proteins, proline treatment is able to supplement the lack of nutrient in human bodies directly. In synthesis industry, besides inducing asymmetric reactions, proline is able to catalyze hydrogenation, polymerization and hydrolysis reactions. The catalyst proline processes a good activity and a high stereospecificity. It is also a flavoring agent which can generate special flavored substance via heated with sugar. In organism, proline is not only an ideal osmotic adjustment substance, but also can clean free radicals to protect plants from osmotic stress. [7]

Protein in beverages can interact with small molecules to influence physical properties.

The amino acid composition of proteins plays a major role in these effects. Proteins that combine with polyphenols to form haze (turbidity) are rich in proline, while peptides that lack proline do not form haze. The more proline, the greater the haze-forming activity. This appears to be the case in beer, wine, and fruit juices. The ratio of protein to polyphenol influences that amount of haze formed. This finding has major implications for turbidimetric analytical methods and for beverage stabilization by adsorption or fining. [8]

2.1.2 Prolinase

Prolinase or PLD for short, is an enzyme which can catalyze the hydrolysis of dipeptides containing proline or hydroxyproline as N-terminal groups. It plays a significant role in recycling proline during collagen synthesis and cell growth. Its concentration level in blood is close to the severity of hepatic damage and chronicity of liver disease. When PLD activity in plasma is over the standard range, it illustrates a liver damage. As for the specific disease, it can be only diagnosed with changes of other indexes namely alanine aminotransferase (ALT) and aspartate aminotransferase (AST). [9]

2.1.2.1 Utilization

It is noticed that free proline as a part of ripening cheese is a flavor agent to improve the taste and texture of many dairy products. [10] The milk casein contains a large amount of proline, therefore they generate many proline-rich peptides during the proteinase action. [11] Peptides with an N-terminal proline residue are usually not hydrolyzed by general purpose aminopeptidases, dipeptidases, or tripeptidases. Thus, lactic acid bacteria express several proline-specific peptidases having distinct substrate specificities. As regards to the proline-rich peptides, they are the main source of the bitterness in cheese and other milk fermented products. [12] In order to optimize the taste and flavor of these dairy products, the specific proline peptidases, namely prolinase, prolidase, proline iminopeptidase, aminopeptidase P

and X-prolyl dipeptidyl aminopeptidase play an influential role in dairy industry. [13-15] Many researchers work on cloning and expressing the gene segment of these enzymes in order to produce them efficiently.

In addition, there is a close relationship between PLD and collagen degradation. It is a significant biochemical factor to reflect the collagen catabolism. [16] It is reported that when liver cirrhosis being caught, the relation between increasing PLD and acute liver injury is not as close as it with liver fibrosis. But some others have just the opposite opinion. However, currently most researchers agree that PLD can influence both of the symptoms. For hepatitis, PLD increased with ALT is predictably caused by inflammation and necrosis; as for cirrhosis, it is possible that hepatic fibrosis can lead to an increasing PLD but a normal ALT.

2.2 Cultivation of recombinant *E. coli*

2.2.1 General Characteristics of recombinant *E. coli*

Escherichia coli (*E. coli*) is a Gram-negative, facultative aerobic, rod-shaped, coliform bacterium of the genus *Escherichia*. [17] Cells are typically 2.0 μm long and 0.25 – 1.0 μm in diameter, with a cell volume of 0.6 - 0.7 μm^3. [18-20] It is motile, fimbriate and nonsporulating which can be commonly found in the intestine of endotherms. As a common prokaryotic organism, most *E. coli* strains are nonpathogenic, but some serotypes can cause serious diarrhea diseases and blood poisoning in their hosts especially infants and baby animals.

2.2.1.1 Metabolism

As a bacterium, *E. coli* belongs to prokaryotic organism with a peptidoglycan-made cell wall, ribosomes, a nucleoid and plasmids working as vectors in genetic engineering. *E. coli*

can live on a wide variety of substrates and uses mixed-acid fermentation in anaerobic conditions, producing lactate, succinate, ethanol, acetate, and carbon dioxide. Since many pathways in mixed-acid fermentation generate hydrogen gas, theses pathways require the levels of hydrogen to be low, as is the case when *E. coli* lives together with hydrogen-consuming organism, such as methanogens or sulphate-reducing bacteria. *E. coli* cells can reproduce very quickly. Under an optimal condition, it takes only 17 to 19 min to complete one regenerating cycle.

E. coli is a kind of facultative anaerobic and chemoheterotrophic bacteria. The nutrients and energy from the other cells can be utilized by *E. coli* for growth and propogation. Given sufficient oxygen, it generates enormous energy via aerobic respiration. Oxygen is always used as the terminal electron acceptor. The enzymes in cytomembrane and cytoplasm are used to hydrolyze glucose and produce carbon dioxide as well as water via processing pyruvic acid into tricarboxylic acid (TCA) cycle.

E. coli is also able to conduct anaerobic respiration and fermentation under an oxygen-free condition. Anaerobic respiration is a process that generates cell energy by coupling membrane-associated electron transfer reactions using an electron acceptor other than O_2. Due to the nitrate reductase in *E. coli*, when taking nitrate as the terminal electron acceptor, it will be reduced to nitrite within an oxygen-free condition.

Fermentation is a method to gain energy for both anaerobic and facultative anaerobic organisms. During this process, electron acceptors are unavailable to support cellular respiration (without O_2, nitrate, nitrite, TMAO, or DMSO present). As fermentation pathways yield very little energy, cells normally take this metabolic process as a last resort. *E. coli* performs a “mixed acid” fermentation under an oxygen-free condition. The fundamental energy source, glucose, can be hydrolyzed into various small molecules namely succinate, ethanol, acetate, formate, lactate, H_2 and CO_2. Similar to the other two pathways, glucose or other sugar is initially metabolized to pyruvate via the glycolytic pathway. Some specific enzymes are able to hydrolyze the pyruvate into Acetyl-CoA and

formate. Under an acidic environment (pH<6.2), formate is able to be separated into CO_2 and H_2. Thus, *E. coli* fermentation can produce both gas and acid. However, unlike the other constant fermentation pathways, this pathway makes alternative end products and in variable amount. In particular, the ratio of the various fermentation products is the response to both the culture medium pH and fermentation substrate nature.

2.2.1.2 Utilization of *E. coli*

Recombinant *E. coli* is commonly applied as a mini factory for producing target proteins massively. However, under a high cell density fermentation condition, one of the most difficulties is the generation of organic acids as by-products. The accumulation of these organic acids influence not only the growth of recombinant cells but also the expression of target proteins. Acetic acid is a typical by-product which can lead to a growth stagnation of the organisms. Currently many researchers proposed several metabolic controlling methods in order to avoid the negative influence of the acetic acid, including 1. block the main pathways of acetic acid generation, 2. limit the carbon flow of glycolytic pathway (EMP), 3. reroute extra carbon flow toward other low poison by-products. [21]

E. coli is a useful organism for studies of bacterial physiology, because it is readily accessible, generally benign, and grew readily on chemically defined media. Thus it came to be used for dissection of biochemical pathways; for studies of bacterial viruses, of bacterial and viral genetics, of the regulation of gene expression, of the nature of the genetic code of gene replication, and of protein synthesis; and, in the present age of genetic engineering, for the manufacture of proteins of commercial value. It is necessary to notice that the strains used in biological engineering is the ones selected by several turns. Because of lacking significant ingredients in cell wall, they cannot live and grow under natural conditions. In addition, even ordinary detergents can kill these strains easily. Thus, even if alive recombinant *E. coli* spreads out from the lab, it will not lead a biochemical crisis to the society directly. [22] Besides, after cultured for several generations, the *E. coli* strains

applied in biological engineering carry various genotypes which can be utilized in molecular clone widely.

E. coli has been applied as an expression host for producing a great variety of recombinant proteins. The vector is constructed by combining the target gene to a plasmid. After imported to the host organism, the target gene will be expressed massively with the multiplication of recombinant *E. coli*. In order to avoid the toxic effects of the foreign gene expression, an inducible promoter sequence is usually inserted to the upstream of target gene to switch on the expression. Various expression systems have been used widely in order to express high-level recombinant protein in *E. coli*. Temperature-inducible expression system and chemically-induced system are most common methods among these expression systems. When the cells are cultivated under 30 ^{0}C, lacI is expressed to combine with operon to repress the transcription. As the temperature rising to 42 ^{0}C, the mutant lacI cannot repress the following expression any more. Therefore, the target protein expression is started at 42 ^{0}C. However, in order to acquire soluble and active protein and avoid heat-shock response of *E. coli*, chemically-induced system is preferred to develop the recombinant protein expression. Moreover, chemically-induced system can accurately express the target protein in a safe and efficient way. Recombinant *E. coli* is able to encode both prokaryotic and eukaryotic genes. While *E. coli* is incapable of performing post-translational modification. Besides, a lack of required organelles leads to the inaccurate and defective structures of some proteins. [23]

2.2.2 Culture conditions of recombinant *E. coli*

2.2.2.1 Temperature and pH

Because of the vigor and vitality of this organism, *E. coli* can survive in a variety of temperature and pH conditions. These two factors have a significant influence on microbial growth and reproduction. The available temperature range for culturing *E. coli* is from 15

to 45 ^{0}C. Due to the heat resistance, part of *E. coli* can still survive after heated at 55 ^{0}C for 60 min or 60 ^{0}C for 15 min. Since most *E. coli* usually infests in the intestine of human and animals, the optimal temperature for growing *E. coli* is 37 ^{0}C. [24]

Culturing temperature has an effect on the production rate and accurate folding of recombinant protein of interest. With a higher temperature, target proteins are usually produced as inclusion bodies, because of the limited time for proper folding. On the other hand, lowering the fermentation temperature slows down the growth rate of the *E. coli* cells and thus reduces the total amount of protein expression. With the same cell density, the target protein concentration can be improved under different temperature during cultivation. The expression level of human growth hormone progressively increased when the culture temperature was increased to 42 ^{0}C at a low cell density (OD_{600}=0.3-1.5). [25] However, some recombinant *E. coli* are cultivated at low temperature in order to produce target protein with increased solubility. Cirkovas and Sereikaite [26] found that after reducing cultivation temperature from 37 ^{0}C to 20 ^{0}C, with the comparable total expression level of mink growth hormone (mGH), some amount of soluble mGH was detected at 20 ^{0}C via the recombinant *E. coli*. Besides, compared to the original culture temperature 37 ^{0}C, more soluble recombinant bovine inhibin-alpha was purified at 22 ^{0}C. [27] Therefore, culturing recombinant *E. coli* under a lower temperature condition can result in a larger proportion of soluble target protein but less amount of total target protein expression. [27] It is necessary to find out a point to balance the soluble protein proportion and total protein expression amount.

The optimal growth pH for *E. coli* is from 6.8 to 8.0. In general, the common pH of *E. coli* culture media is between 7.0 and 7.5. When pH is below 6.0 or above 8.0, the growth rate of *E. coli* will slow down apparently. Both the growth of recombinant *E. coli* and formation of metabolic product are related to the pH in the medium. Besides, the dissociations of nutrients in culture medium and the separations of intermediated metabolites influenced by the pH value would result in an improper adsorption and

utilization of nutrients and an inaccurate formation and secretion of metabolic products. On the other hand, the pH value of medium can affect the charging conditions of both enzymes and substrates in recombinant cells. It influences the synthesis of enzymes and thus would lead to a change to the cell metabolism and permeability. Therefore, culturing recombinant *E. coli* under improper pH has a harmful effect on the cell growth and enzyme production. It was found that when pH value was 7.2 and temperature was 37 ^{0}C, glutathione synthetase systems had the highest enzyme production and the total dry cell weight reached its maximum. [28]

2.2.2.2 Culture media

Culture media can be separated as synthetic medium and complex medium. They provide carbon source, nitrogen source, mineral salt, microelement, water and other nutrients for the organism's growth and reproduction. In general, carbon source is commonly acquired from sugar, low molecular weight alcohols, fat and organic acid. Taking glucose as the main carbon source, *E. coli* is able to grow fast but generates lots of toxic by-products. It is found that glycerol can replace glucose to reduce the accumulation of acetic acid effectively which is much better for the high cell density fermentation. [29] Mineral salt in culture media is related to the enzyme activity and osmotic pressure stability. [30] Specifically, a certain concentration of phosphate can promote the growth of recombinant cells and magnesium can restrain the self-dissolving of cells as well as increase the stability of plasmids. [31] As a microelement, Fe^{2+} is helpful to *E. coli* growth and reduce acetic acid production. [32]

A diverse range of culture media are applied to grow recombinant cells, according to the demands of a particular experiment of application. Luria Broth (LB) medium is widely used in laboratory researches, while various rich media are used to increase protein, plasmid, and cell yield. [33] Increasing plasmid yield was implemented via prolonging the exponential phase by the development of Terrific Broth (TB) in 1987. [34] TB is richer than

LB including glycerol and potassium phosphates. These components can promote a high yield of bacteria and reduce the cell mortality caused by a sudden pH drop. [35] In addition, several new forms of culture media are conducted by researchers. Sekar, et al, [36] exploited tender coconut water (TCW) as a natural and cheaper growth medium for recombinant *E. coli.* After supplemented with 25 mM ammonium sulphate, the high demand of nitrogen sources was satisfied resulting in a further increased biomass by 2 to 10 folds. Besides, several recombinant proteins could be successfully expressed in TCW with the equivalent amount in LB.

In general, the plasmid vector is built with an antibiotic-resistance gene sequence. The recombinant cells can successfully express the specific antibodies to the antibiotics for surviving. In order to guarantee the purity of host *E. coli*, a certain concentration of antibiotics is added to the culture media in order to kill those cells carrying no recombinant plasmids.

2.2.3 Recombinant *E. coli* induction

2.2.3.1 Lactose operon model

Lactose operon model is commonly combined with a target gene in plasmid in order to control the transcription process. Therefore, the overexpression of target protein can be avoided at the beginning of the fermentation. Specific control of the lac genes depends on the availability of the substrate lactose to the bacterium. The proteins are not produced by the bacterium when lactose is unavailable as a carbon source.

The lac operon consists of three structural genes, a promoter, a terminator, regulator and an operator. The three structural genes are: lacZ, lacY and lacA encoding β-galactosidase, β-galactoside permease and β-thiogalactoside transacetylase, respectively. As shown in Figure 2.1, [37] in front of lac operon, there is a sequence named lacI which can be transcribed and translated to lac repressor, LacI. The lac repressor has a binding site which

can be combined with the operator in lac operon. The operator is located between promoter and structural genes. When repressor protein binds to the operator, as a result, RNA polymerase is blocked from combining with promoter and the following transcription of genes is hindered. While the combination of the repressor protein and operation sequence cannot last permanently. The half-life period of this binding around 10 to 20 minutes. During this period, RNA polymerase is allowed to proceed the transcription of all the genes into mRNA and thereby leading to a small amount of β-galactosidase expression and β-galactoside permease.

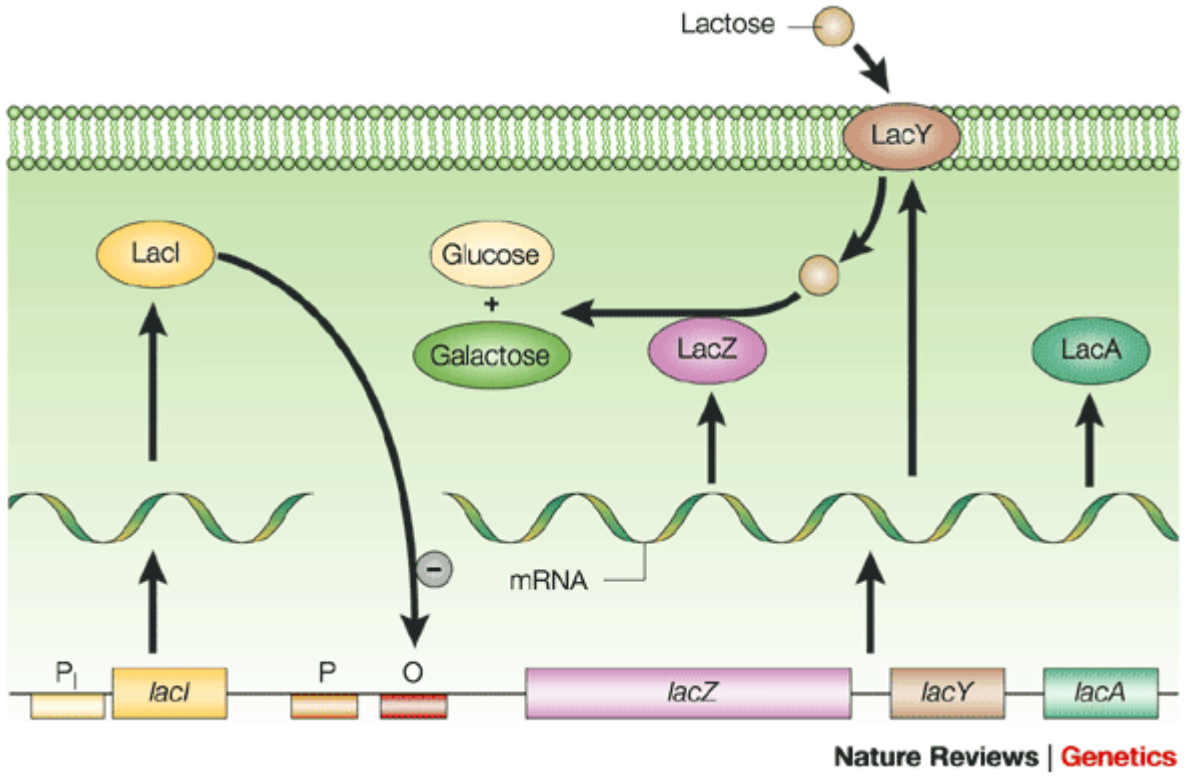

Figure 2.1 The lactose operon of *E. coli* [37]

The genes lacZ, lacY and lacA are transcribed from a single promoter (P) that produces a single mRNA from which the three proteins are translated. The operon is regulated by Lac repressor, the product of the lacI gene, which is transcribed from its own promoter (PI). The repressor inhibits transcription by binding to the lac operator (O). Repressor binding to the operator is prevented by the inducer.

However, the activity of lac repressor can be controlled by lactose. The existence of

small amount of β-galactosidase rearranges the molecular structure of the lactose resulting in the formation of allolactose. This small molecule is able to connect with and deactivate the repressor. Due to the presence of the lac repressor protein, the target gene introduced to recombinant plasmid following structural genes cannot be expressed without lactose. When cells are grown with lactose, an allosteric shift occurs leading to the repressor being unable to bind to the operator. After the repressor protein left the operator, the promoter can connect to RNA polymerase and switch on the transcription of structural and following genes. This allows a higher level of protein encoded.

2.2.3.2 Inducer

In molecular biology, an inducer is a molecule that regulates gene expression. An inducer can bind to protein repressors or activators. [38] Activators generally bind poorly to activator DNA sequences unless an inducer is present. Activator binds to an inducer and the complex binds to the activation sequence and activates target gene. Removing the inducer stops transcription. The maltose operon in *E. coli* is a typical example of the inducer binding with an activator. Only after binding with the inducer, maltose, the activator protein is able to trigger transcribing the DNA via RNA polymerase. [39]

Inducers function by disabling repressors. The gene is expressed because an inducer binds to the repressor. The binding of the inducer to the repressor prevents the repressor from binding to the operator. RNA polymerase can then begin to transcribe operon genes. As mentioned in 2.2.3.1, lactose operon is a common system with repressor combined inducer.

Originally, lactose is the inducer to switch on the expression system which is nontoxic and cheap, whereas the enzyme produced by lacZ is able to hydrolyze the rest lactose into galactose and glucose. Thus, the concentration of lactose cannot be maintained at a stable level in order to ensure the target protein generating during the whole process. Lacking of inducer is likely to result in an interruption of the protein producing and a waste of materials.

Besides, lactose cannot enter into the cells without lactose permease. And it is necessary to be converted to allolactose by β-galactosidase before inducing the system. Using lactose as a inducer is more complex, troublesome but less efficient. Gradually, several synthetic chemicals are produced to replace the lactose. Because of the comparable structure to lactose, isopropyl-β-d-thiogalactoside (IPTG) has become one of the most widely applied inducer to the lac operon system. The popular inducer, IPTG, is unbiodegradable, highly inducing but expensive. Initially, IPTG is not able to be metabolized by organism which can provide a reliable system without introducing interfering substances. In addition, the constant high concentration of IPTG can guarantee a sufficient overexpression of the recombinant protein. However, a higher concentration of IPTG cannot cause a higher accumulation of recombinant cells and target proteins. Specifically, IPTG is a little toxic to the organisms. Some cells are likely to be killed once the IPTG concentration beyond a certain amount. Moreover, a large amount of IPTG in broth results in few soluble protein and massive inclusion bodies. Hence, the optimal concentration of IPTG is not the more the better. Sometimes, a lower one can still satisfy the requirements.

2.2.3.3 Inducing timing

It is believed that the yield of the foreign gene products has a positive correlation to the total gene products per unit volume. While the total gene products per unit volume are positively related to the cell concentration and the average expression level of each recombinant cell. Induction means switching on the mode to take advantage of the limited materials and energy to express target protein rather than propagating rapidly. Thus it is significant to find out a proper inducing moment in order to gain the maximum protein production with a sufficient cell concentration. Traditionally, optical density (OD) is applied as an indicator to show the growth pattern of recombinant *E. coli*. It is also used to estimate an inducing moment to achieve an ideal expression level. It was found in a research that starting induction at a higher OD_{600} reading was likely to acquire a higher

expression level of hepatitis D virus antigen (HDVAg). It was the optimal inducing timing when OD_{600} was between 0.7 and 1.2 with 0.25mmol/L IPTG at 37 ℃. [40] Most inductions are started during the exponential phase of the recombinant cells. Some researchers reported that the optimal inducing timing was from the middle to the end of exponential phase. [41] If adding IPTG too early, the amount of the bacteria was too small to produce sufficient target protein. Besides, the toxic IPTG would extremely slow down the growth rate leading to an inadequate target protein expression. The expression levels of recombinant polygonatum cyrtonema lectin at different bacterial concentration induced by 0.5 mM IPTG showed the optimal OD_{600} was about 0.6. [42] The recombinant *E. coli* cells producing hemoglobin were grown at 37 ^{0}C when OD_{600} reached 0.6-0.8 before adding IPTG. [43] Huang [44] started the inductions of rH-PepR1 and rH-PepR2 with OD_{600} at 0.5 under a low temperature cultivation at 16 ^{0}C. However, the inducing moment should not be set too late of the growth phase. Approaching to the stationary phase, numerous cells lysed and died with a comparable amount of cells generated and produced. The percentage of alive and available host cells which can encode target genes are much smaller than that in log phase.

Considering the various temperature conditions, microbial strains and working volumes the maximum cell concentrations and lengths of log phase of each recombinant strains are totally different. Though the time period when OD_{600} is between 0.7 and 1.2 could be referred as an ideal inducing moment, a further test is necessary to find out a specific optimal cell concentration for the best expression quantity.

2.3 Fermentation redox potential and dissolved oxygen

2.3.1 Fermentation Redox potential

2.3.1.1 Basic information

Redox potential (ORP) reflects the ability of a solution losing or gaining electrons. A higher ORP represents the solution is oxidizing and has a tendency to gain electrons. While a lower ORP indicates a reductive solution which in prone to give out electrons. ORP is measured in volt (V) or millivolt (mV). Because the absolute potentials are difficult to accurately measure, ORP is defined relative to a reference electrode. ORP of aqueous solutions are determined by measuring the potential difference between an inert sensing electrode in contact with the solution and a stable reference electrode connected to the solution by a salt bridge.

ORP electrode is commonly made of various metals namely nickel, silver and gold. These medals allow electrons to move around inside their lattice structure. Among those materials, platinum is an optimal one because of a high ORP and a high sensitive measurement. Besides, the platinum oxide formed on surface of electrode is able to carry electrons which do not influence the electrode sensitivity.

The growth of micro-organisms is influenced by several environmental factors. The most common are pH, water activity, temperature, and organic acid concentration. Redox potential is also a physico-chemical factor required to be taken into consideration. [45] For some species of Salmonella and Clostridium, ORP-related growth limits have been demonstrated and seem to be intrinsic species parameters [46-47].

Generally, organism broth cannot maintain a redox balance during a fermentation. These cells receive nutrients from culture media which are the reactants for redox reactions. Then the inside redox reactions are connected with intracellular metabolic process to generate energy for cells growth and expression. As a result, the broth is oxidizing with a reduced

ORP. Therefore, the ORP detection is necessary for indicating the intercellular reactions in order to improve the fermentation efficiency. As an index to evaluate the fermentation environment, ORP is sensitive enough to indicate the trace amount of oxygen in anaerobic conditions. Besides, some proteins cannot be folded correctly without living in a certain value of ORP. It was found by Lin, Yun-Huin [48] that ORP can indicate the clavulanic acid (CA) formation better than DO. With ORP control, productivity of CA was improved by 96%.

It is reported that bacteria are very susceptible to changes in the redox potential of their growth medium-each species has a range of ORP within which it will grow or to which it can be adapted to [49]. It could be extrapolated that there exists a correlation between fermentation redox potential profile and *E. coli* growth pattern. Previously, an acute decrease in ORP (up to -550 ~ -600 mV) has been observed during the *E. coli* growth under aerobic respiratory [50-51] and anaerobic fermentative [52] conditions as well. Such a decrease might be resulted with the secretion of some redox-active metabolite into the culture medium, lowering external pH [53], or determined by processes on the bacterial membrane [52]. In addition, the decrease in redox potential is also used to determine a transition of culture from the logarithmic into the stationary phase during the growth [50-51,53].

2.3.1.2 Correlation between ORP and DO

ORP is a value when electron change system of electrode comes to a balance. It is defined as the following Nernst equation:

$$E = E_0 + \left(\frac{RT}{nF}\right)\ln\left(\frac{\alpha_O}{\alpha_R}\right) = E_0 + \left(\frac{RT}{4F}\right)\ln pO_2 + \frac{RT}{F}\ln[H^+] \quad \text{.....(2.1)}$$

E_0 is the standard reduction potential; R is the universal gas constant; T is the temperature; F is the Faraday constant; n is the number of electrons; α_O is the activity of the oxidized form; α_R is the activity of the reduced form; pO_2 oxygen pressure in solution. [54]

This value is related to DO, pH and temperature. During a typical fermentation, with a

controlled temperature and pH, ORP can only be influenced by DO level. Because of this correlation, it is feasible to improve fermentation production by controlling ORP. During the ethanol fermentation by yeast, it is tricky to control DO level at an ideal amount. On one hand, a trace of DO is helpful to keep yeast active with ethanol around. [55] On the other hand, because of the anaerobic respiration, EMP will be repressed apparently by an over amount of oxygen leading to a reduced production. [56] A traditional DO sensor is not sensitive enough to indicate a trace of oxygen in fermenter. Thus, ORP control during ethanol fermentation is a delicate method to maintain a certain amount of DO for an ideal yield of ethanol.

DO in culture media is related to the oxygen dissolved from the air and the oxygen consumed by the organism. From the equation shown as before, it is found that with a fixed temperature and pH, DO is the only factor determining ORP. Once the amount of oxygen dissolved from the air is controlled and remains constant, the ORP profile during the fermentation would demonstrate the oxygen consumption process of organism directly and accurately. The oxygen is utilized by aerobic respiration for producing energy in order to accomplish cell reproduction, metabolic reactions and product-oriented fermentation. The ORP profile is able to indicate the activities of intracellular reactions indirectly via the variation of the oxygen uptake. It contributes to understand the intracellular activities during fermentation and optimizes the growing conditions for a higher production.

2.3.1.3 Pervasive Applications of ORP

ORP is widely applied in many areas, namely marine exploration, biological engineering, environmental protection and ethanol industry. In oceans, geophysical exploration approaches are applied to study geological structure and search mineral resources. Geophysical field is observed during this series of researches, namely gravity, magnetism, heat flux and natural earthquake. However, because of the moving water, researchers must conduct new instruments and methods for exploration differing from those on land. Among

those methods, measuring ORP is a simple and direct way for exploring mineral reserves. It was discovered in 1970s [57] that hydro carbons tended to cause redox reactions by moving upside vertically. The rocks above oil and gas field are rich in reducing power which ends up to a low ORP. Thus, it is feasible to locate the oil and gas field by measuring ORP.

The activity of some recombinant proteins can be influenced by environmental redox potential, though the principle is still not clear [58]. For instance, the phosphatase PhoA (*E. coli*) could not be expressed in an active form under reduced conditions. While the active phosphatase was detectable when expressed under oxidized condition [58]. It was also reported that some recombinant protein such as alkaline phosphatase and chicken lysozyme produced by *E. coli* can have renaturation and the activity recovery directly depended on either dissolved oxygen tension or redox potential [59].

Similar to pH, DO and temperature, fermentation ORP is another culture condition controlled to improve efficiency and productivity in microbial synthesis. Recombinant *A. pullulans* is used as a mini factory for producing polymalic acid (PMLA) by Wan [60]. They conducted a research on the relation between PMLA synthesis and cells growth. It was found that the decreased growth rate of organism, at the end of log phase, was caused by a high PMLA concentration and a week reducing broth. It was proved that the yield of PLMA was improved obviously with a controlled fermentation ORP below 70 mV in both repeated-batch and cell-recycle fermentation. Therefore, it is believed that a low fermentation ORP in broth, with a strong reducing power, is able to promote the oxidizing reactions inside organisms.

Natural water is rich in various elements including both reducing and oxidizing substances. In general, ORP of sea and fresh water is 0.4 V indicating a healthy and contaminant free oxidizing condition. The optimal ORP for aerobes is 300 mV to 400 mV or at least over 100 mV. The ORP in water is widely used as an index to evaluate the water quality. Besides, maintaining ORP within a certain range can effectively accelerate

contaminants degradation in polluted water. Sulfides in water are detrimental to both water quality and human health. These sulfides are prone to be oxidized to elemental sulfur by colorless sulfur bacteria (CSB), which is known as a valid approach for treating waste water. It was concluded that both removing rate of sulfide and formation rate of sulfate ion decreased with a reducing ORP. [61] Within various model volumes, the maximum production rate of elemental sulfur can be reached by adjusting a suitable ORP.

As mentioned in section 2.3.1.2, ORP is able to be used for maintaining a trace DO level in broth during ethanol fermentation in order to improve the productivity. On the other hand, it is available to weaken the inhibitors influence on yeast fermentation with ORP regulation. Lignocellulose is one of the most high-yield reusable resources in the world which can be applied in producing biofuel and bio-based chemicals. [62] It is necessary to preprocess the lignocellulose by hydrolyzing cellulose, hemicellulose and lignin. However, many toxic by-products, namely acetic acid, furfural and aldehydes, have inhibiting effects on the following ethanol fermentation. These inhibitors are proved to reduce the amount of biomass and ethanol yield. However, with inhibitors in the broth, both biomass and ethanol yield can be increased by regulating ORP to an oxidation state. In addition, ORP regulation helps remove inhibitors in the system. The added redox agents can react with inhibitors directly and promote the inhibitor removal by yeast cells themselves. [63]

2.3.2 Dissolved oxygen

2.3.2.1 Basic information

Dissolved oxygen (DO) is defined as the molecular oxygen from the air dissolving in water. The DO level has a close relationship with atmospheric pressure, oxygen partial pressure in the air, water temperature and water quality. Naturally, temperature condition is a significant factor to DO, because the oxygen content in the air always remains stable. The lower the temperature is, the higher the DO level is.

In natural condition, DO mainly comes from two sources. Firstly, before DO level reaching the point of saturation, oxygen in the air keeps permeating into water. In addition, aquatic plants are able to release oxygen via photosynthesis underwater. However, once water contaminated by organism, the natural DO level cannot satisfy the oxygen demand resulting in providing an oxygen-free environment for anaerobic bacteria accumulation. As a result, the water become dark and stank.

2.3.2.2 Fermentation and dissolved oxygen

DO is a significant factor to the aerobic fermentation. Recombinant *E. coli* takes advantage of the DO for aerobic respiration. The fermentation produce rate can be increased with a faster oxygen solution and a higher oxygen utilization rate. During the fermentation, with a reduced DO level, the growth rate of recombinant cells slows down and stability of the recombinant plasmid decreases, especially at the end of fermentation. The overexpression of the foreign gene requires a great quantity of energy which calls for a more active respiration and a larger DO supply. Therefore, maintain a high DO level is beneficial to both the bacteria growth and recombinant protein expression. [64] The yield of alive cells can be promoted by increasing the agitation rate and adding air pumps to supply more DO to the system.

Oxygen is required in every growing process of *E. coli.* The concentration of DO makes a great effect on both organism growth and product formation, especially during a high cell density fermentation. In the middle of fermentation, recombinant cells accumulate exponentially which results in a huge oxygen consumption. Once the DO supply cannot meet the demand, acetic acid would be produced rapidly and massively by recombinant *E. coli*. Therefore, as mentioned in Section 2.2.1.1, the by-product acetic acid cannot only restrain the protein synthesis but also slow down the organism growth. The worse is that some cells can even autolysis due to a high concentration of acetic acid.

In order to maintain an optimal DO level, several methods are developed to improve the

dissolution of oxygen. For instance, a higher DO level can be accomplished by increasing the air pressure inside a fermenter, replacing the natural air supply with pure oxygen and adding hydrogen peroxide (H_2O_2) to the culture media. Besides, due to the ability of carrying oxygen, gene of hemoglobin combined with other target gene is introduced to recombinant *E. coli* to avoid lacking of DO. [65] During the research on recombinant L-asparaginase fermentation, it was found that with an addition of 5% n-dodecane, which was a carrier of oxygen, to the culture media, the available oxygen in broth was increased significantly and the expression of L-asparaginase was improved as well. Based on this progress, pure oxygen was supplied to the system resulting in an 83% promotion in target protein expression quantity. [66] However, it is noticed that a higher DO level can lead to a better production only within a certain limitation. According to a recombinant cecropin-X fermentation, the expression of target protein can be potentially reduced by an excessively high DO level. Specifically, when DO level is between 20% and 40% saturation, the recombinant bacteria generate rapidly with an extremely low production of the imported target gene. While a limited oxygen supply is more beneficial to the overexpression of these *E. coli* cells. Therefore, in reality, the DO level is set at a point not too much over the critical value in order to avoid the harmful effects from insufficient or over-supplied oxygen. [67]

Depending on the *E. coli* strains and product features, the optimal DO level varies seriously among different saturations. Even for one strain and fixed growth conditions, the optimal DO level of recombinant *E. coli* varies in different growth phases. During a fed-batch culture of *E. coli* BL21/pET28b-glnA, DO level was used as a signal to control the nutrient feeding. DO level was kept between 20% and 40% saturation by increasing agitation rate in the batch culture period. Once nutrients are insufficient, DO level was controlled at 20% to 50% saturation by feeding supplements. [68] It was reported by Sanjoy K. [69] that the DO level went down obviously after adding inducing agent. Therefore, the start of target protein expression implied a rise in oxygen demand and consumption. At the

end of the fermentation, DO level went up slowly and maintained at a constant value because of the equal rates cell mobility and natality. Most authors conducting recombinant *E. coli* researches did not set DO level in the fermenter as a variable but only mentioned that a supply of vigorous aeration was essential. Since high cell density cultivation and target protein overexpression ask for a great deal of DO, the oxygen concentration can hardly reach the critical value with a traditional tool supplying the air.

3. Hypotheses and Objectives

Besides the fast growth kinetics and the available high cell density cultivation, a sufficient knowledge of genetic information is another reason for *E. coli* becoming a popular host organism for expressing recombinant proteins. A high density growth of *E. coli* is preferred in order to acquire a high yield of bacterial biomass and recombinant protein production. During the growth period, especially when target protein starts expression, recombinant cells call for a great deal of energy from aerobic respiration. To maintain an aerobic growth condition, the high oxygen demand becomes an urgent problem. To increase the oxygen concentration in air supply, many researchers mix the sparging air with pure oxygen [70]. On the other hand, previous researches also reported that there was a negative effect on *E. coli* when propagated under high dissolved oxygen concentration [71]. Thanks to the SoxRS regulon, the increasing dissolved oxygen concentration is no longer a fatal factor to the growing cells [72]. However, the existing of some possible sub-lethal effects that may potentially affect the quality of recombinant protein being produced and the culture stability should be considered. Currently, most researchers did not concentrate on the amount of air supply during recombinant protein expression by *E. coli*. Thus quantify the required dissolved oxygen is worthy a further research. Besides, it is feasible to correlate the DO level in fermenter with recombinant cells growth profile or even target protein expression level in this project. Therefore, the target protein production can be optimized via controlling DO level. In order to maintain it at a required amount, several reasonable methods have been conducted by researchers. Traditionally, increasing agitation rate and flow rate are the most common and convenient approaches to control DO level. [67] Besides, regardless the limited budget, replacing air by pure oxygen is another effective way. [73] Some researchers even insert a hemoglobin gene to the recombinant plasmid in order to carry more DO for fermentation. [65] In this project, increasing agitation rate and flow rate are two main methods taken in to consideration.

Measuring OD of growing cultures is a common way to quantify various important

culture parameters like cell concentration, biomass production or changes in the cell morphology. During the target protein expression period, OD is widely used to indicate the growth pattern of host cells and to show the proper timing for induction. In reality, within a critical amount, OD is proportional to cell mass but not the live cells. In addition, the measurement error can be magnified during the dilution process. Thus the value of OD cannot show the real stage of organism growth [74]. During the process of organism accumulation and target protein production, the nutrients metabolism leads to a complex electron equivalent inside the organism and results in a change of ORP in the culture medium. Based on the previous efforts made in cultivation of recombinant *E. coli*, very few researches were focusing on the influence of fermentation ORP profile. In this research, we propose to experimentally correlate and characterize fermentation ORP profile during the course of recombinant *E. coli*. cultivation. Based on the correlated profile, a potential induction point is expected to be found for maximizing the production of target protein. In addition, an optimal moment for production harvest is expected to be identified as well. According to the difference in data measurement, ORP is able to be measured and recorded in succession automatically by computer via an ORP sensor. As we expect, it is possible to develop a program to regulate the whole fermentation process by recognizing the special features of inducing timing and harvest moment.

Objectives of this research is: 1) to correlate the fermentation ORP profile with the optimal induction point as well as harvest moment of recombinant *E. coli*; 2) to correlate the culture DO level with recombinant *E. coli* growth rate and target protein expression; 3) to improve and control the DO level during fermentation by switching agitation rate and aeration rate; 4) to develop a program which can monitor and control the fermentation process automatically.

4. Experimental Design, Materials and Methods

4.1 Experimental design

In order to find out the correlation between ORP profile and growth pattern of recombinant cells, the fermentation ORP and OD reading would be recorded in this project. Based on the observation, a short fluctuation was found during the optimal induction timing. Besides, the ORP profile witness a fluctuated growth at the end of the fermentation. The experiment was designed to be repeated at least three times to ensure the reliability of these observed correlation. It was also tested that this correlation would not be affected by the inducer IPTG, antifoam solution or other required conditions of the fermentation. As the DO level was an important condition to the recombinant *E. coli* growth and protein expression, it was introduced to be monitored in order to improve the biomass accumulation and protein production. The correlation between fermentation DO level and recombinant cells growth pattern was assessed and analyzed. Two methods-adding an extra air pump (2.6 L/min) and increasing agitation rate were supposed to increase the system DO level to meet the demand. As mentioned in *Pichia* fermentation process guidelines [75], the optimal DO level for this project was set at 40% saturation.

4.2 Experimental materials

4.2.1 Strains and media

The recombinant *E. coli* strain pKK-PepR2/E. coli TOP10F' was obtained from Dr. Takuji Tanaka's lab at University of Saskatchewan. The construction of this strain has been reported in Huang's thesis [6]. It carries gene of prolinase via a vector pKK223-3 using tac promoter system. With 1 mM IPTG induction, it produces a 34.6 kDa protein which is soluble and active at 16 ^{0}C. Both inoculum and fermenter media were LB medium

consisting of 10 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl. The pH of media was adjusted to 7.5 by adding 1M NaOH. After autoclaved at 121 ^{0}C for 15 minutes, the media was mixed with 150 μg/mL of ampicillin for culturing only the recombinant strain. All chemicals were purchased from VWR International (Edmonton, AB, Canada) and Fisher Scientific (Ottawa, ON, Canada).

4.2.2 Fermenter, control system and experimental setup

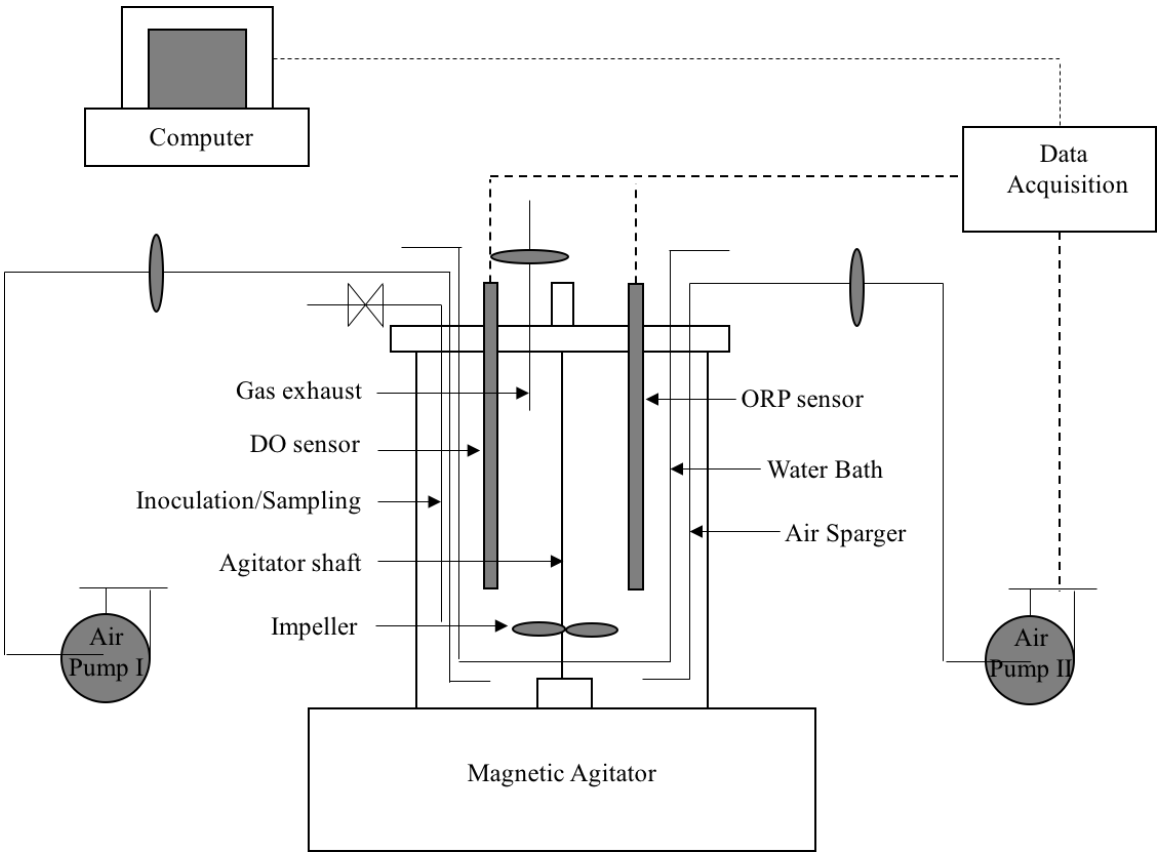

Figure 4.1 Flow diagram of fermentation system used in this study

A jar fermenter (Model: Omni Culture, New York, NY, USA) with two liter working volume was used in this study. It was equipped with a detachable stainless steel lid to maintain a sterilized environment for the fermentation. The agitation rate was maintained at 400 rpm and forced aeration was achieved by supplying small bubbles from the air

sparger at the bottom. The stable fermentation temperature was maintained by a water bath tube and the required air was provided by an air pump (Model: Whisper 60, Tetra, Blacksburg, VA, USA) working at 2.6 L/min. ORP probe purchased from Mettler Toledo Inc. (Model: Pt4805-DPAS-SC-K8S, Billerica, MA, USA) and DO sensor from Mettler Toledo Inc. (Model: InPro6800, Billerica, MA, USA) were installed in the fermenter in order to collect data through a multi-parameter transmitter (Model: M400, Mettler toledo, Billerica, MA, USA). The electronic signals were then sent to a custom-built controller (ORP/DO sensor DAS/SSR, Chemical Engineering, U of S) and processed by a PC (Personal Computer) via LabView software (Version 2017, National Instrument, Austin, TX, USA). Once the DO level was below the set value, LabView system would sent a feedback signal to the controller in order to trigger out the second air pump (Model: Whisper 60, Tetra, Blacksburg, VA, USA) and provided more sterile air for the fermentation. The fermentation process was graphically described in Figure 4.1 above.

4.3 Cultivation and measurement

4.3.1 Cultivation of recombinant E. coli

From a -80 ^{0}C frozen stock, a pinch of frozen culture was scratched out and inoculated in 2 mL of LB broth with 150 μg/mL ampicillin as activated culture. After incubated on a shaker with 250 rpm at 37 ^{0}C overnight, the fully grown starter culture was inoculated into a sterilized 200 mL LB medium supplemented with 150 μg/mL ampicillin under condition of 37 ^{0}C, 250 rpm overnight. This overnighted culture was then inoculated to the jar fermenter with 2 liter working volume and cultivated by LB medium with 150 μg/ml ampicillin at 16 ^{0}C by water bath. The culture had a forced aeration by sparging membrane-sterile air at the bottom of the jar. Traditionally, after inoculation, the absorbance at 600 nm of the broth was monitored by a UV-VIS spectrophotometer (Model: UVmini-1240, Mandel, Shimadzu, Japan) every 4 hours. Five-milliliter samples were taken from the

fermenter. When the absorbance reading reached 0.7, 1 mM IPTG (Fisher Scientific, Ottawa, ON, Canada) was added to the fermenter for inducing target protein expression. The culture lasted for 72 more hours and OD was measured every 6 hours after induction until the growth reached the stationary phase.

In order to improve the fermentation control, a new method was conducted in this experiment. Redox potentials acquired by the control computer were used to guide and determine when to induce protein expression and when to harvest cells. When the slope of ORP profile dropped noticeably and potentials fluctuated within ±2 mV for at least half an hour, the recombinant prolinase expression was triggered by adding 1 mM IPTG. While if the redox potentials went up with fluctuations after a long-term (over 50 hours) steady stage, fermentation was stopped and cells were harvested for further processing.

4.3.2 ORP and DO measurement

The redox potentials and DO levels were detected by an ORP probe and a DO sensor. Then those data were sent to a control computer via a signal transmitter and a controller. They were recorded by LabView every 10 minutes.

Recombinant *E. coli* requires a large amount of oxygen for cells accumulation and protein production. DO provided by a single air pump and an agitation rate at 400 rpm cannot meet the requirement. In order to improve the cells growth and recombinant prolinase expression, DO level was controlled under two different methods. With a 400-rpm agitation rate, when DO level was below the set point, a signal was sent to the controller to start the second air pump. More air was provided to the fermenter to keep fermenter DO at a derived level. On the other hand, with the only air pump, the agitation rate would be increased manually once DO level dropped below the set point.

4.4 Sampling and quantification

4.4.1 Extraction of crude prolinase

After induction, 20 mL fermentation broth was taken as a sample every 6 hours till the end of fermentation. The samples were immediately centrifuged at 6000 g (Model: Sorvall superspeed centrifuge with GS-3 rotor, Thermo Scientific, Waltham, MA, USA), 30 min, 4℃. The supernatant was discarded and the precipitate was resuspended in 5 volumes of ice-cold buffer (20 mM Tris-HCl pH7.5) per weight of wet cell pellet. The suspended cells were disrupted with ultra sonication (Model: Sonifier 450, Branson Ultrasonics Co., CT, USA). They underwent 20 sec burst at 4-5 output level with 40 sec intervals for 8 times in an ice bath. Then the disrupted cells were centrifuged at 12000 g (Model: Sorvall superspeed centrifuge with ss-34 rotor, Thermo Scientific, Waltham, MA, USA), 20 min, 4 ℃, for separating the crude extract in supernatant from cell debris in sediment.

4.4.2 Determination of the expression for pKK-PepR2

4.4.2.1 Materials

Bradford Protein Assay kit (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) was used to determine total protein concentration of the culture. The experimental protocol provided by the kit was followed to construct a calibration curve that correlate total protein concentration with absorbance reading measured at 595 nm. All chemicals used in this study were commercially available ACS grade, and were purchased from VWR International (Edmonton, AB, Canada) and Fisher Scientific (Ottawa, ON, Canada).

4.4.2.2 Prolinase expression

Crude extract was analyzed on 10% SDS-PAGE to examine the expression of recombinant protein in the host. A standard protein purchased from Bio-Rad Laboratories Ltd, (Mississauga, ON, Canada) was used as a marker to find out the target prolinase. The recipe of each required solution was provided in Table 4.1.

Crude extract samples and standard protein solution were prepared as the same as total protein concentration. The equal volume of dye buffer was mixed with crude extract and the marker, before boiled in 100 ^{0}C water bath for 5 minutes. Then the mixers were put into the prepared stacking and resolution gel with running buffer. After connected to the electrophoresis apparatus, the system was run under constant voltage at 200 V until the bromophenol blue indicator arrived 1 to 2 cm away from the edge of the gel. After removed from the electrophoresis cell (Model: Mini-Protean II, Bio-Rad Laboratories Ltd., Mississauga, ON, Canada), the gel was dyed by blue silver stain for 2 to 4 hours before washed overnight. Checked the bands of each sample and compared them to the marker.

Table 4.1 Chemicals, materials and solutions used in the experiment

Blue Silver Stain Constituent	 Concentration (w/v)
Ammonium Sulfate	10%
Phosphoric Acid	10%
Methanol	20%
Dye Coomassie Brilliant Blue G-250	0.12%

Dye Buffer Constituent	 Concentration (w/v)
SDS	2%
BPB	0.01%
2-mercaptoethanol	2% (v/v)
Glycerol	50% (v/v)

Running Buffer Constituent	 Concentration(w/v)
Tris Base	3%
Gylcine	14.4%
SDS	1%

Resolving Gel: (2 pieces) Constituent	 Concentration
Water	2720 µl
10% SDS	80 µl
1.5M Tris-HCl Buffer pH8.8	2000 µl
30%Acylamide (30:0.8=acrylamide: bisacrylamide)	3200 µl
Ammumion Rersulfate	15-20 crystals
TEMED (Addition will start polymerization)	24 µl

Stacking Gel (2 pieces) Constituent	 Concentration
Water	2440 µl
10% SDS	40 µl
0.5 M Tris-HCl Buffer pH6.8	1000 µl
30%Acylamide (30:0.8=acrylamide: bisacrylamide)	520 µl
Ammumion Rersulfate	10-15 crystals
TEMED (Addition will start polymerization)	8 µl

4.4.3 Enzyme activity of the recombinant prolinase

4.4.3.1 Materials

Required chemicals were purchased from VWR International (Edmonton, AB, Canada) and Fisher Scientific (Ottawa, ON, Canada). Pro-Leu was ordered from Sigma-Aldrich Canada Co. (L8753, Oakville, ON, Canada). Ninhydrin solution was prepared according to Table 4.2.

4.4.3.2 Proline quantification

Proline released from substrate Pro-Leu was assessed by a colorimetric assay that detects proline as a colored proline-ninhydrin complex using light absorbance at 500 nm. A series of proline solutions were prepared in order to construct a standard curve. Twenty microliter of solution containing 0 to 0.4 μmol proline were transferred into 200 μL of ninhydrin solution (see Table 4.2). This mixture was heated at 95^0C for 5 min under constant temperature control (Model: 1162 Chiller/Heating circulating water bath, VWR Polyscience, Edmonton, AB, Canada), and was then chilled on ice. The mixtures were measured at ABS_{500} using a spectrophotometer (Model: Genesys 10S UV-VIS, Thermo Scientific, Ottawa, ON, Canada). Different proline concentration correlated with absorbance were made as a proline standard curve.

Table 4.2 Composition of ninhydrin solution

Ninhydrin solution Constituent	Concentration
Ninhydrin	15% (w/v)
Glacial acetic acid	0.03% (v/v)
N-butanol	100% (v/v)
HCl	Adjusted pH to 1

4.4.3.3 Enzyme activity quantification

Compared to the previous proline standard curve, the concentration of proline hydrolyzed by recombinant prolinase in each sample could be calculated and recorded. A mixture consisting of 60 μL of water, 10 μL of 10x buffer (20 mM Tris-HCl pH7.5) and 10 μL of 20 mM Pro-Leu was incubated in 37^0C water bath (Fisher Scientific Isotemp™, Ottawa, ON, Canada) for 5 min. Twenty microliters of each crude extract were mixed with the pre-incubated mixture to initiate reaction, and 20 μL was transferred from the reaction mixture to 200 μL of ninhydrin solution (see Table 4.2) at every one minute for four times

(or with longer time interval if the enzyme activities were low). After heated at 95 ^{0}C for 5 min and chilled on ice, the absorbance of mixtures was measured at 500 nm. The recorded absorbance was converted into the amount of liberated proline in accordance with the standard curve of proline determination previously. The rate of liberated proline implied the hydrolysis activity of recombinant prolinase. Enzyme reaction rate was then calculated from the slope ($\mu mol/mg \cdot min$) of the linear regression between the amount of liberated proline and reaction time.

5. Results and Discussion

The fermentation ORP was recorded along with OD reading in order to correlate this relationship. A short fluctuation during the reduced stage of a ORP profile was observed. A series of experiments were thus conducted in order to validate the repeatability of this observation. Then dissolved oxygen (DO) level was introduced to the data collection to analyze the correlation between DO level and pKK-PepR2 growth pattern. Lastly, the recombinant prolinase activity was measured to ensure the proposed method did not have any negative influence on the expression of target protein.

5.1 Observation

The recombinant *E. coli* was cultured in a 2-L LB media supplemented with 150 μg/mL ampicillin at 16 ^{0}C in water bath. The agitation rate was maintained at 400 rpm and forced aeration was achieved by sparging membrane-sterile air (pore size of membrane is 0.1 μm) near the bottom of the fermenter.

In order to increase the biomass and target protein production, the recombinant *E. coli* was induced in the beginning of exponential phase. From the previous research [6], it was found that this microorganism started to grow exponentially when the absorbance reading of the broth reached the range between 0.5 and 0.7. A typical profile of optical density and redox potential measured at 16 ^{0}C is shown in Figure 1.

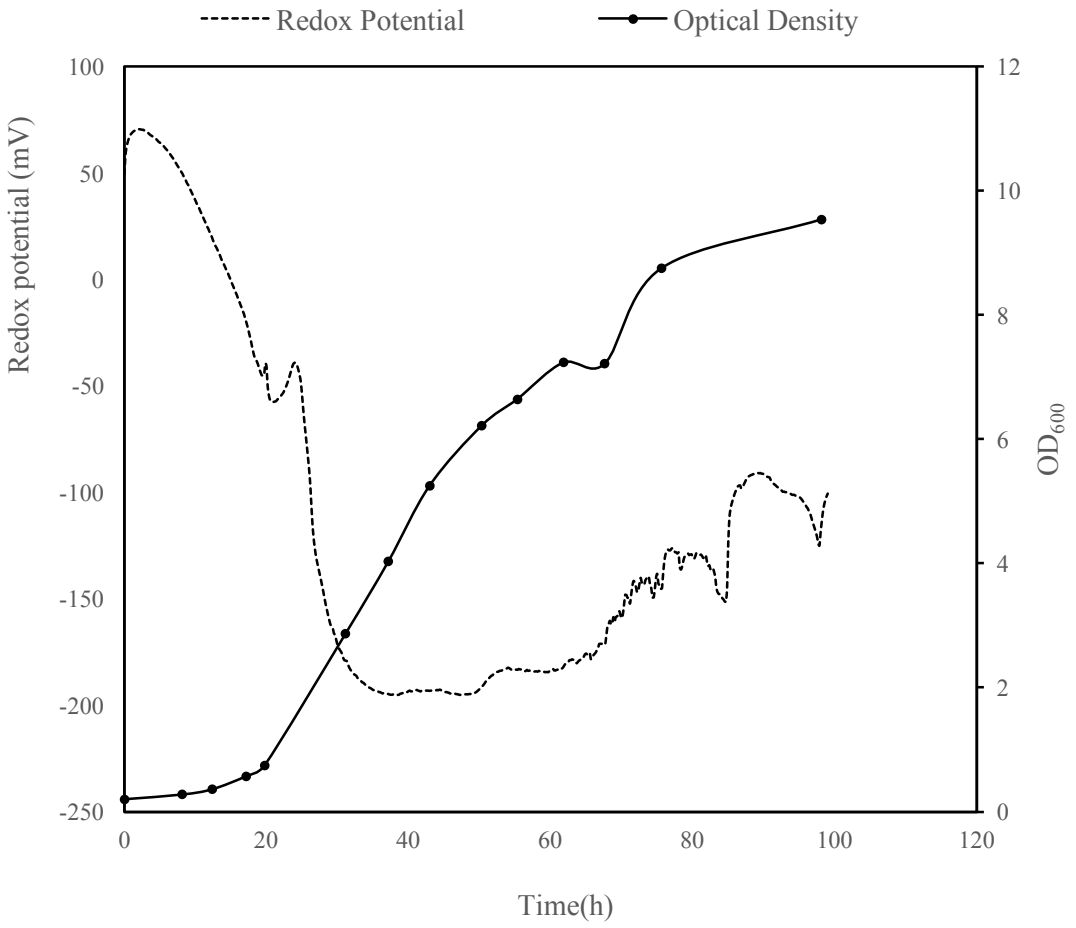

Figure 5.1 Typical profiles of OD_{600} and ORP at 16 ^{0}C.

According to the OD profile illustrated in Figure 5.1, the lag phase lasted around 19 h at 16 ^{0}C before OD_{600} reaching 0.7. The cells took additional 60 h of propagation before entering stationary phase. On the other hand, ORP decreased exponentially from 50 mV to -200 mV with an obvious fluctuation at about the 20th h. Then the oxidizing power and reducing power came to a balance at around 30th h and this period lasted for more than 30 hours. With the growth stepped into the stationary period, the ORP went up gradually.

It was discovered that the induction point (OD_{600}=0.5~0.7) and the fluctuation during the ORP decline period happened at almost the same time. It was predictable that the fluctuation during the decline period of the ORP profile could be used to guide the addition

of inducing agent during recombinant *E. coli* fermentation.

Since the amount of active target prolinase and alive recombinant cells reached maximum during stationary phase [6], the pKK-PepR2 was harvested at the 72th hour after induction. It is obvious from Figure 5.1 that with the growth turned into stationary phase, ORP profile increased gradually after about 70 hours. As a result, the continually raising ORP indicated the fermentation approaches to the end. The ORP gradually increasing period could be set as a harvest moment of the fermentation.

5.2 Correlation between redox potential profile and the growth pattern of recombinant *E. coli*

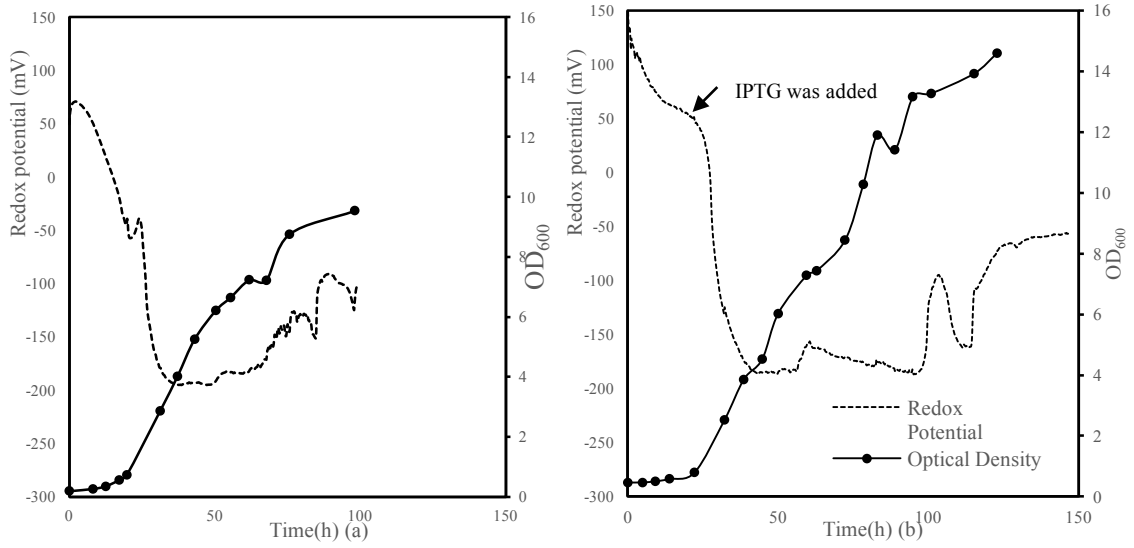

Figure 5.2 Profiles of OD_{600} and ORP at 16 ^{0}C (a) without induction, (b) with induction by 1mM IPTG based on ORP profile.

In this research, the recombinant *E. coli* was cultivated in the same environment as described in Section 5.1. The inducing agent was added to fermenter when the ORP profile reached around the 20th hour. The absorbance reading and ORP were recorded as in Figure 5.2 (b). As compared to Figure 5.1, same experimental conditions were repeated. After a

20-h lag stage, the OD_{600} gradually increased to around 0.7 and the recombinant cells started the exponential stage which lasted for about 80 hours. On the other hand, a noticeable slope change between 0 and 20 h in ORP profile was seen, where the corresponding OD_{600} reading is about 0.7. Then the redox potential reduced rapidly to around -200 mV with in 20 hours and the power of reduction and oxidation came to a balance until the end of the fermentation. It is practical to follow the redox potential profile to indicate the growth pattern of the recombinant *E. coli*. The visible slope change in ORP profile was used as an inducing timing for IPTG addition and the raising ORP was known as a harvest moment.

Inducer IPTG is added to the fermenter to inactivate the repressor in order to trigger the transcription and start the target protein expression. Previously, the correlation between OD_{600} and ORP was observed under an IPTG free condition (Figure 5.1 (a)). Comparing to Figure 5.2 (b), where IPTG was added, both figures portrait similar OD and ORP profiles. That is, the timing for IPTG addition and the moment for cell harvesting were nearly the same. This indicates that the addition of IPTG to the recombinant *E. coli* culture were not alter both OD and ORP profiles noticeably.

It could be concluded that, the ORP profile could be used to replace OD profile, and be used to guide the progress of recombinant *E. coli* fermentation.

5.3 Control of dissolved oxygen level and the growth pattern of recombinant *E. coli*

Dissolved oxygen (DO) level during fermentation has a significant effect on the growth of recombinant *E. coli* cells. In order to improve the expression of recombinant prolinase, the DO level in the fermenter was monitored by a DO sensor. The relationship between DO level and cell growth was shown in Figure 5.3. During the lag phase of the growth, the provision of DO in the fermenter was sufficient as indicated by 100% DO saturation. As the cell entered the exponential phase, the DO level decreased drastically to 0% within 5

hours. Then the DO level kept at 0% until the end of the fermentation. The lack of DO would affect cell growth and metabolism. It is postulated that a higher DO level could accelerate cell growth and shorten the fermentation period. Methods to increase DO level during recombinant *E. coli* cultivation were attempted in order to improve cell growth and prolinase expression.

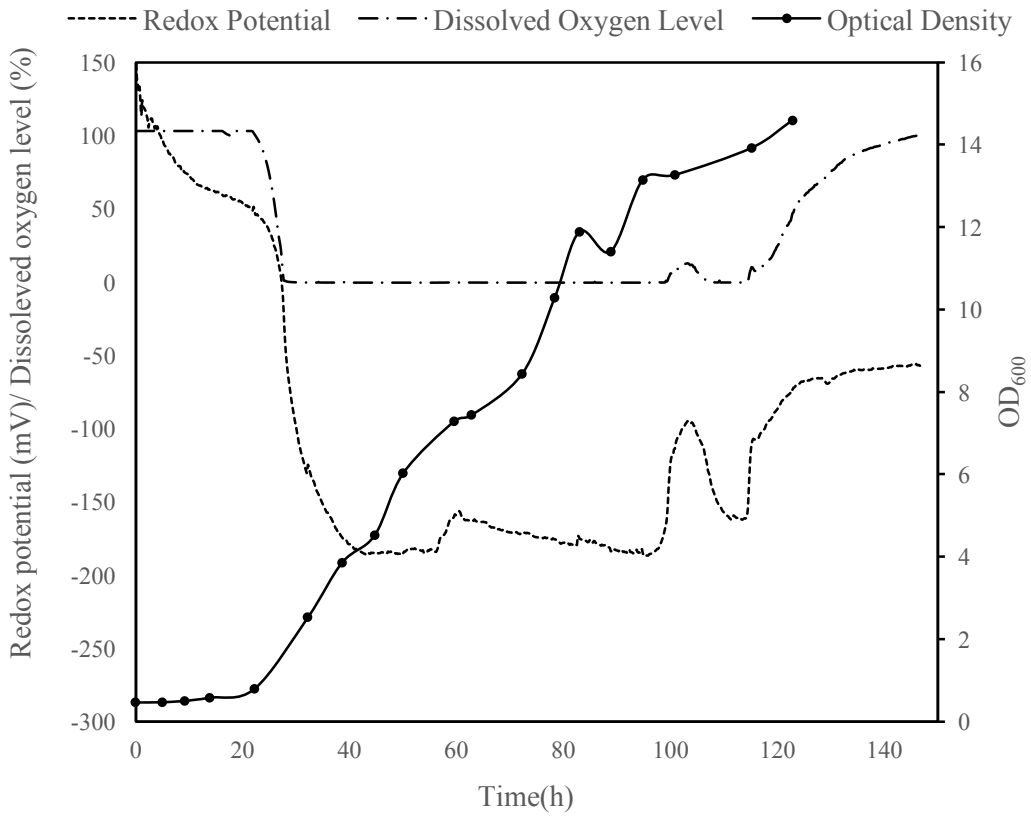

Figure 5.3 Profiles of OD_{600}, ORP and DO level during the growth of recombinant *E. coli* at 16 ^{0}C with one air pump under a constant agitation rate of 400 rpm.

From other study [75], it was reported that a minimum DO level should be maintained during the growth of *Pichia pastors*. We postulated that the same analogy should also apply to this investigation. As a result, keeping the DO level at 40% was set as a target. To do so, an air pump (2.6 L/min) was installed to the fermentation system and the agitation rate was kept at 400 rpm. From Figure 5.4, it was noticed that after the induction the recombinant

cell still required a large amount of oxygen for cell metabolism and protein expression. After stopping the extra air supply, the DO level in the fermenter could not suffice the demand of cultivated cells and the DO level decreased to 0% saturation instantly. Compared to Figure 5.3, it was found that without sufficient air supply, the cell growth rate slowed down immediately and kept at a low level. When the air supply was resumed, the cell growth rate increased instantaneously. Therefore, the growth of recombinant *E. coli* required a large amount of oxygen. The air supply with a single air pump (2.6 L/min) was insufficient.

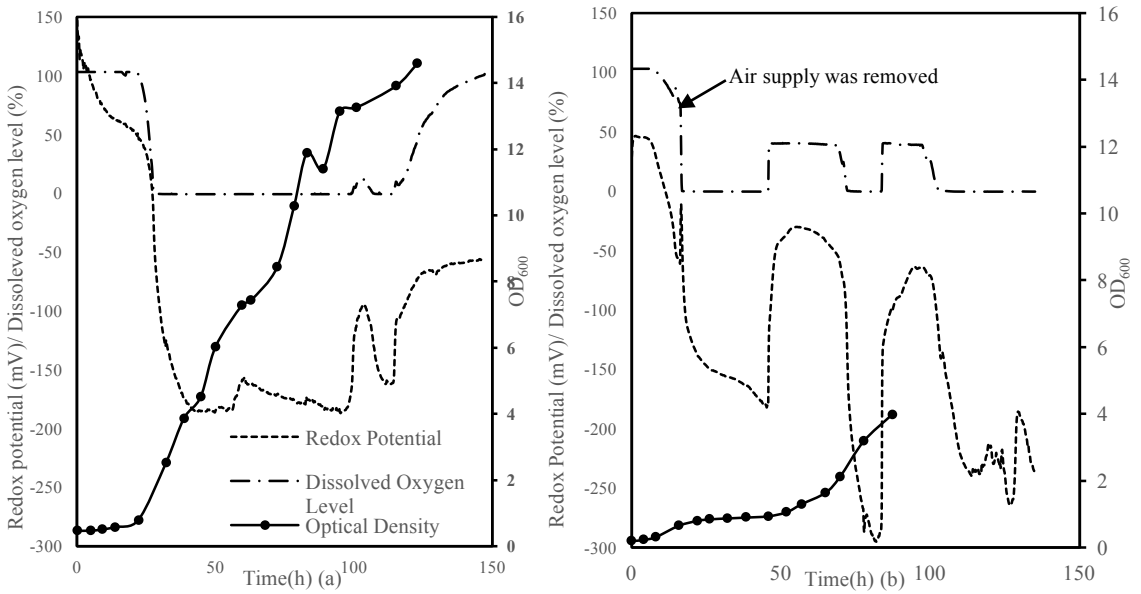

Figure 5.4 Profiles of OD_{600}, ORP and DO level during the growth of recombinant *E. coli* at 16 ^{0}C with one air pump and agitation rate at 400 rpm (a) without control; (b) no air supply after induction, supply recovery with a controlled DO level at 40%.

In order to provide more DO to the fermenter, one more air pump (2.6 L/min) was added to keep DO at the derived level. When the cells entered the exponential phase at around the 20th hour (Figure 5.5(b)), the DO level declined drastically. While the level decreased below 40% DO saturation, the second air pump was turned on. With the extra air supply, the DO level maintained at 40% saturation for only about 4 hours. Notice that the DO level

still could not meet the requirement. It is concluded that adding an extra air pump (2.6 L/min) could not control fermentation DO level at the derived level.

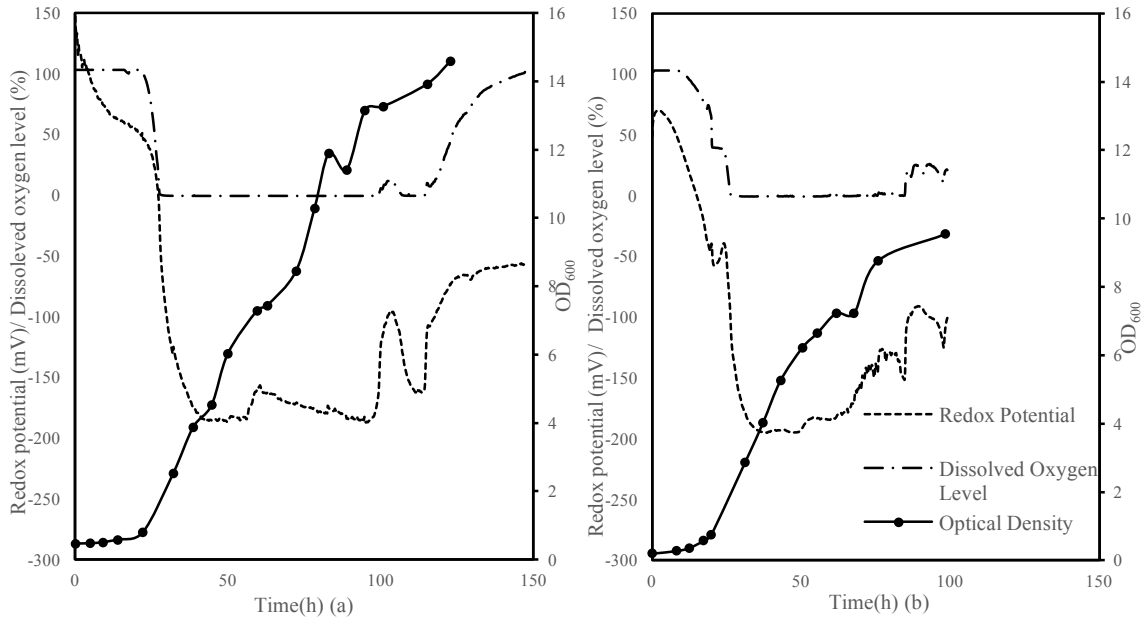

Figure 5.5 Profiles of OD_{600}, ORP and DO level during the growth of recombinant *E. coli* at 16 ^{0}C with one air pump and agitation rate at 400 rpm (a) without control; (b) with a controlled DO level at 40% by an extra air pump.

In the following research, a new method of increasing agitation rate while keeping constant aeration rate was conducted to increase the DO level. When DO level was below 40%, the agitation rate was increased in order to reinstate the DO level. As seen in Figure 6, when DO level fell below 30% at about the 47th hour, the agitation rate was increased from 400 rpm to 450 rpm. The DO level maintained at 30% for about one hour and decreased again until reached 0% saturation. After that, even when the agitation rate was increased from 450 rpm to 500 rpm and to 550 rpm, the DO level was always kept at 0% saturation. Note that because of the design and propeller used in the fermenter, the maximum tolerable agitation rate was set at 550 rpm. Therefore, during the period DO level at 0%, both of the two methods, i.e., adding an extra air pump and increasing agitation rate could not meet the oxygen demand by recombinant *E. coli* grown under exponential phase.

It is also noticed that though the DO level was not kept at the derived amount, the fermentation period was shortened after conducting the two DO level improvement methods. The extra air pump helped shorten the fermentation cycle for around 10 hours and also lower the OD reading from OD_{600}=14 to 10 at the end of fermentation. On the other hand, the higher agitation rate shortened the exponential phase for about 30 hours but imposed unnoticeable influence on biomass concentration. In conclusion, the extra air supply method could shorten the fermentation cycle resulting in a lower concentration of biomass and protein production. The higher agitation rate could accelerate recombinant cell propagation rate without reducing the biomass amount, but the extra shear force caused by agitation could be harmful to the cell survival rate.

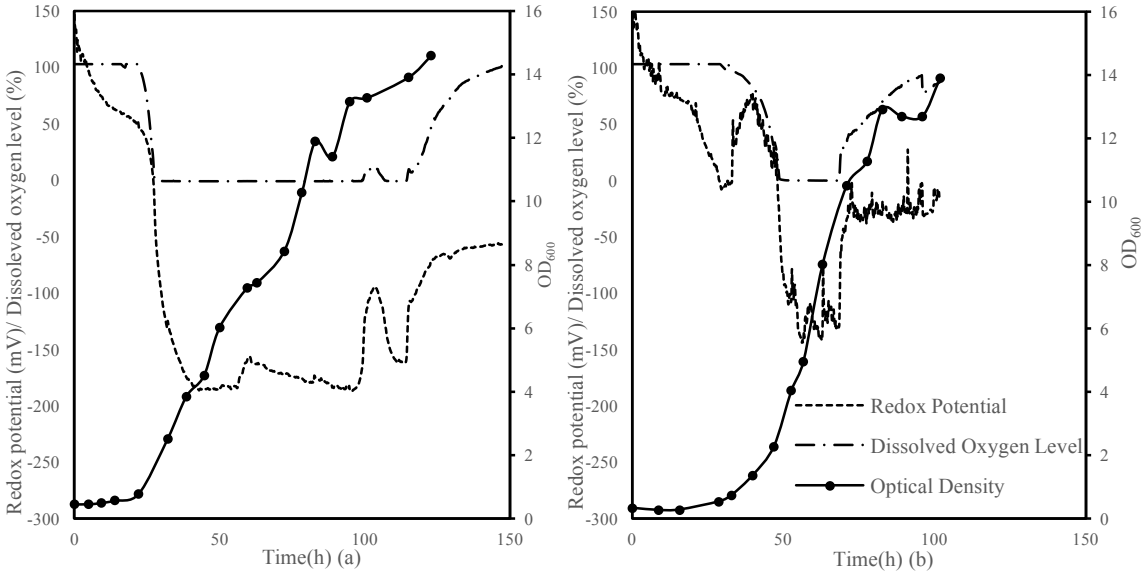

Figure 5.6 Profiles of OD, ORP and DO level during the growth of recombinant *E. coli* at 16 ^{0}C with one air pump and agitation rate at 400 rpm (a) without control; (b) with a controlled DO level at 40% by changing agitation rate.

5.4 Correlation between redox potential profile and target protein expression

5.4.1Recombinant prolinase expression

Instead of using OD reading as an indicator to initiate induction, a real-time ORP profile was taken to guide the timing of IPTG addition (see Figure 5.2). After the induction, samples were withdrawn, labelled as Number 1 to Number 10, to determine protein expression. After ultrasonication and centrifugation, the total protein concentration of each crude exact was measured by Coomassie brilliant blue G-250. As shown in Figure 5.7, the target protein expression was quantified from the collected samples by running SDS-PAGE gel.

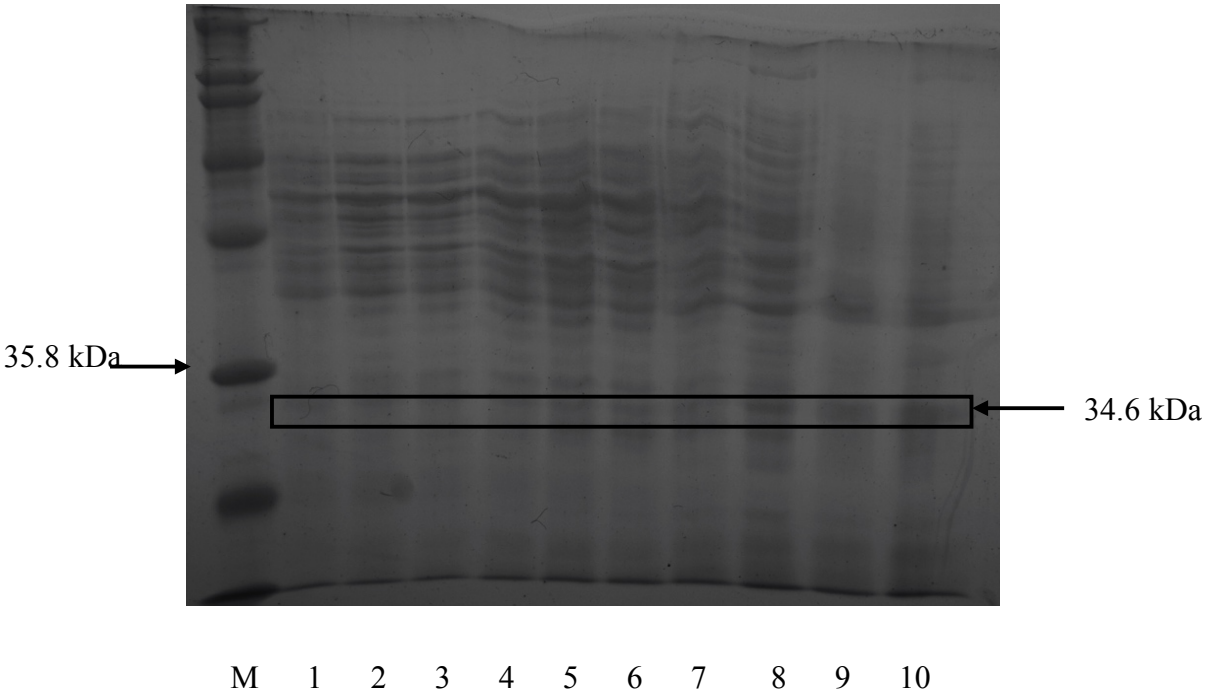

Figure 5.7 Coomassie Brilliant Blue G-250-stained 10% SDS-PAGE gel showing pKK-PepR2 crude extract diluted with a 5-volume 20mM Tris-HCl buffer (pH7.5) of different samples. M: protein marker. Lane 1-10: Sample 1-10.

The target recombinant prolinase was 34.6 kDa. According to the marker protein provided, it was apparent in Figure 5.7 that a band appeared below the standard 35.8 kDa. This band became wider and thicker from Sample 1 to Sample 10. Although there is 1.2 kDa difference in molecular weight, it was regarded this band belongs to the recombinant prolinase. As a result, the activities of these samples were examined by ninhydrin reaction method.

5.4.2 Correlation between redox potential profile and recombinant prolinase activity

As an enzyme, prolinase can cleave L-prolyl-amino acid bonds and produce proline. The complex of proline-ninhydrin has a visible color which can be detected by light absorption at 500 nm. A standard curve correlating absorption reading and proline concentration was provided in Figure 5.8. In this curve, ‘x’ indicates proline amount (μmol) and y is the absorbance at 500 nm.

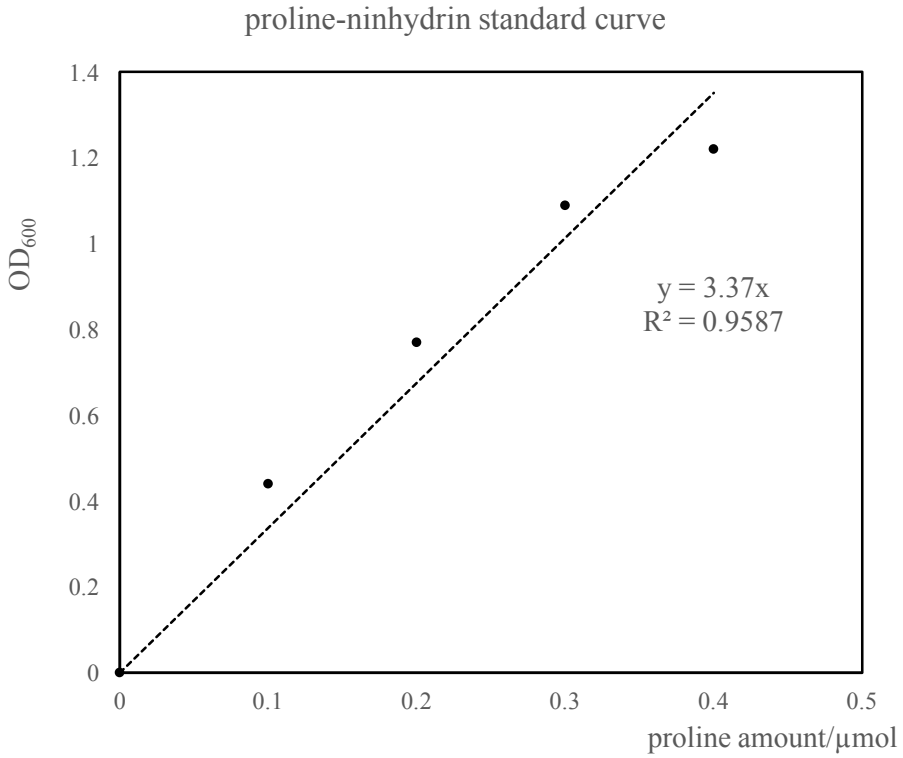

Figure 5.8 Standard curve of proline-ninhydrin. The linear regression was y=3.37x (R^2=0.96)

The prolinase activity of Sample 1, 6 and 10 were chosen to be tested by ninhydrin reaction method. Sixty-microliter ddH_2O, 10 µL 10x Tris-buffer (20 mM Tris-HCl, pH7.5) and 10 µL 2 mM Pro-Leu, were mixed and kept in 37 ^{0}C water for 5 minutes. After adding 20µL sample to the mixture, immediately, 20 µL of the liquid was taken to the 200 µL ninhydrin solution in every minute. It was then boiled in 95 ^{0}C water for 5 minutes before chilled on ice. From the absorbance reading (OD_{500}), the proline amount could be quantified from Figure 5.8. Note that the proline produced by per amount of recombinant prolinase in every minute was defined as a unit in order to compare the enzyme activity.

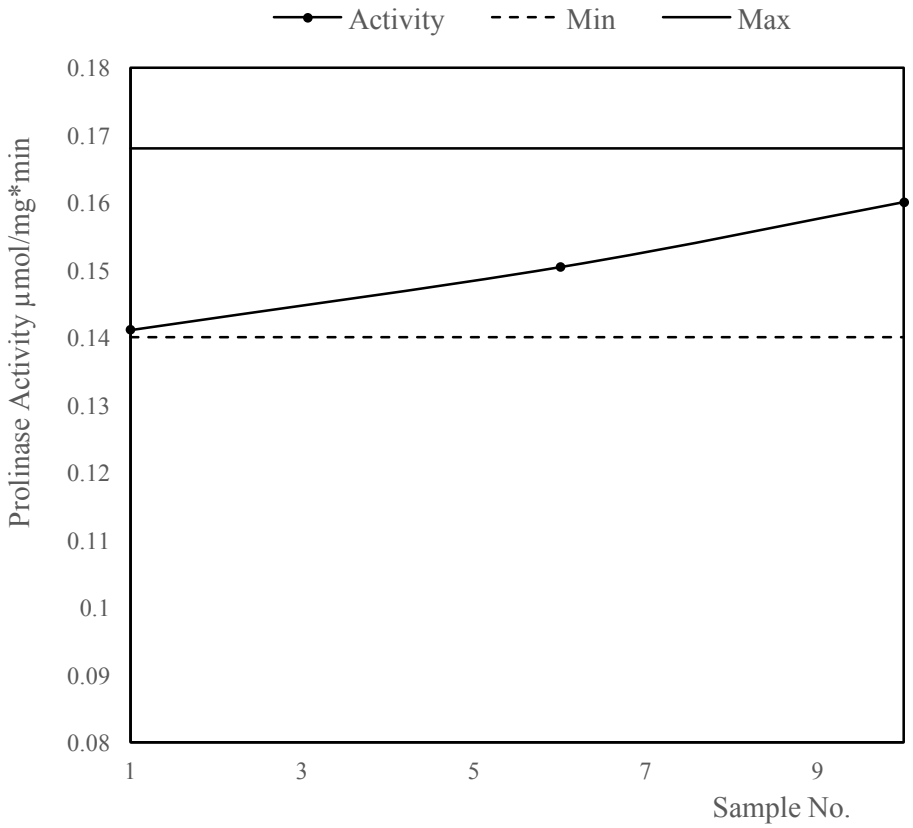

Figure 5.9 Recombinant prolinase activity of each sample. Sample Number:1,6 and 10. Prolinase activity: $\mu molg/mg \cdot min$. From Huang's [6] research, the activity of prolinase under the same condition with 2mM Pro-Leu substrate was tested as 0.154 ± 0.014, which meant the activity fluctuated between 0.14 and 0.168 µmol/mg*min.

From Figure 5.9, it was observed that the protein activity was slowly increased from Sample 1 to Sample 10. And the activities were all within the range between 0.14 and 0.168 $\mu molg/mg \cdot min$. Using ORP profile as an indicator for induction and harvest moment did not influence the target protein expression and activity. When the prolinase activity profile was superimposed to profiles of ORP and DO level (see Figure 5.10), it becomes apparent that ORP profile could be used as an indicator 1) to guide when to add inducing agent, and 2) to guide when to harvest cells.

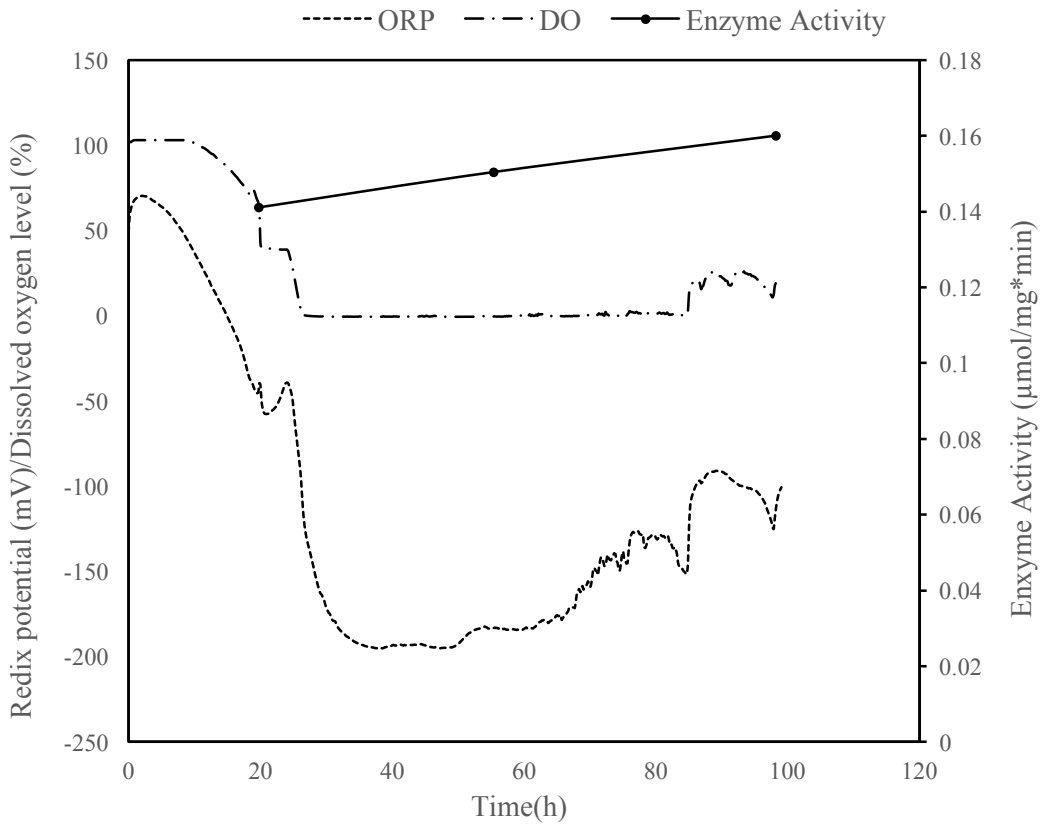

Figure 5.10 Sample No.1, 6, 10 recombinant prolinase activity with ORP and DO level profiles.

6. Conclusion

In this study, a correlation between the fermentation ORP profile and recombinant *E. coli* growth pattern was illustrated and analyzed. The ORP profile could replace the OD reading as an indicator to portrait the growth pattern of a recombinant *E. coli*. Specifically, different portions of the ORP profile correlated to the corresponding stages of growth patterns. Moreover, these features could be used to guide the timing for induction and harvest during the fermentation.

To be specific, with the increasing of OD reading, ORP profiles declined noticeably and kept at the minimum before a fluctuated growth which represented the end of fermentation. And during the optimal inducing timing, ORP profile experienced an obvious lower deceasing slope. Therefore, thanks to this correlation, the growth pattern of the recombinant *E. coli* can be estimated directly from the ORP profile during cultivation. This research also illustrated that the inducer IPTG (the inducing agent) did not impose any obvious impact on the correlation between ORP profile and recombinant *E. coli* growth pattern.

Dissolved oxygen level plays a crucial role in culturing *E. coli*. It has been demonstrated in this research that insufficient air supply would slow down the growth rate of recombinant *E. coli*. And it is essential to provide sufficient DO to the fermenter. The earlier fermentation process used one air pump (2.6 L/min) and set agitation rate at 400 rpm. Two methods, adding an extra air pump (2.6 L/min) and increasing agitation rate to 550 rpm, were implemented to the fermentation system in order to prolong the period with required DO level at least 40% saturation. But the length of this period was so short that was negligible compared to the whole fermentation process. Thus, a new effective method is suggested to be conducted to the system in the further research in order to improve the recombinant protein production.

Last but not least, the recombinant prolinase was analyzed including quantity and activity. It was noted that recombinant prolinase was expressed and accumulated during

the fermentation. Moreover, the activity of recombinant prolinase expressed via this research was in the range as previously reported by other researcher [2] and gradually increased during the fermentation. In summary, ORP profile could be used as an indicator to guide when to add inducing agent and when to harvest.

7. Future work

Based on the experiments and analysis have been done in this study, there were several recommendations and potential expansion of current work I would like to recommend for future work.

Currently, the growth pattern of recombinant *E. coli* was represented by OD profiles in order to determine the inducing timing and harvest moment. This method was a periodic measurement which is labor-intensive and time-consuming. Besides, taking samples frequently made it more likely to introduce contaminants to the fermentation system. Alternatively, using ORP profiles as an indicator to show the growth pattern of recombinant *E. coli* would avoid all the above-noted disadvantages. The fermentation ORP was continuously measured via an ORP sensor and recorded by a computer. It was found from this research that there existed a correlation between fermentation ORP and *E. coli* growth pattern. To be specific, during the optimal inducing timing, the slope of ORP profile witness a recognizable decreasing for at least half an hour. In addition, the ORP profile experienced a fluctuated rise at the end of fermentation which could be identified as an optimal harvest moment. Therefore, it is feasible to develop a strategy in order to monitor these ORP features and control the fermentation automatically, for example, determine when to add inducing agent and when to stop the fermentation. As thus, without frequent sampling the recombinant *E. coli* would be cultured in a closed and contamination free environment. Besides, the cost of labor and time would be saved as well.

Under the optimal recombinant *E. coli* culture conditions, unattended DO level has imposed a restriction to the growth of recombinant *E. coli* cell rate and the protein expression. As described in this research, two methods had been tested but neither of them could maintain DO at a desired level as cells enter exponential phase. In the further research, an improved method is supposed to solve this problem. The previously tested two methods could be combined together. That is the DO would be controlled by a variable agitation rate initially until it reached its maximum and then under the maximum agitation rate the

fermentation system would switch to aeration control. An extra stirrer could also be installed to the agitator shaft in order to improve the air distribution uniformly. Meanwhile, in the further research, the respiratory quotient (RQ) could be monitored in order to study cell's respiratory physiology during the recombinant *E. coli* fermentation.

ORP has been used as a process parameter to study its effect on carbon flow and energetic flux in microorganisms. [76] ORP is defined as the net balance between oxidizing and reducing powers during a fermentation process. The main components are DO and NADH which can alter the redox state in a fermenter. In addition, the fermentation ORP can reflect the intracellular activity and the DO level of the fermentation broth. [77] Once the DO level is controlled, any change of intracellular activity of the recombinant cells could be correlated to the changes of fermentation ORP. It was found previously that each species, or even each strain, has a preferable ORP range during fermentation, within which cell growth is optimal. Moreover, extracellular ORP was proved to affect the synthesis or stability of certain enzymes which could change metabolic fluxes and ATP yield of microbes. [76] Thus, with a fully understanding of the correlation between ORP profiles and intracellular activity of recombinant *E. coli*, the ORP-equipped fermentation process would create a better cultivating environment for recombinant *E. coli* towards higher productivity.

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