The Influence of Controlling Redox Potential on Plasma Membrane Fatty Acid Composition during Very High Gravity Fermentation

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ABSTRACT

Fatty acid components on yeast plasma membrane were critical in maintaining proper cell activity during bioethanol fermentation. The alteration of fatty acid composition on yeast plasma membrane was recognized as an adaptive response to several environmental stress including osmotic pressure, ethanol inhibition and nutrients limit. These stresses were exacerbated under very-high-gravity condition in which excessive fermentable sugar was provided in feedstock. Controlling redox potential was proved beneficial in improving yeast performance under very-high-gravity condition. Fatty acid synthesis and desaturation pathways involved dissolved oxygen as well as balance between NAD^+/NADH and $NADP^+$/NADPH which could be influenced by the regulation of redox potential in media. In this study, fatty acid composition profiles under different glucose concentrations and different redox potential control level were examined. Its connection with yeast cell growth, ethanol productivity and other metabolites' concentrations were studied as well to reveal any causal correlation between redox potential control, membrane fatty acid composition and yeast activity.

Two glucose concentrations used in this study were 200 g/L and 300 g/L which represented normal and very high gravity respectively in bioethanol fermentation. In 300 g/L fermentation, three redox conditions were adopted while two different redox conditions were used in 200 g/L fermentation. Biomass concentration, ethanol productivity and fatty acid composition were observed to be affected by both gravity and ORP control strategy. Final biomass concentrations were 4.302 g/L in 200 g/L glucose with no ORP control condition and 7.658 in 200 g/L glucose with ORP controlled at -100 mV condition. In 300 g/L glucose fermentation, final biomass concentrations were 3.400 g/L for no ORP control, 4.953 g/L for -150 mV ORP control and 5.260 for -100 mV ORP control. Ethanol productivities were 2.574 g/Lh for 200 g/L glucose without

ORP control and 3.780 g/Lh for 200 g/L glucose with -100 mV ORP control. In 300 g/L glucose fermentation, ethanol productivity decreased to 1.584 g/Lh when no ORP control was imposed. ORP control at -150 mV could improve the ethanol productivity to 1.693 g/Lh while -100 mV ORP control was able to further enhance the ethanol productivity to 1.829 g/Lh. Fatty acid composition was observed to shift to more saturated components when no ORP control was applied. Such trend of saturation was increased by higher gravity condition. ORP control was shown to change this tendency to saturation and help restore fatty acid components on plasma membrane to a more balanced distribution.

Keywords: very-high-gravity fermentation, redox potential control, fatty acid composition, membrane, biomass, ethanol productivity

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ABBREVIATIONS

ATP	Adenosine triphosphate
DO	Dissolved oxygen
DNA	Deoxyribonucleic acid
FAS	Fatty acid synthase
GHG	Greenhouse gas
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
ORP	Reduction-oxidation potential
OD	Optical density
VHG	Very high gravity

NOMENCLATURE

SI	Saturation index
$FA_{C16:0}$	concentration percentage of palmitic acid (C16:0)
$FA_{C18:0}$	concentration percentage of stearic acid (C18:0)
$FA_{C16:1}$	concentration percentage of palmitoleic acid (C16:1)
$FA_{C18:1}$	concentration percentage of oleic acid (C18:1)

1 Introduction

1.1 Background

Biofuels have been recognized as potential replacements for traditional fossil fuels during past several decades. Developed from brewing industry, biofuel production technologies did not gain enough attention until many countries were haunted by severe fuel shortage during WWII. Later, research related to biofuel production boomed again because of fluctuating crude price during several oil crises. Since the public concern more about the exacerbating global warming and greenhouse effect, biofuel as an eco-friendly fuel solution started to develop rapidly in the last decade.

Among varied forms of biofuels, bioethanol as a relatively well developed and easy-to-apply biofuel solution, has long competed with fossil fuels in transportation sector. The production cost of bioethanol nowadays is still slightly expensive in most regions compared with its fossil fuel competitors. However, as the low-cost crude oil reserves continue to deplete and new technical breakthroughs are being deployed in bioethanol production, we can expect such gap to narrow down or even be eliminated.

In recent years, researchers developed several prospective improvements for current bioethanol industry. Such improvements covered interdisciplinary researches including plant science, molecular biology, microbiology and engineering, etc. Among these improvements, an innovation in fermentation process defined as very-high-gravity (VHG) fermentation showed potential in reducing production cost and enhancing productivity. Different from traditional bioethanol fermentation process, VHG fermentation employs higher feedstock concentration which allows it to achieve higher titer at the final stage. This could help save energy and time in downstream

ethanol recovery, thus significantly reduce the operational cost and energy input of bioethanol plants. Such a high feedstock concentration in the VHG fermentation process on the other hand induces a harsh environment for microorganism to propagate. Thus, some research efforts were undertaken to resolve this problem to improve the bioethanol productivity under varied forms of environmental stress. Our lab in past few years developed a novel fermentation strategy incorporating on-line reduction-oxidation (redox) potential control into VHG fermentation process. It has been proved that such strategy helped maintain ethanol productivity and yeast proper growth in VHG media.

In this thesis, the influence of redox potential control on yeast plasma membrane fatty acid composition was examined to illustrate any possible explanation for enhanced cell viability and bioethanol productivity during VHG fermentation. Because of the essential role plasma membrane plays in protecting yeast cells from environmental perturbations, it is reasonable to believe that changes happen on the composition of plasma membrane could contribute to the improvement of cells' defence to stresses *in vitro*.

1.2 Thesis organization

Chapter 1. The introduction of the thesis that stated description of thesis organization

Chapter 2. A literature review that provided necessary background knowledge regarding bioethanol prospective, very-high-gravity fermentation technologies, fatty acid composition of yeast plasma membrane and principles of redox potential. The knowledge gaps as well as the objectives of this thesis were also presented in this chapter.

Chapter 3. An introduction of the experimental design, materials and methodology used in this project

Chapter 4. Discussion of experimental results using figures, tables and statistical tools based on the raw data acquired.

Chapter 5. Conclusion summarized from data analysis and discussion.

Chapter 6. Recommendations and future work

2 Literature review

2.1 Review of Biofuel backgrounds

It was reported that a long-term increase in global energy consumption can be expected as a result of sustained growth in global economy and population (U.S. Energy Information Agency, 2015). Such increase requires a more reliable and diverse energy supply solution which can hardly be fulfilled by single non-renewable fossil fuel resources. Besides, the heavy environmental stress brought by the consumption of fossil fuels started to emerge and exacerbate in recent years. The most notorious one was the global warming and greenhouse effect caused by excessive greenhouse gas (GHG) released by burning of fossil fuels i.e. coal, crude and natural gas. It should also be noticed that in spite of recent technology advances, margins from cultivation continued to shrink and jobs lost chronically in agricultural sector. Such situation seriously threatens global food security and thus increased investment and policy reforms are necessary in order to solve the poverty problem in rural area as well as boom productivity in food production (Godfray et al., 2010). Years of efforts have been devoted into searching alternatives to petroleum-derived fuels to alleviate both the world's reliance on non-renewable resources and the burden on environment by burning of fossil fuels (Gray, Zhao, & Emptage, 2006). After decades of developments, varied forms of renewable resources including solar, hydraulic, wind, geo-thermal and biomass, etc. nowadays can be harnessed to produce energy to meet the increasing global energy demand. Despite of the recent fluctuation of oil price, renewable fuels are destined to become an integral part of the future energy solutions concerning their sustainable and eco-friendly characteristics (Stephanopoulos, 2008). Compared with other forms of renewable energy solutions, biofuel is more suitable for transportation sector because it not only requires fewer modifications to the widely used gasoline or diesel engines but provides competitive energy density for vehicles.

There are currently two separate conversion pathways for biofuel production show equally potential for further development and commercialization. Biological conversion utilizes the classic fermentative pathways of microorganism to convert biomass feedstock into varied types of biofuels including bioalcohols (mainly bioethanol and biobutanol), biogas and biodiesel (Luque, Campelo, & Clark, 2011). Chemical conversion mainly produces biodiesel or syn-gas through either thermochemical conversion or transesterification reaction (Demirbas, 2007). Both conversion processes have their own advantages as well as disadvantages largely depending on different production scenarios. Chemical process basically could utilize a broader range of feedstock, hold enhanced productivity and produce biofuels with high energy density. But the main product from chemical conversion, biodiesel, confronts several defects compared with crude derived diesel. The products from biological process require few modifications to current engine design, thus are easily to blend with gasoline (Zainura, 2013).

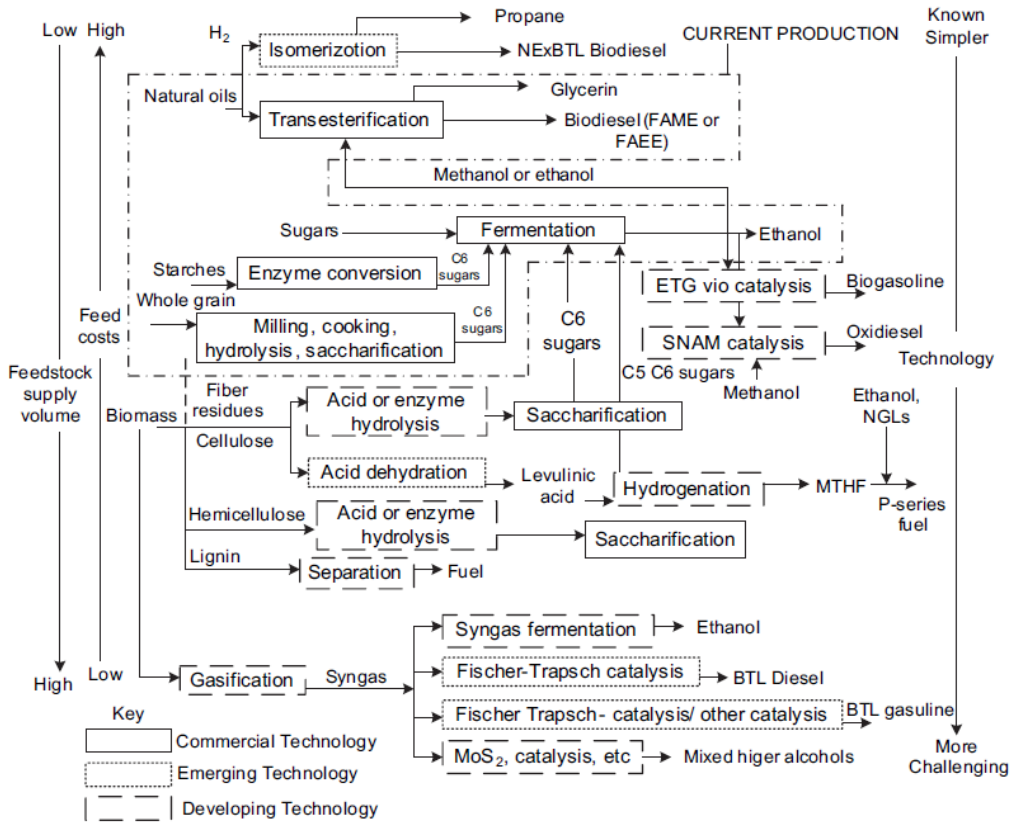

Figure 2.1 Varied forms of biofuels under development (Nigam & Singh, 2011).

However, the feedstock costs for both conversion processes are still higher compared with the crude currently used in refinery industry. This problem is especially amplified for first-generation biofuel production which mainly relies on crops, vegetable oils and animal fats as its feedstock resources (Viesturs & Melece, 2014). Besides, the reliance on food products as feedstock ignited increased concern over the controversy between biofuel production and global food security (Rosegrant & Cline, 2003). Despite the fact that there are still certain disadvantages exist in first-generation technologies, commercial production has been deployed quickly in the first decade of twenty first century driven by the favourable government policy and unstable oil price (Doku & Di Falco, 2012; Sorda, Banse, & Kemfert, 2010). In addition to these efforts, some researchers shift their interests onto the development of a new generation of biofuel technology. Compared

with traditional biofuel, the next-generation biofuel has several advancement including: utilizing agricultural residues and non-crop biomass as feedstock in production, integration of production process and further mitigating GHG emission in biofuel production life cycle (Taylor, 2008). Although there are some technical barriers lying between laboratory research and commercial demonstration, it is expectable that such barriers could be eliminated by increased investments in research and development from both public and private sectors (Sims, Mabee, Saddler, & Taylor, 2010).

2.2 Review of Bioethanol Fermentation

2.2.1 1st-generation bioethanol fermentation

Bioethanol, among all forms of liquid biofuels, is a well-developed and widely-commercialized biofuel solution. The most common feedstock utilized in commercialized bioethanol plants today is corn in the North America and sugarcane in Brazil respectively. The two countries possess over half of the total share of global bioethanol supply. Thanks to easily acquired feedstock and support from government in infrastructure building, bioethanol in Brazil is very competitive with gasoline in retail price. This overturned many people's stereotypical image of bioethanol as an expensive alternative energy solution with limited application potential and thus proved that bioethanol, converted from cheap feedstock and supported by proper policies, is actually quite promising in commercialization (Luo, van der Voet, & Huppes, 2009). Unlike Brazil, corn derived bioethanol in North America is relatively costly in production as prices of bulk agricultural products increased significantly after the deployment of many commercial bioethanol plants. The same dilemma was witnessed in Europe and China as well since producers in both regions apply the similar conversion technology used in North America (McAloon, Taylor, Yee, Ibsen, & Wooley). This situation, combined with other concerns around food security and GHG emission, drives the scientists and

researchers to pursue a cheaper and more realizable bioethanol production solution defined as 2nd-generation bioethanol.

2.2.2 2nd-generation bioethanol fermentation

The main difference distinguishes the 2nd-generation bioethanol from its predecessor is the utilization of cheaper feedstock including agricultural residues, non-crop plants and municipal wastes, etc. to achieve a drastic curtail in production cost. This could help reduce the pricing of bioethanol in many regional markets and grant bioethanol more advantages when competing with fossil fuels in transportation sector. Although some bottlenecks existed makes the 2nd-generation bioethanol still costly in production, private sector, under the support from government, have deployed several pilot plants to demonstrate the application potential of 2nd-generation technology. Based on the positive feedbacks acquired, some corporation established their plan for commercial plants which was estimated to be completed and fully operational before 2020 (Advanced Ethanol Council, 2014). Lots of research efforts have been devoted into 2nd-generation technology improvements from upstream to downstream including but not limited to: genetic modification of cellulosic and hemi-cellulosic material, cost effective pre-treatment, robust microorganism development, process optimization and wastewater treatment during ethanol recovery (Gray et al., 2006). The ultimate purpose of these researches is to reduce the cost of 2nd-generation bioethanol production to an acceptable scale for commercialization.

2.3 Very-high-gravity fermentation

Very-high-gravity (VHG) fermentation is defined as “the preparation and complete fermentation of mashes containing 27 g or more dissolved solids per 100 g of mash, which corresponds to 300 g per litre of mash” (Thomas, Hynes, Jones, & Ingledew, 1993). Starting with medium containing sugar over 250 g/L, fermentation system can achieve a final ethanol concentration of more than 16.1% (v/v). Such kind of fermentation is favorable in industrial fuel alcohol production for its capability to not only reduce the costs of downstream recovery process but also enhance process productivity through increasing final concentration of fermentation product (Puligundla, Smogrovicova, Obulam, & Ko, 2011).

Table 2.1 Advantages of VHG fermentation (Puligundla et al., 2011).

Feature	Low or normal gravity	Very high gravity
Feedstock concentration		
Brewing-wort gravity	11–12°P	18°P or more
Fuel ethanol-mash or fermenting medium gravity	16–20 g, max. 24 g/100 ml dissolved solids	30 g or more, max. up to 39 g/100 ml
Final ethanol concentration		
Ethanol content of beer	4–5% (v/v)	14–16% (v/v)
Fuel ethanol	10–12% (v/v)	Increased to >15 or 18% (v/v)
Plant capacity	Fixed	Increased because of more fermentor space created through removal of insoluble matter
Plant efficiency		
Labour costs	Fixed/litre ethanol production	Relatively decreased
Energy costs	Fixed/litre ethanol production	Relatively reduced (about 4% savings) due to less water in fermentor and in still to process; Avoidance of energy loss due to handling insolubles
Potable water savings		
Starch-to-water ratio	1:3	1:2 or 1:1.8, depending on substrate savings up to 58.5%
Effluent	10–15 l/l ethanol	6–9 l/l ethanol recovered
Enzymes' (liquefying & saccharifying) activity	Low activity due to high dilution rate	High activity due to decreased starch-to-water ratio
Enzymes' stability	Less stable	Highly stable due to increased substrate concentration
Spoilage bacteria	Acetic and lactic acid bacteria thrive well, decreases fermentation efficiency	Spoilage bacteria cannot survive better under high osmotic conditions
Co-products/by-products		
Quality	Feed grade by-products	Food quality co-products/distillers grains
Spent yeast	Low protein yeast	High protein spent yeast
Fermentor downtime	Fixed hours	Relatively reduced hours, high-productivity ethanol production

These advantages only hold true in a constant cellular system free from influence of harsh fermentation environment while actual situation shows that yeast cells confront varied forms of stresses during VHG fermentation. Due to the high osmotic stress caused by excessive sugar in the medium, yeast cells suffer a prolonged lag phase at the early stage of fermentation. Sluggish fermentation and undermined productivity was thus observed as a result of osmotic influence of the media (D'Amore, Panchal, Russeil, & Stewart, 1988; Panchal & Stewart, 1980). Yeast cells' growth is also inhibited as ethanol enriches in medium. Once a certain concentration threshold is reached which varies among different yeast species, cell viability decreases dramatically, so is

fermentation productivity. Many hypothesises regarding the inhibition mechanism of ethanol have been proposed, yet no conclusive explanation could be provided so far because of the complicated interaction within the yeast metabolic network (D'Amore & Stewart, 1987). Besides the role of osmosis and ethanol, other factors including media viscosity, water activity and nutrient limitation need to be taken into consideration in order to optimize the performance of VHG fermentation. Some research efforts have been put into illustrating yeast metabolism and its reaction to environmental fluctuations. Meanwhile, varied process control strategies are developed in parallel from engineering perspectives to fulfill the mission of enhancing yeast cells' growth in medium including: external energy input, chemical reagents supplementation, aeration etc.(Liu, Xue, Lin, & Bai, 2013). D'Amore et al. (1991) found that trahalose might be an essential component in shaping yeast stress tolerance in high gravity environment. In their study, a series of strains were studied and the result showed that those osmotolerant strains possessed higher intracellular trahalose concentration. Their study also showed that high intracellular trahalose strains were more resistant to stress brought by extreme temperature (D'Amore, Crumplen, & Stewart, 1991). Other researchers such as Zhao et al discovered the application of yeast flocculation in improving yeast resistance to environmental stresses (Zhao & Bai, 2009). Lately, Lam et al. (2014) reported that the elevation of extracellular potassium concentration and PH could help enhance yeast's general resistance to multiple alcohols (Lam, Ghaderi, Fink, & Stephanopoulos, 2014). This makes it possible to regulate yeast metabolism effectively by a single parameter during complex industrial fermentation process. It is worth noticing that redox potential (ORP) changes simultaneously as cells react to the deteriorating extracellular environment. Such correlation makes it feasible to control yeast cells' growth in the fermentation process by manipulating system's ORP parameter

(Lin, Chien, & Duan, 2010). From industrial perspective, combined with up-to-date technology limits, aeration is a much more favorable strategy for its reasonable cost performance.

2.4 The Role of Plasma Membrane Fatty Acids in Yeast Metabolism

2.4.1 Plasma membrane and yeast tolerance mechanism

It is commonly agreed during the evolution of brewing industry that yeast cannot overproduce ethanol to a limitless extent. The maximal yield of fermentation is restricted by each strain's tolerance to ethanol, which is the ultimate target product in bioethanol industry. Unlike traditional brewing industry concerns not only ethanol content but also flavouring additives, yeast biomass and aromatic by-products, the purpose of fuel ethanol industry is pure and straightforward: produce as much ethanol as possible in a limited production period through an economically feasible process. Hence, several parameters including productivity, yield, biomass and cell viability, etc. are of critical importance in bioethanol industry.

Researchers and engineers in field have long realized the inhibitive effect ethanol imposed on yeast cells and researches related to this topic have lasted for several decades with some results being recognized. Casey et al. (1986) and D' Amore et al. (1987) tried to define the significance of ethanol tolerance using common parameters which were tractable during production process and proposed the potential targets of ethanol toxicity based on existing knowledge of yeast metabolism but left detail ethanol toxicity and cell tolerance mechanism unexplained (Casey & Ingledew, 1986; D'Amore & Stewart, 1987). Following these results, some researchers proposed that cell membrane, also known as plasma membrane, might play a critical role in yeast tolerance (D'amore, Panchal, Russell, & Stewart, 1989; Ingram, 1986). As more advanced analytical technologies were applied and researchers' understanding of yeast physiology and metabolism went into details, it was discovered that the alteration of plasma membrane fluidity and

permeability under the influence of ethanol could be the result of undermined cell viability and activity (Swan & Watson, 1997). Researches also revealed that many components on plasma membrane namely H^+-ATPase, transport proteins and lipids might be involved in antagonizing the damaging effect brought by excessive ethanol (Aguilera, Peinado, Millan, Ortega, & Mauricio, 2006; Hervé Alexandre, Rousseaux, & Charpentier, 1994). Since plasma membrane is the primary target exposed to different kinds of stresses and it plays critical role in nutrition uptake and proton flux control, it is reasonable to presume that plasma membrane could be influential in shaping yeast stress tolerance.

It should be noticed that besides plasma membrane, other potential targets related to yeast tolerance were investigated at the same time. Ding et al. (2009) summarized updated progresses in studying yeast response to ethanol in their recent review paper that mentioned several factors influencing cell tolerance mechanism such as activity of heat shock proteins and fluctuation of stress related transcriptional factors (Ding et al., 2009). With the help of novel genetic analytical techniques such as DNA microarray and real-time PCR, researches focusing on genetic dissection of yeast cell's response to ethanol were performed. Such works were expected to facilitate scientists in building a detail image of the tolerance mechanism from transcriptional level. Ogawa et al. (2003) found in their research that several stress-responsive genes were highly expressed in an ethanol-tolerant mutant when compared with its parent strain. Their finding was further validated by exposing this mutant in high ethanol condition and discovering that those highly expressed genes were over-expressed even more under such exposure (Ogawa et al., 2000). Several research groups applied DNA microarray technique to acquire so called global gene expression information from different perspectives including short-term ethanol inducing, comparison between a mutant strain and its parent and step-wise cultivation under increased

ethanol concentration (H Alexandre, Ansanay-Galeote, Dequin, & Blondin, 2001; T. N. Dinh et al., 2009; Liu, Lin, & Bai, 2013). Based on previous research fruits, Stanley et al. (2010) redefined that the yeast response to stress in essence was a regulation of cellular activities facing challenging environment to maintain basic survival. This required the overexpression of genes related to energy producing pathways i.e. glycolysis and mitochondrial function and inhibition of genes associated with energy consuming growth-related pathways in order to restore the metabolic balance impaired by extracellular stresses (Stanley, Bandara, Fraser, Chambers, & Stanley, 2010). Another review work from Ma and Liu (2010) categorized hundreds of reported genes according to their function and transcriptional targets (Ma & Liu, 2010). This helped the following researchers continue to progress with a comprehensive gene ontology knowledge.

It is recommended that researchers should be aware of the overlapping and interaction of multiple genes under complex network at genome level. This makes it difficult to improve the yeast tolerance by only modifying few related genes since such modification would easily be buffered by unpredictable interactions within the network. However, the information generated from gene-related studies is critical to accurately explain the tolerance forming mechanism and thus useful in examining yeast tolerance under the regulation of other parameters.

2.4.2 Fatty acid composition in plasma membrane

Fatty acid is not the sole component of plasma membrane yet it is indispensable in membrane composition. Fatty acid components are susceptible to ethanol's influence because the lipophilic character of ethanol allows it to insert into structure of plasma membrane easily and interact with lipid components in membrane. Such hypothesis has been tested by Ingram in his study using an *E. coli* strain (Ingram, 1976). The results showed that cell growth was inhibited when exposed to ethanol and did not resume until fatty acid composition in membrane started to change. This

observation was explained as an adaptive response of *E. coli* to ethanol. Later in his review paper, Ingram systematically explain the detail changes he found in fatty acid composition in deferent strains including *S. cerevisiae* (Ingram, 1986). You et al. (2003) reported that unsaturated fatty acids components on membrane i.e. oleic and palmitoleic acid may contribute to yeast tolerance to ethanol. Their study supported the hypothesis that ethanol tolerance in yeast derived from elevated oleic acid share in lipid membrane composition (You, Rosenfield, & Knipple, 2003). Recent researches performed by Dinh et al. (2008) suggested that yeast maintain its growth activity in the stepwise increasing ethanol cultivation through an adaptive alteration of fatty acid composition and cell size (Thai Nho Dinh, Nagahisa, Hirasawa, Furusawa, & Shimizu, 2008). Some researchers believed that the alteration in fatty acid composition i.e. increase in unsaturated fatty acid content renders cell membrane tight and formal which might help antagonize the fluidizing effect induced by ethanol. Aguilera et al. (2006) reported a statistical correlation among oleic acid and ergosterol content, ATPase activity and ethanol tolerance through comparison among five separate wine strains under 4 % v/v ethanol treatment (Aguilera et al., 2006). Huffer et al. (2011) argued that despite of fact that membrane lipids tended to shift into more saturated and unbranched ones in the presence of alcohols, such shift did not necessarily enhance the tolerance of the strain. Actually, the strain observed with supreme tolerance appeared to maintain its membrane fluidity after ethanol exposure (Huffer, Clark, Ning, Blanch, & Clark, 2011). Serrano et al. (1988) suggested that activation of ATPase lying on plasma membrane required the involvement of a negatively charged polar head coupled with unsaturated fatty acyl chains (Serrano, Montesinos, & Sanchez, 1988). This discovery showed the potential impact the alteration in fatty acid composition could bring on other membrane proteins' proper function.

Though our research concentrate on studying the impact of fatty acid content on yeast physiology under varied stresses, it should be noticed that other components might play an equally important role in fulfilling this goal. Sterol components, especially ergosterols, have been investigated recent years since it was observed by some researchers that varying sterol level on cell membrane correlated with yeast tolerance (Dickey, Yim, & Faller, 2009). An up-to-date research proved that yeast cell tended to increase both ergosterol and unsaturated lipids to maintain a proper thickness of membrane as ethanol accumulated during anaerobic fermentation (Vanegas, Contreras, Faller, & Longo, 2012). Besides, amino acids as mentioned above, when inserted in membrane could confer certain tolerance on yeast (Ding et al., 2009).

2.4.3 The influence of dissolved oxygen on membrane fatty acid composition

The fatty acid composition in yeast cell membrane is relatively simple: four species including palmitic acid, palmitoleic acid, stearic acid and oleic acid consists over half of total fatty acid content with minor species including C14 and C26 acting as protein modifier or incorporating into membrane lipids (Tehlivets, Scheuringer, & Kohlwein, 2007). As is shown in Figure 3, the synthesis of fatty acid in yeast started from the carboxylation of acetyl-CoA catalyzed by acetyl-CoA-carboxylase (ACC) located in both cytosol and mitochondria encoded by *ACC1* and *HFA1* respectively. The product from the initial step i.e. malonyl-CoA is then catalyzed by fatty acid synthase (FAS) in cytosol to form a saturated acyl chain. Following elongation and desaturation is carried out by varied elongase and acyl-CoA Δ9-desaturase. Both enzymes locate in endoplasmic reticulum which makes it a critical organelle for fatty acid synthesis together with mitochondria and cytosol.

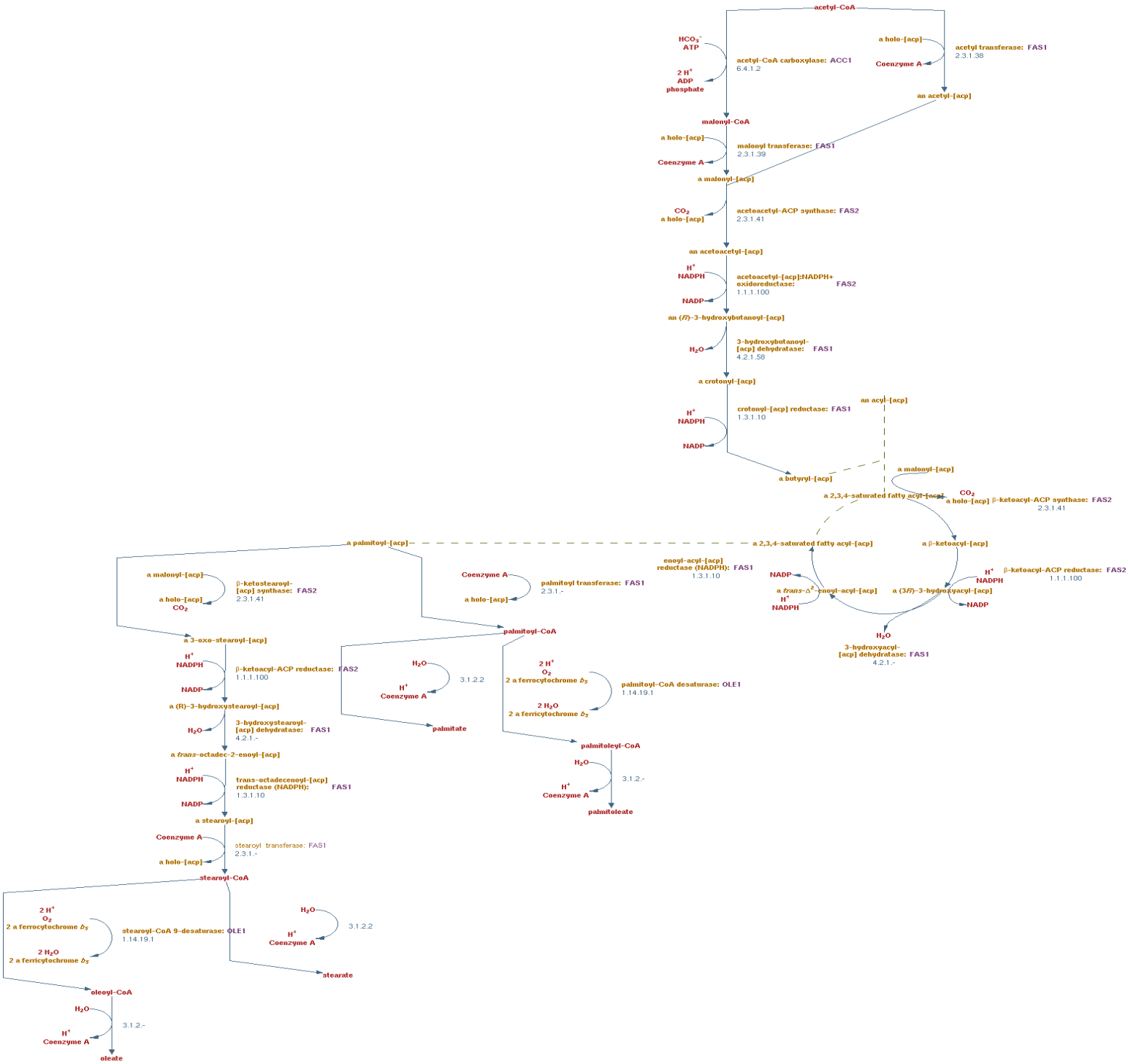

Figure 2.2 Fatty acid biosynthesis pathways summary (Saccharomyces Genome Database, 2015)

In addition to the information presented above, there are other perspectives which are not included in the previous discussion or figure should be noticed. First, the general fatty acid biosynthesis process can be briefly described in the following figure:

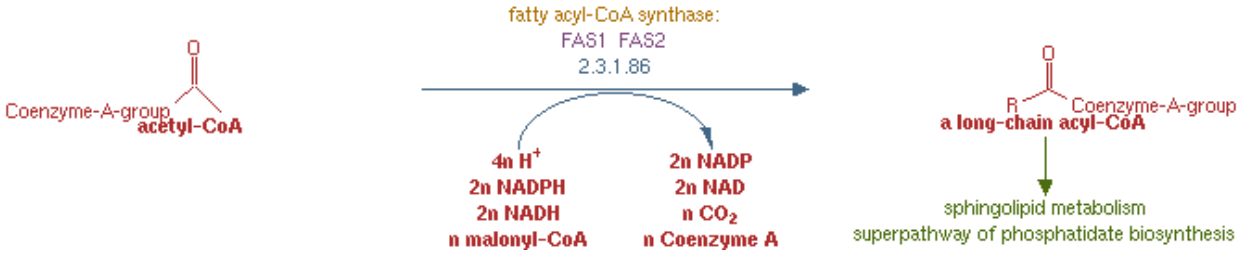

Figure 2.3 Long chain fatty acid synthesis process in a concise description (Saccharomyces Genome Database, 2014)

Fundamental building block malonyl coenzyme A (malonyl-CoA) is added onto acetyl-CoA step by step to form the basic long chain fatty acid component, mostly palmitic acid ($C_{16:0}$). This basic fatty acid is then further modified into other long chain saturated and unsaturated fatty acids. Besides malonyl-CoA, both Nicotinamide adenine dinucleotide (NAD^+) and Nicotinamide adenine dinucleotide phosphate ($NADP^+$) are involved as reducing agents in the synthesis pathway and its succeeding elongation and desaturation pathways. The preceding acetyl-CoA generating pathway involves the consumption of ATP and conversion of NADH to NADPH. These pathways altogether make intracellular fatty acid synthesis network a reducing and energy consuming role in overall cell metabolism.

It is known that synthesis of unsaturated fatty acids and sterols requires the incorporation of molecular oxygen. From pathway perspective, desaturation pathways which generates palmitoleic acid and oleic acid consume oxygen molecules as an essential electron acceptor. From genetic perspective, gene involved in the synthesis of Δ9 fatty acid desaturase is regulated by both oxygen

and fatty acid concentration (Volkmann & Klein, 1983). It is proved that without the supplementation of essential unsaturated fatty acids and sterols, most yeast strains including *S. cerevisiae* cannot reproduce under anaerobic condition. Thus, oxygen availability is a rate limiting factor in fatty acid biosynthesis pathway. It should be noticed that as a facultative anaerobe, yeast holds the ability to switch its metabolic pathways regarding oxygen availability.

2.5 Principles of redox potential

2.5.1 Redox potential detecting and control

Although oxygen is indispensable for cell growth and metabolism, it is a challenge for industry to accurately monitoring intracellular oxygen profile in an economic way. The common method adopted by industry is to monitor dissolved oxygen (DO) level using a DO probe in media and infer the intracellular oxygen distribution based on the DO information acquired. In pharmaceutical industry where aerobic cultivation of microorganism is applied to pursue a high cell density for target protein and antibiotics production, DO level is a critical parameter under control for optimal microorganism performance. However, DO level is quite low in micro-aerobic and anaerobic conditions which makes it hard to be detected by common DO probes and hence unable to be controlled on-line. Noticing that even if the dissolved oxygen level is significantly lower in micro-aerobic and anaerobic conditions, oxygen in media is still the prior electron accepter and thus influential to redox homeostasis in media (Pham et al., 2008). As an alternative solution, redox potential (ORP) profile acquired in media using ORP probe is employed as a parameter reflecting intracellular redox homeostasis and metabolism.

2.5.2 Redox profile of very-high-gravity fermentation

ORP detected in media can be affected by several factors including reducing substrates, DO level, and cell metabolism, etc (Liu, Xue, et al., 2013). Under very-high-gravity condition, *S.*

cerevisiae tends to synthesize more stress protectants such as glycerol and trahalose to alleviate the detrimental effect imposed by varied stresses. Synthesis of these protectants could detract NADH from ethanol production and thus cause an imbalanced redox profile. Lin et al. (2010) explained the details of ORP profile under very-high-gravity condition by proposing the bathtub curve hypothesis (Lin et al., 2010). At very early stage of very-high-gravity fermentation, dissolved oxygen due to its strong electron affinity was rapidly consumed, leading to a sharp decrease in ORP value. As dissolved oxygen continued to deplete, *S. cerevisiae* switched its metabolism from aerobic respiration to anaerobic fermentation. Such switch combined with the accumulation of ethanol would trigger the synthesis of many stress protectants which consumed reducing agents NADH in their synthesis pathways. This could further drove the ORP value to decrease during very-high-gravity fermentation and yeast growth and viability would be undermined in turn. The ORP value would rebound after the hampered metabolism and death of yeast cell starting to occur at the later phase of fermentation as extracellular environment deteriorated.

2.6 Knowledge Gap

VHG fermentation technology is quite promising for industrial deployment if negative impacts derived from osmotic pressure, water activity and product inhibition could be eliminated or at least partially relieved. Many studies dealing with these challenges from different perspectives including genetic modification of strain character, nutrient supplementation and cell immobilization, etc. have been reviewed in previous sectors. Recently, redox potential was reported as a useful parameter to reflect subtle changes in yeast metabolic profiles between different phases. The control of redox potential during VHG fermentation has been proved effective in boosting productivity, cell growth and viability. Principles of redox potential control strategy focused on explaining its direct influence on restoring intracellular redox homeostasis. However, other factors in yeast metabolism that could be affected were overlooked and thus need to be examined in detail.

Plasma membrane is a critical constituent involved in shaping yeast stress tolerance. Many components on plasma membrane including fatty acids are subjected to alteration brought by external parameter. Since the regulation of redox potential in VHG fermentation not only affects extra- and intracellular redox conditions but also alters the dissolved oxygen profile through sparging sterile air into media. As the synthesis of fatty acids in yeast cell is deeply influenced by molecular oxygen abundance, it is reasonable to assume that the control of redox potential in media could also changes the fatty acid composition on yeast plasma membrane. Although some researches have been reported regarding the adaptive behavior of yeast in altering its plasma membrane fatty acid composition under the presence of ethanol in varied concentration, a real-time monitoring of such adaption is missing and thus needed.

2.7 Research Objectives

Based on the knowledge gap mentioned above, this project aims at studying the influence of redox potential control on plasma membrane fatty acid composition during VHG fermentation. The proposed objectives of this project includes:

I. Performing VHG fermentation experiments at two different substrate concentration with or without redox potential control strategy.

II. Determining yeast performance and productivity under varied conditions

III. Exploring the differences in plasma membrane fatty acid composition under varied conditions

IV. Revealing the potential influence of the control of redox potential on plasma fatty acid composition

V. Revealing the possible influence of the alteration in plasma membrane fatty acid composition on productivity, cell growth and viability

3 Experimental Design, Material and Methods

3.1 Experimental Materials

3.1.1 Strain and media

The active dry yeast (Ethanol Red^{TM}) was obtained from Lesaffre Yeast Corp. (Milwaukee, MI, USA). The YPD agar medium (10 g/L yeast extract, 10 g/L peptone, 20 g/L dextrose, and 20 g/L agar) used for preparing plate culture for strain purification was purchased from BD Bioscience (Mississauga, ON, Canada).

The pre-culture medium used in this study was 100 mL solution consisted of: 200 g/L glucose, 10 g/L yeast extract, 0.2% (v/v) of $MgSO_4 \cdot 7H_2O$ stock solution and 0.5% (v/v) of urea stock solution. The glucose used in this study was purchased from MP Biomedicals (Solon, OH, USA) and the yeast extract powder was purchased from HiMedia Laboratories (Mumbai, India).

The fermenter medium consisted of: 200 g/L or 300 g/L glucose, 10 g/L yeast extract, 1 g/L sodium glutamate, 0.2% (v/v) of $MgSO_4 \cdot 7H_2O$ stock solution, 0.25% (v/v) of KH_2PO_4 stock solution, 0.5% (v/v) of urea stock solution, 0.2% (v/v) of $(NH_4)_2SO_4$ stock solution and mineral salts including 0.1% (v/v) of each stock solution: H_3BO_3; $MnSO_4 \cdot H_2O$; $CuSO_4$; KI; $FeCl_3 \cdot 6H_2O$; $CaCl_2 \cdot 2H_2O$; $ZnSO_4 \cdot 7H_2O$ and Na_2MoO_4. The concentrations of the stock solutions used in the fermenter medium and pre-culture medium were provided in Table 3.1. All chemicals used for solution preparation were of reagent grade or higher purity.

Table 3.1 Name and concentration of each stock solution used for preparation of media in the experiments

Media Constituent	**Concentration (mM)**
KH_2PO_4	735
$MgSO_4 \cdot 7H_2O$	1000
Urea	1600
$(NH_4)_2SO_4$	1000
H_3BO_3	24
$MnSO_4 \cdot H_2O$	2
$CuSO_4$	10
KI	1.8
$FeCl_3 \cdot 6H_2O$	100
$CaCl_2 \cdot 2H_2O$	82
$ZnSO_4 \cdot 7H_2O$	1000
Na_2MoO_4	1.5

3.1.2 Fermenter and control system

A 2-litre jar fermenter (Model: Omni Culture, New York, NY, USA) was used in this study. A detachable stainless steel lid was placed on top of the fermenter to maintain sterility during the fermentation. The fermenter was equipped with an ORP probe purchased from Mettler-Toledo (Model: Pt-4805-DPAS-K8S/225, Billerica, MA, USA) to monitor the on-line ORP profile during fermentations. The electronic signal acquired by ORP probe was then processed through the controller (Model: NI PCI-6013) and sent to the control computer. ORP control was achieved using LabView software (Version 2013, National Instrument, Austin, TX, USA) installed in the

computer. The LabView system sent the feedback signal to controller again and triggered the air pump (Model: Elite 802, Hagen Corp., USA) to sparge sterilized air into the media. The fermentation process was graphically described in Figure 3.1 below.

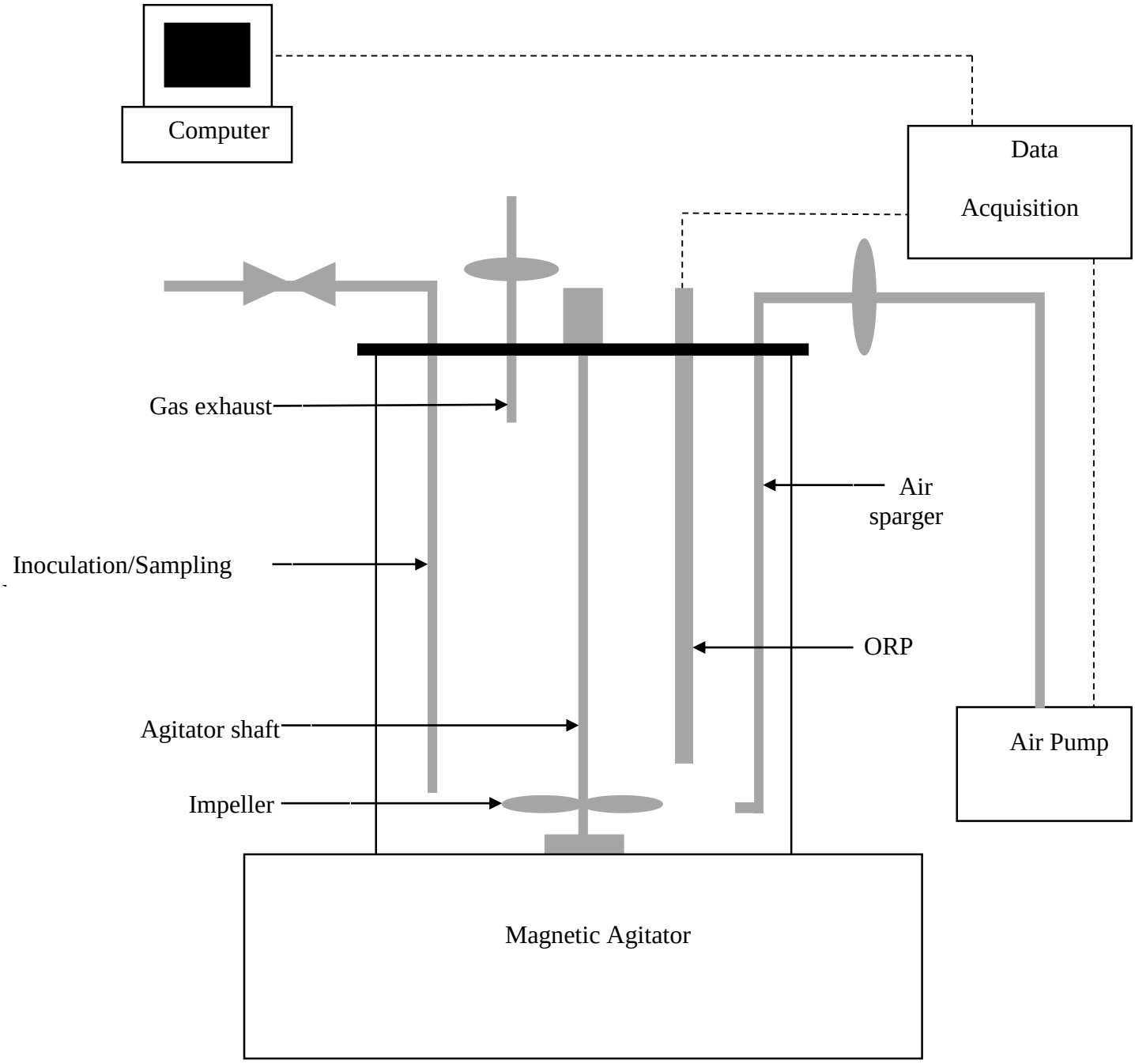

Figure 3.1 Flow diagram of fermentation system used in this study

3.2 Experimental Methods

3.2.1 Strain and fermentation conditions

3.2.1.1 Experimental strain and its purification

The dry yeast was rehydrated in 50-ml pre-culture media first and then transferred to YPD agar media in sterilized petri dishes and cultivated at 32 °C for at least 18 h. In order to purify the yeast strain used in experiments, two sub-culture were performed as described below. A single colony was selected from the plate culture on YPD agar media and dissolved in 50-ml pre-culture media again. The liquid culture was transferred to YPD agar plates for further cultivation and the same procedure was repeated once more for better purification of the strain. The purified yeast strain was then stored on YPD agar media at 4 °C for later experiments.

3.2.1.2 Fermentation conditions

Fermentation was carried out in a sterilized jar fermenter with a 2-litre working volume. The agitation rate was kept at 200 rpm for all batches of experiments. The temperature of the fermenter was maintained at 32 °C by circulating 32 °C water through the heating/cooling coil in the fermenter. The initial glucose concentrations were set at 200 g/L and 300 g/L respectively. The yeast strain was pre-cultured in the pre-culture media for 18 h with 150 rpm at 32 °C before inoculum. The inoculum volume was controlled at 5 % of the working volume [11]. Samples were taken every 4-8 hours during experiments and separated in several aliquots for different analytical purposes. All experiments in this study were performed in triplicates and the trend of each condition was presented in average with the variation defined by standard deviations.

3.2.1.3 ORP control strategy

ORP potential in the media was monitored and controlled through LabView software combined with ORP probes. The direct control of ORP in media was realized by sparging sterilized air (air

filtered by 0.2 μm PTFE membrane) into the fermenter. The flow rate of the air pump was set at 1300 ml/min. In order to balance the elevated air pressure in the fermenter brought by sparging air into the media, a gas exhaust tube coupled with a PTFE air filter was installed on the fermenter. The control levels were set at without control, -100 mV and -150 mV respectively for varied concentrations in experiments. Selection of these control level was based on previous experience in our lab (Lin et al., 2010).

3.2.2 Sample processing and data analysis

3.2.2.1 Analytical instruments and parameters

Samples taken during fermentation went through several analytical processes. A high-performance liquid chromatography (HPLC) system (Infinity 1260 Binary LC system, Agilent Technologies, Canada) equipped with an organic acid column (ICSep-ION-300, Transgenomic, USA) and a refractive index detector (RID) was used to determine the concentration of major metabolites including: glucose, ethanol and glycerol etc. during bioethanol fermentation. A gas chromatography (GC) system (7890B GC system, Agilent Technologies, USA) coupled with a FFAP column (DP-FFAP, 0.25 mm × 30 m × 0.25 μm, Agilent Technologies, USA) and a flame ion detector (FID) was used to determine the fatty acid composition of yeast plasma membrane. An UV-VIS spectrophotometer (UV Mini-1240, Shimadzu, Japan) was employed to acquire optical density (OD) values for biomass analysis. The hemacytometer and microscope used to detect cell viability during the fermentation was purchased from Hausser Scientific (Bright-Line 1490, Hausser Scientific, USA) and Zeiss (Primo Star, Carl Zeiss, Canada) respectively.

3.2.2.2 Analysis of major metabolites including carbohydrates and alcohols

A 1-ml portion from the fermentation media was sampled every 4 to 8 hours depending on varied conditions applied. The sample was centrifuged at 8000 rpm and 5 °C for 10 min. After centrifugation, the supernatant was extracted and stored at -80 °C for HPLC analysis of concentration of major metabolites including glucose, ethanol and glycerol. The analytical conditions of the HPLC system were set at 0.5 mL/min flow rate, 65 °C column temperature, 35 °C detector temperature. Mobile phase used in this study was 8.5 mM H_2SO_4 solution. The injection volume was set at 10 µl with a dilution rate of 5. The chromatograph was collected by Agilent ChemStation software and analyzed by comparing the acquired peak area with the pre-established calibration table of each metabolite.

The calibration table was established through analysis of a standard solution with known concentration. The standard solution was diluted by HPLC-grade water (Arium® Pro, Sartorius AG, Germany) in serial and 5 different concentrations including 100%, 75%, 50%, 25% and 10% of original concentration of standard solution were acquired. Each concentration was analyzed in triplicates by HPLC system and a calibration table corresponding concentration against signal peak area would thus be created.

3.2.2.3 Analysis of fatty acid composition on yeast plasma membrane

Another 20 to 40-ml portion from the fermentation media was acquired during each sampling in experiments. The sample was centrifuged at 4000 rpm and 5 °C for 20 min. The supernatant was removed and the biomass was washed once with HPLC-grade water and centrifuged once more for 10 min at 5 °C. After removing the water, the biomass is frozen at -80 °C for later GC analysis of major fatty acid components. The method used to extract fatty acid components from frozen samples is a combination of some previously established methods (Bligh & Dyer, 1959;

Folch, Lees, & Sloane-Stanley, 1957; Huffer et al., 2011). Generally, the cells were re-dissolved in a chloroform-methanol-water mixture solution with an approximate volumetric ration of 8:4:3. The solution was centrifuged using 3000 rpm at 5 °C until two phases fully separated. The lower phase containing mostly fatty acids was extracted and transferred to a glass vial placed on a block heater (customized picture shown in Fig 3.2) to evaporate at 70 °C overnight to remove all the liquid and precipitate fatty acids. The extracted fatty acids were methylated in an acidic methanol environment for 2 hours. The fatty acyl methyl esters (FAMEs) were then extracted by 1 ml reagent grade hexane (Fisher Scientifics, Canada) and analyzed by GC system. The GC analysis was performed using helium as carrier gas with column pressure controlled at 11 psi which corresponds to flowrate of 0.584 ml/min. The ramping strategy started at 110 °C for 5 min, then increased to 240 °C at 10 °C/min and maintained at 240 °C for 10 min. The FID detector temperature was set at 240 °C. The chromatograph was analyzed by comparing with the pre-established standard curve of each fatty acid component including palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0) and oleic acid (C18:1). Creation of the calibration table was similar to the serial dilution method described in the above HPLC analysis section.

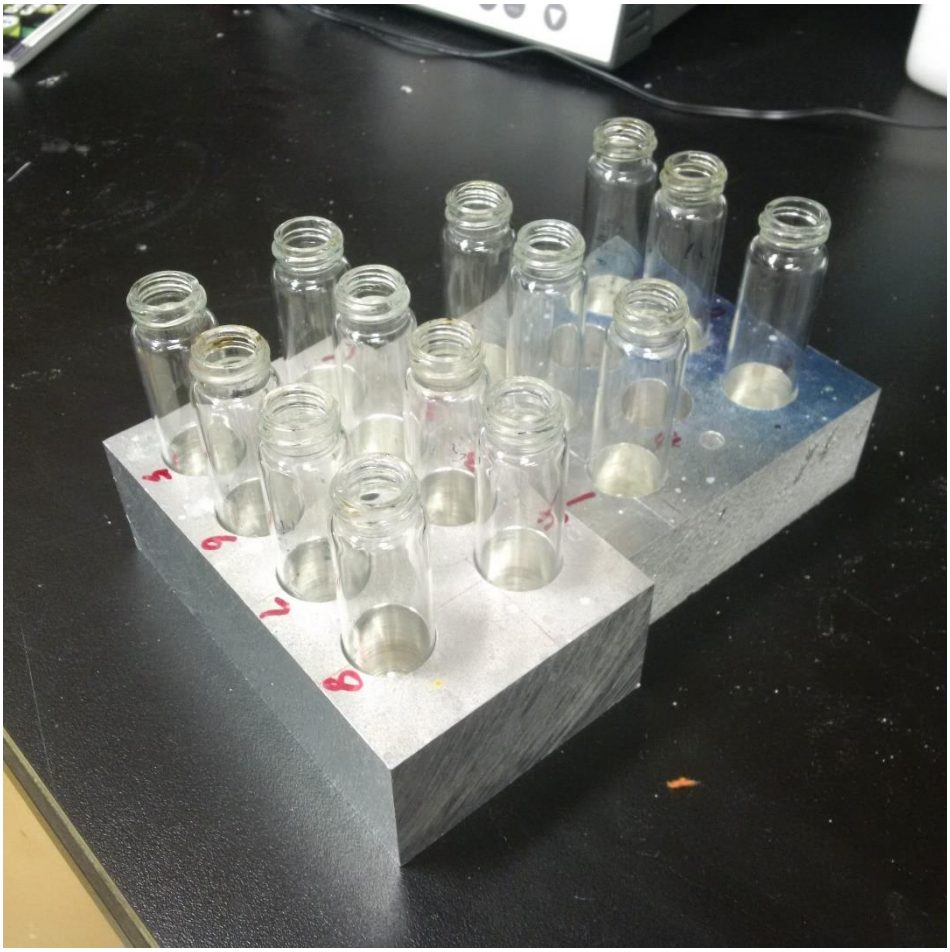

Figure 3.2 Customized block heater for fatty acid extraction

3.2.2.4 Analysis of biomass and cell viability

A 1-ml portion from the fermentation media was taken to determine biomass and cell viability profiles. Half of the portion was diluted by 3 to 30 times depending on biomass concentration and measured by spectrophotometer. An optical density (OD) reading was acquired and recorded. Later the OD value was used to determine the biomass concentration in the fermentation media by comparing with a pre-established biomass/OD value standard curve. The other half of the portion was diluted by 1 to 20 times and dyed with methylene violet stain. Dead cells would be dyed due to undermined cell membrane constitution and function. This helped distinguish dead cells from

viable cells and determine the cell viability by dividing viable cells' number with total cells' number.

4 Results and Discussion

4.1 Redox Potential Profiles under Varied Fermentation Conditions

4.1.1 Redox potential trends under normal gravity (~200 g glucose/l) and very high gravity (~300 g glucose/l) condition

In definition, redox potential measures the tendency of a chemical species in acquiring electrons and thereby being reduced. The redox potential of media thus represents the balance between reducing and oxidizing agents existed in media. Due to the reducing character of glucose, the redox potential of media solution for bioethanol fermentation would maintain mostly negative throughout fermentation process. The redox potential of the media could be affected by other factors including: pH, temperature, microbial growth and dissolved oxygen level.

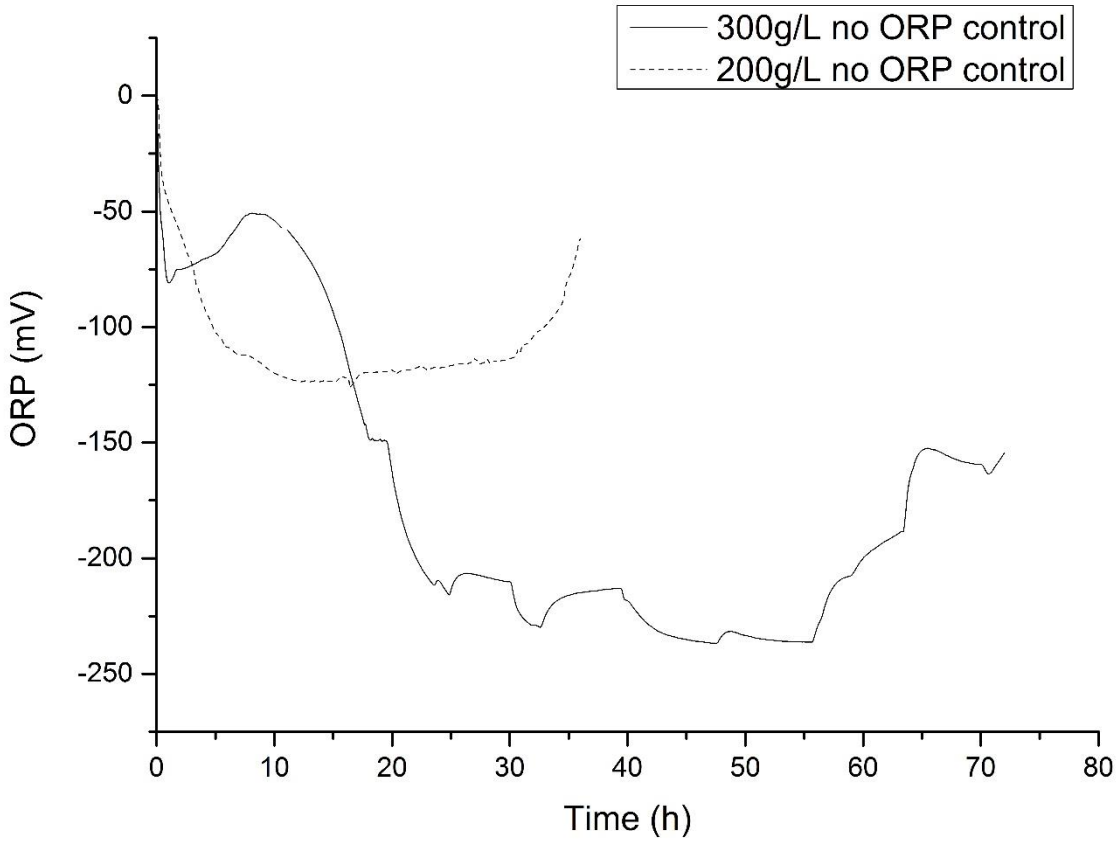

Figure 4.1 comparison of ORP profile under two glucose concentrations without ORP control

Fig 4.1 recorded the ORP patterns acquired in this study under two separate glucose concentrations when no extracellular disturbance and regulation were implemented during fermentation. Started from around zero, ORP level sank quickly after the inoculation of yeast into fermenter. As previously proposed by Chien et al. (Lin et al., 2010), such a plunge in ORP level might result from the inference that dissolved oxygen in media cannot balance the excessive electrons released via active adaption and growth of yeast cells. It was found that ORP level tended to decrease faster under normal gravity (200 g glucose/l) condition compared with the one under high gravity (300 g glucose/l) condition. As was shown later in Fig 4.3, such difference could be explained by the more vigorous growth of yeast under normal gravity. Yeast under very high

gravity condition otherwise proliferated quite sluggishly because of serious osmotic stress and scarcity of dissolved oxygen in media.

Another finding worth noticing was that ORP level under very high gravity condition would be reduced into much lower region compared with normal gravity condition. ORP level during very high gravity fermentation could reach as low as -250 mV while under normal gravity condition, ORP level could only decrease to around -130 mV. This situation did not match the recorded biomass profile which revealed when no ORP control was applied, less biomass was formed under VHG condition compared with normal gravity condition. Thus it was not likely that yeast growth brought such difference. It might be other organic acids formed during fermentation that caused such difference in ORP level at two separate glucose concentration since formation of these organic compounds would result in a net reduction of NAD^+ to NADH. Another possible reason was that when exposed to anaerobic condition, yeast had to use fermentation pathway to re-oxidize net NADH produced during glycolysis. Usually such duty was assumed more efficiently by respiration when enough molecular oxygen was prepared. Under higher glucose concentration, yeast's glycolysis tended to accelerate but fermentative route inhibited which could possibly lead to a rapid depletion of NAD^+ and in turn resulted in lower ORP level as observed in this study.

4.1.2 Effects of redox potential control strategy

Noticing such trend mentioned in Section 4.1.1, a certain ORP control strategy was developed to facilitate yeast cells in balancing intracellular redox situation and thus enhance their proper function in media.

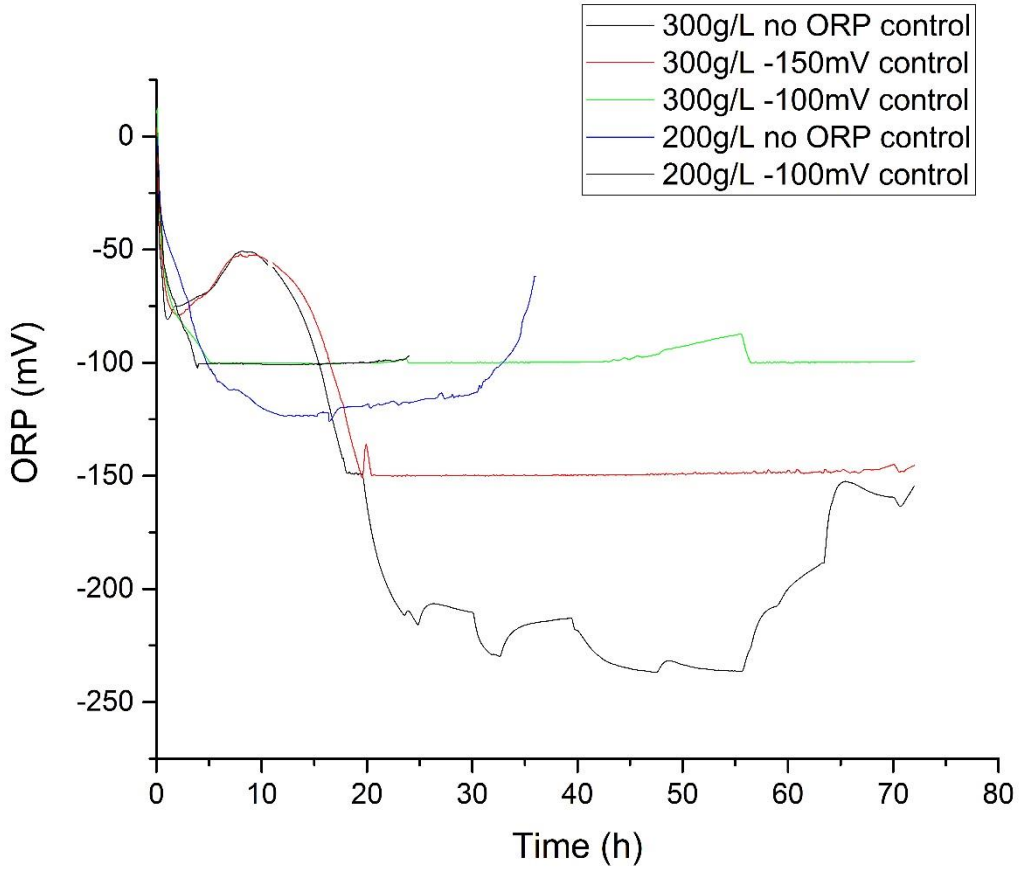

Figure 4.2 ORP control effects at different level under two glucose concentrations

Fig 4.2 showed the effects of ORP control on different pre-set levels. Since the ORP cannot go below -150 mV during fermentation of 200 g glucose/l, proposed 200 g/L controlled at -150 mV condition was not performed. As was presented in Fig 4.2, -100 mV ORP control started around 5 hours after inoculation which was similar in both glucose concentrations. -150 mV ORP control started around 20 hours after inoculation in 300 g/L glucose concentration. It should be noticed that the difference in regulation starting time was critical in later sections to compare the effect of different ORP control level on fermentation performance.

4.2 Biomass and Cell Viability of Yeast under Varied Fermentation Conditions

Biomass concentration and yeast cell viability data acquired in this study was summarized in this section. Despite of similar biomass and robust cell viability at the very beginning of each experiment, the final values varied significantly from each other. The final biomass and cell viabilities of 200 g glucose/L fermentation with or without ORP control were 7.659±0.235 g/L with cell viability at 93.91±1.35 % and 4.302±0.133 g/L with cell viability at 81.76±4.04 % respectively. In 300 g glucose/L fermentations, the final biomass and cell viability data was recorded as follows: final biomass and cell viability was 3.400±0.264 g/L and 74.33±6.44 % for no ORP control condition; final biomass and cell viability was 5.260±0.065 g/L and 66.05±14.86 % for -150 mV ORP control condition; final biomass and cell viability was 4.953±0.053 g/L and 68.22±5.19 % for -100mV ORP control condition. Besides easily observed differences of final biomass and cell viability profiles among different conditions, other minutiae regarding time-course trends and the influence of ORP control would be examined in the following subsections.

4.2.1 The influence of media's gravity on yeast biomass and viability

The comparison of biomass and viability profiles during fermentation of different glucose concentration was presented in Fig 4.3. It can be inferred from the figure that in spite of varied control conditions, biomass inclined to accumulate at very early stage of fermentation. However, the growth rate was affected deeply by different glucose concentrations. As was shown in Fig 4.3, biomass under normal gravity condition could grow up to 4.302 g/L which was 26.53 % higher than biomass under VHG condition. Viability profiles nonetheless did not show enough differences between two gravities. In both gravities, cell viability would drop significantly as ethanol accumulated in media. The cell viability under 300 g glucose/L condition was slightly

lower than 200 g glucose/L one was probably explained by different ethanol concentrations achieved in each fermentation condition.

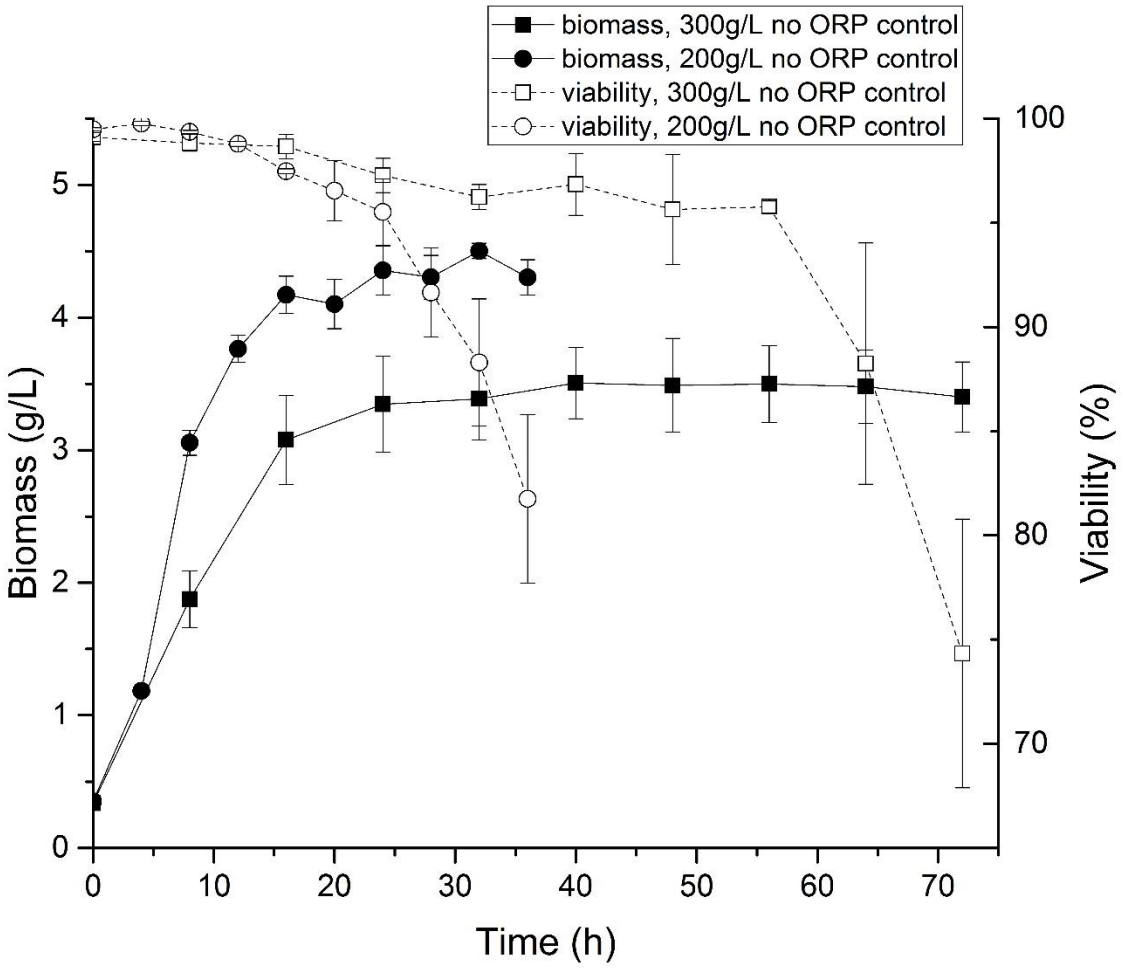

Figure 4.3 Biomass and viability profiles under different glucose concentrations without ORP control (sample points shown in this figure represented the average value and error bar represented standard deviation of three replicates which applied in all figures displayed below)

The same difference was observed in Fig 4.4 which described the comparison between 300 g glucose/L with -100 mV ORP control and 200 g glucose/L with -100 mV ORP control conditions. Under ORP control, the final biomass during 200 g glucose/L could reach as much as 7.659 g/L while higher gravity would reduce this amount by 35.33 % to 4.953 g/L. During the beginning 16

hours of fermentation which was the growth period of yeast, biomass exposed to normal gravity accumulated in a faster rate compared with the situation under VHG condition. It was believed that such variance in biomass would contribute to the disparity in ethanol productivities under different gravity conditions.

Many factors might contribute to the influence of glucose concentration observed in this study. Although ORP control helped to relieve the adverse effect brought by very high gravity in our study as was explained above, it cannot totally remove the inhibition of high glucose concentration on yeast cell growth since even under -100 mV ORP control, biomass profiles still differentiate between two gravity conditions. Previous work proved that high osmotic pressure caused an increase in intracellular ethanol concentration and thus repression on yeast proliferation (D'Amore et al., 1988). In our study, it was unlikely that intracellular ethanol induced such inhibition as it did not accumulate as much as to play an influential role inhibiting yeast growth at the early stage of fermentation. More likely it could be that high osmolarity limited the nutrients uptake which led to the inhibition on yeast growth. Besides, high osmotic stress would decrease water activity in media. Even though yeast could survive a wide range of water activity or even complete dehydration, the activity and growth rate would be undermined without a favorable water content presented in media. This might explain the difference observed in biomass and cell viability profiles under varied conditions.

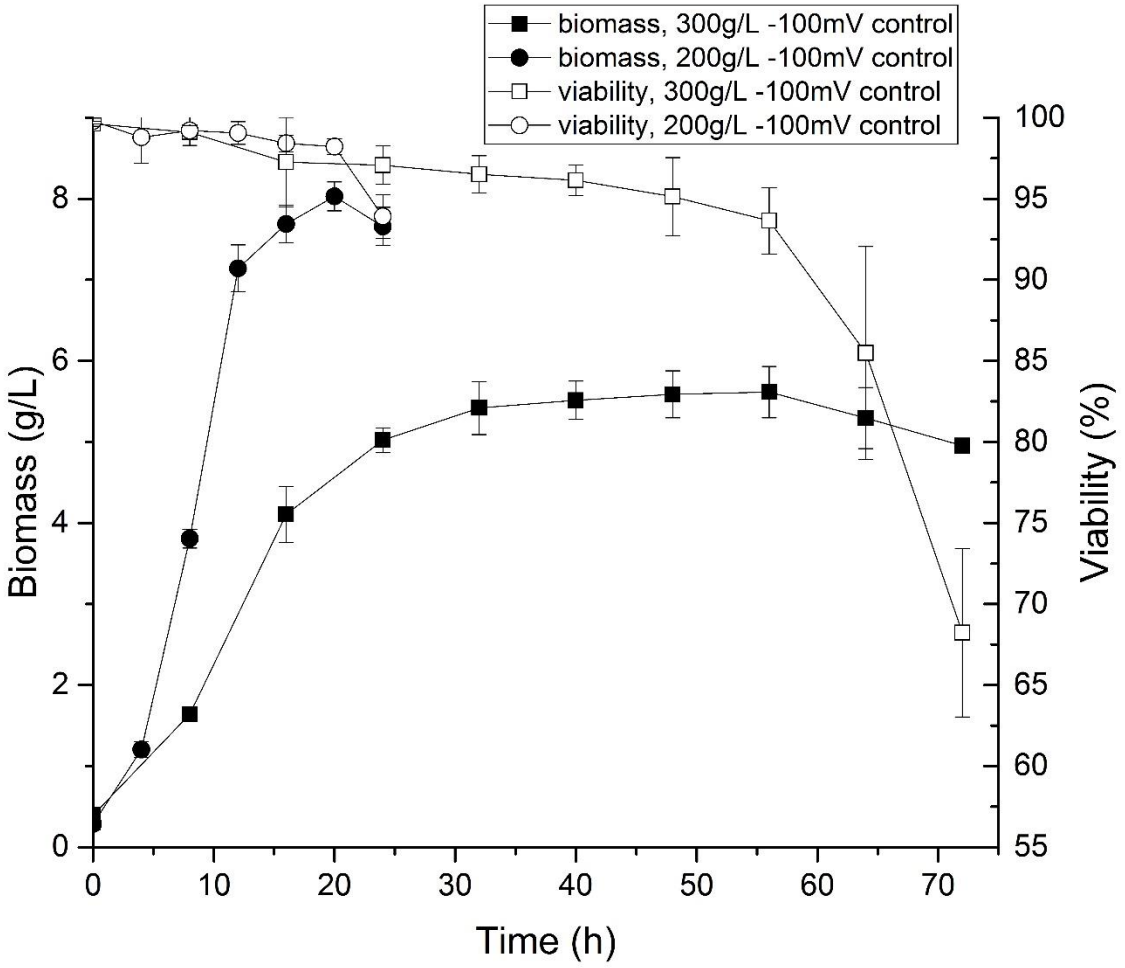

Figure 4.4 Biomass and viability profiles under different glucose concentrations with ORP controlled at -100 mV level

4.2.2 The influence of redox potential control on yeast biomass and viability

Fig 4.5 and Fig 4.6 displayed the positive effect of ORP control during fermentation of different glucose concentration. It could be observed in Fig 4.5 that compared with no ORP control group, biomass tended to grow faster and accumulate more when exposed to ORP control at -100 mV level. The final biomass in 200 g/L glucose with -100 mV ORP control was 7.659 g/L which was 78.01 % higher than no ORP control group. During 200 g glucose/L fermentation without ORP

control, cell viability would drop quickly at the last phase of fermentation as toxic effect of ethanol exacerbated as its concentration increase. This problem was not observed in -100 mV ORP control group. The cell viability maintained at a robust level over 90 % till the end of fermentation. All these differences combined would render the ORP control fermentation at 200 g glucose/L faster and more productive without significant loss on yield and final ethanol titer.

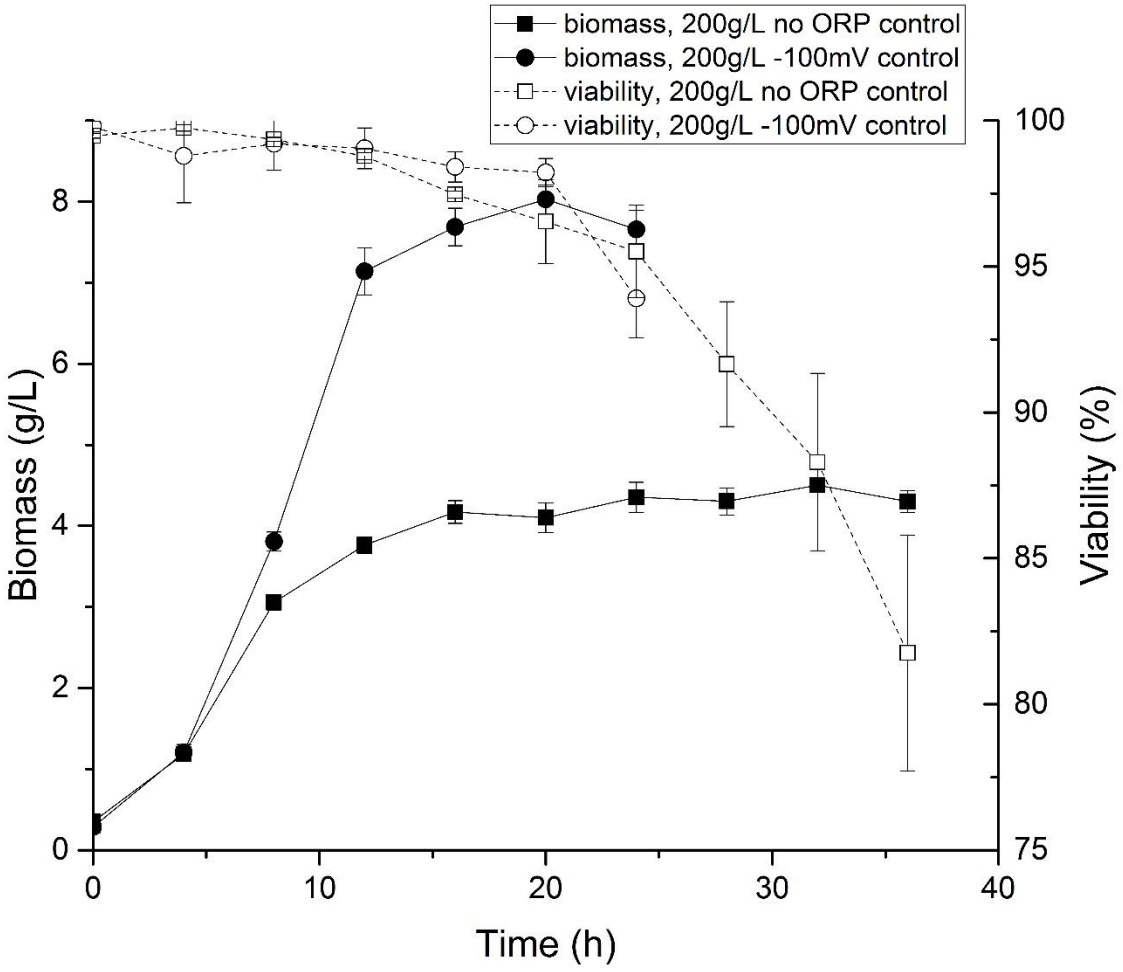

Figure 4.5 Biomass and viability profiles of 200 g/L glucose fermentation with or without ORP control

In 300 g glucose/l fermentation, two different ORP control level were adopted which made it possible to compare not only the effect of the presence of ORP control during VHG fermentation

but the difference between two ORP control levels. Fig 4.6 showed that the presence of ORP control had similar influence on biomass growth in VHG condition. Final biomass concentration with ORP control was higher than no ORP control group. There was no significant difference in final biomass between two ORP control level, but average growth rate of -100 mV ORP control group was higher compared with -150 mV ORP control group in the early phase of fermentation. This could be explained by the observation that ORP control at -100 mV started 15 hours earlier than -150 mV which was recorded in Fig 4.2. It can be seen that after -100 mV ORP control was triggered around 8 hours after inoculation, biomass profile started to distinguish from no ORP control group at next sample point which was 16 hours after inoculation. Since -150 mV ORP control hadn't started until 20 hours after inoculation, biomass profile of -150 mV ORP control group didn't distinguish from on ORP control group during first 3 sample points. It was only after the ORP control started at the 20^{th} hour that biomass profile in -150 mV ORP control group started to differentiate from no ORP control group. Such comparison revealed that ORP control could directly improve yeast proliferation under varied gravity conditions but this improvement couldn't last indefinitely after exceeding certain ORP level. Despite of the fact we observed in biomass profile in 300 g glucose/L fermentation, cell viability summarized in Fig 4.7 showed no significant difference among three ORP conditions. This inconsistency between biomass and cell viability indicated that while ORP control was beneficial for yeast growth at early phase of fermentation, it couldn't really enhance yeast tolerance to ethanol as cell viability decreased when ethanol accumulated in all three groups.

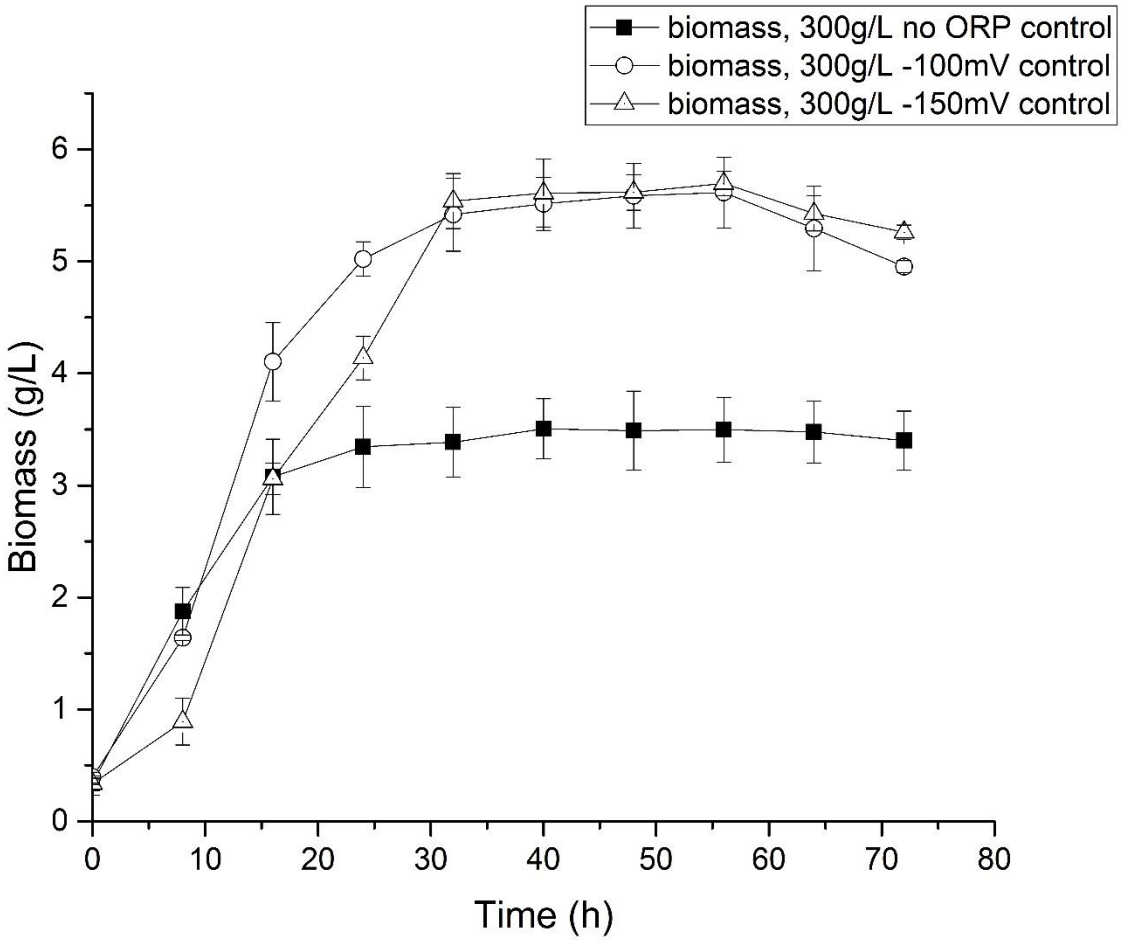

Figure 4.6 Biomass profiles of 300 g/L glucose fermentation without ORP control, with -100 mV ORP control and -150 mV ORP control

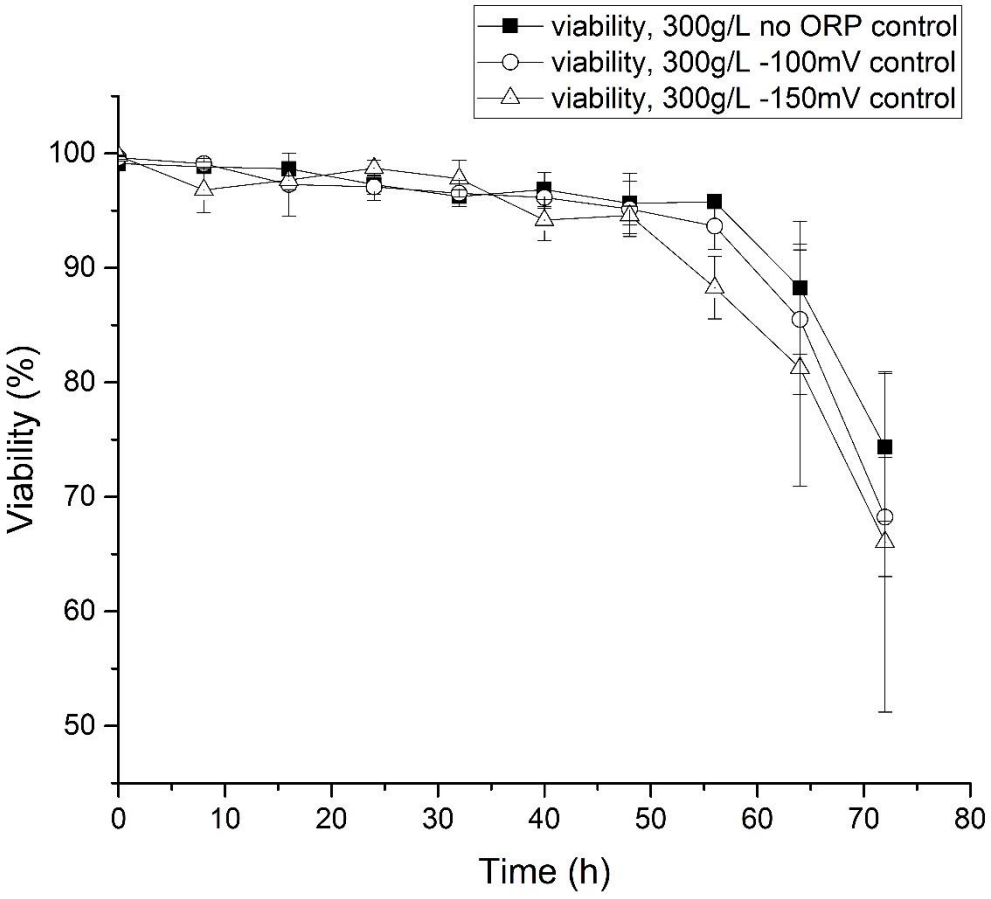

Figure 4.7 Cell viability profile of 300 g/L glucose fermentation without ORP control, with -100 mV ORP control and -150 mV ORP control

4.3 Metabolic Profiles under Varied Fermentation Conditions

Table 4.1 listed information of some metabolites during fermentation under different conditions. From the table we could figure out that ORP control was beneficial in improving fermentation productivity. In 200 g glucose/L fermentation, the productivity of -100 mV ORP control fermentation was 27.24 % higher than no ORP control situation. Similar trend was observed in 300 g glucose/L fermentation as well. Although lower than normal gravity condition, the productivity of -150 mV ORP control fermentation was 42.26 % higher than no ORP control fermentation. The productivity could be slightly reduced by 11.12 % under -100 mV ORP control condition compared with -150 mV condition.

Table 4.1 Summary of metabolic profiles under different fermentation conditions

ORP (mV)	**Time (h)**	**Glucose (g/L)**		**Ethanol (g/L)**		**Yield (g/g)**	**Ethanol Production rate (g/Lh)**
		Initial	**Residue**	**Initial**	**Final**		
300 g glucose/l							
No control	72	288.96±13.69	32.64±9.35	2.02±1.32	116.05±3.73	0.445	2.770
-150mV	72	288.18±7.85	3.51±4.96	1.44±0.76	123.38±1.53	0.439	3.941
-100mV	72	308.49±3.27	20.06±5.63	3.53±0.28	135.21±1.80	0.456	3.503
200 g glucose/l							
No control	36	199.72±3.90	0.32±0.55	2.28±0.72	94.95±1.33	0.465	4.343
-100mV	24	206.87±2.60	0.04±0.07	2.06±0.93	92.78±1.37	0.437	5.526

The trend for ethanol yield was not clearly expressed in this study. It was shown in the table that ORP control might slightly undermine the ethanol yield compared with no ORP control situations. Nonetheless the yield of -100 mV ORP control 300 g glucose/L fermentation was the highest among all three conditions in 300 g glucose/L.

4.3.1 The influence of media's gravity on metabolic profiles of fermentation

Fig 4.8 showed ethanol and glycerol concentrations under two gravities with or without ORP control. It can be seen on (a) and (c) of Fig 4.8 that ethanol accumulated faster at lower gravity

condition when ORP condition maintained the same. Although more ethanol was produced during the fermentation of 300 g/L glucose, such high final titer was achieved on the sacrifice of process productivity. This trend corresponded with the average ethanol productivity data listed in Table 4.1. Another perspective worth noticing was the glycerol profiles between two gravities. Albeit that terminal glycerol concentration in 300 g glucose/l fermentation was much greater than 200g/L condition, it should be mentioned that there was no significant difference between the glycerol production rates. Based on the hypothesis of Noti et al. (Noti, Vaudano, Pessione, & Garcia-Moruno, 2015), it might be the result of re-balance between intracellular and extracellular glycerol concentration. According to their explanation, when exposed to high-gravity induced hyperosmotic pressure, some yeast strains were capable of retaining intracellular glycerol to antagonize the adverse stresses from the harsh environment. Later during fermentation, intracellular glycerol would be released to the media as ethanol hadn't accumulated yet and osmotic stress ameliorated. This might help partially illustrate the similar glycerol productivities but quite diverge final concentrations during the fermentation of two glucose concentrations.

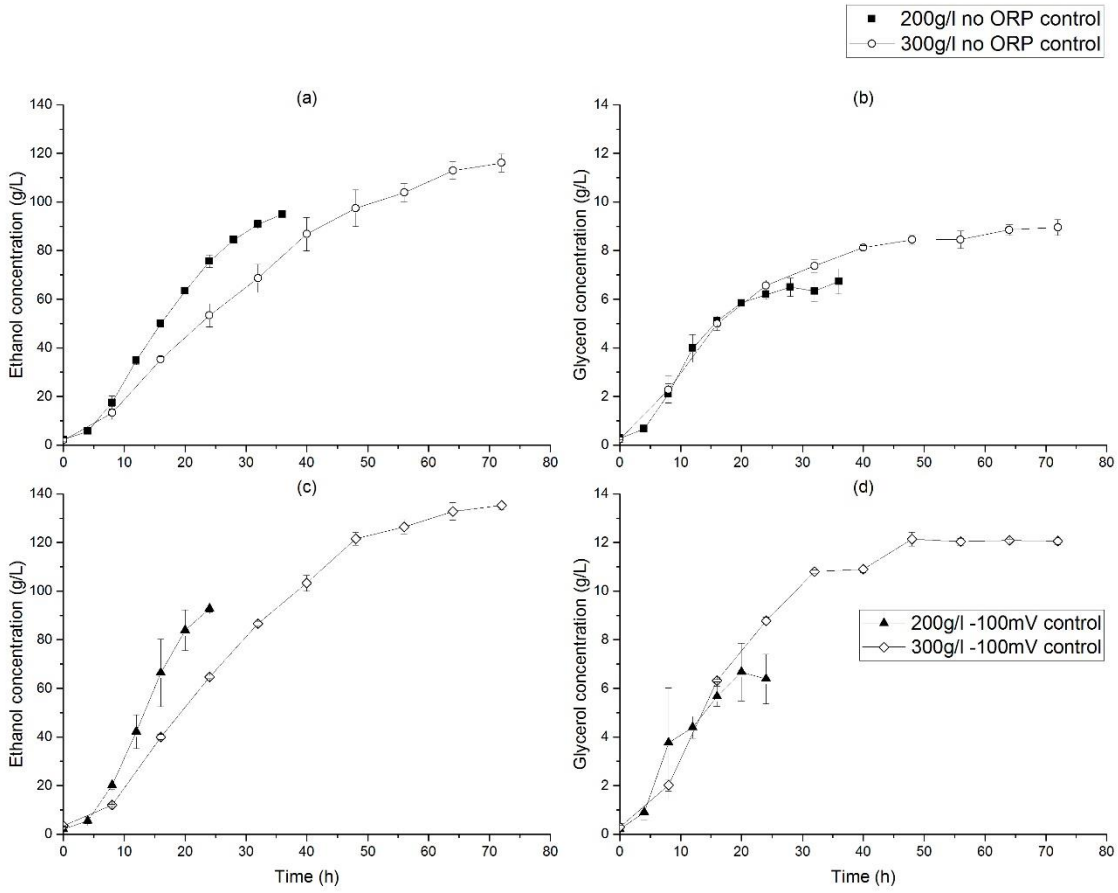

Figure 4.8 Comparisons of Glycerol and Ethanol concentration on varied conditions: (a) Ethanol concentrations of two gravities without ORP control (b) Glycerol concentrations of two gravities without ORP control (c) Ethanol concentrations of two gravities with -100 mV ORP control (d) Glycerol concentrations of two gravities with -100 mV ORP control

4.3.2 The influence of redox potential control on metabolic profiles of fermentation

Analysis of metabolic profiles with different ORP control levels in 300 g glucose/l fermentations showed that both glucose consumption rate and ethanol productivity was increase corresponding to the increase of ORP control level. Another interesting finding was that glycerol concentration tended to increase to a higher level in -100 mV ORP control fermentation of 300 g glucose/l while it remained similar between -150 mV ORP control and no ORP control conditions. This might indicate that excessive air sparged into the media could accelerate the synthesis of glycerol. However, such excessive did not enhance ethanol tolerance significantly in this study. It was more likely that synthesis of glycerol was a tool for yeast to utilize excessive oxidizing force and maintain its intracellular redox balance. Thus it should be cautious when ORP control level was set too high since this could diverge carbon source more into by-product instead of ethanol.

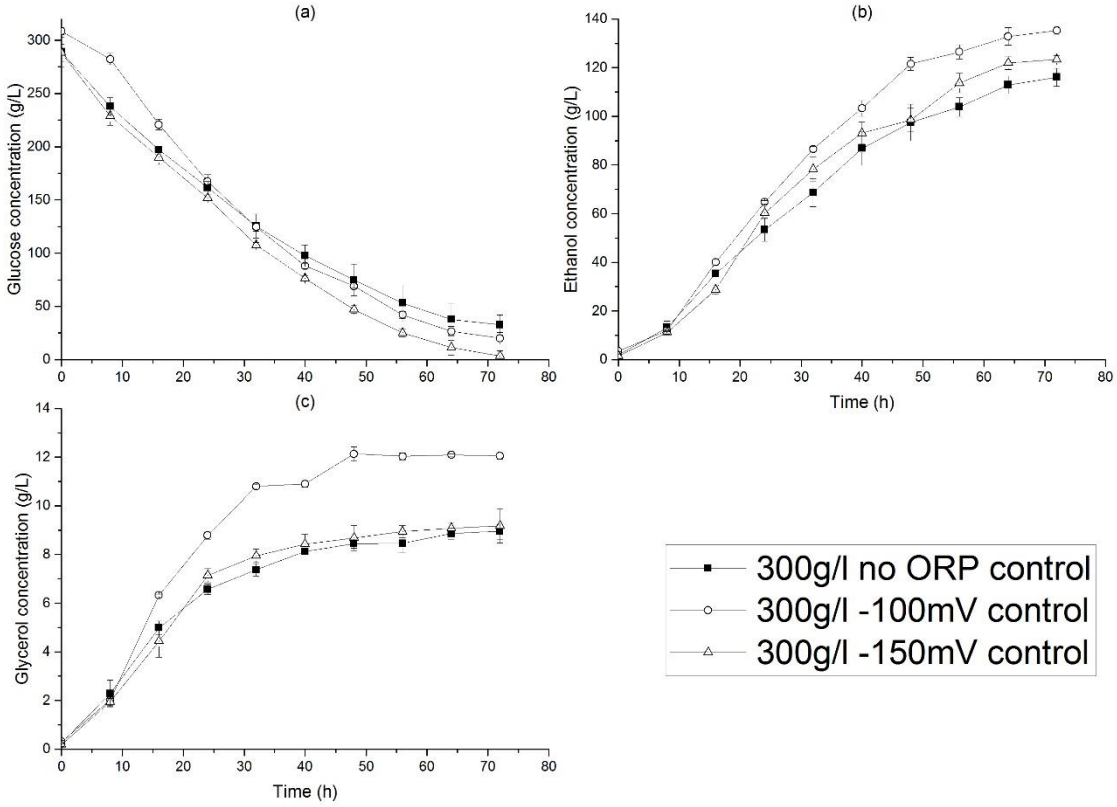

Figure 4.9 Comparison of glucose, ethanol and glycerol concentration in 300 g/L fermentation with no ORP control, -100 mV ORP control and -150 mV ORP control (a) glucose concentration profiles under three conditions (b) ethanol concentration profiles under three conditions (c) glycerol concentration profiles under three conditions

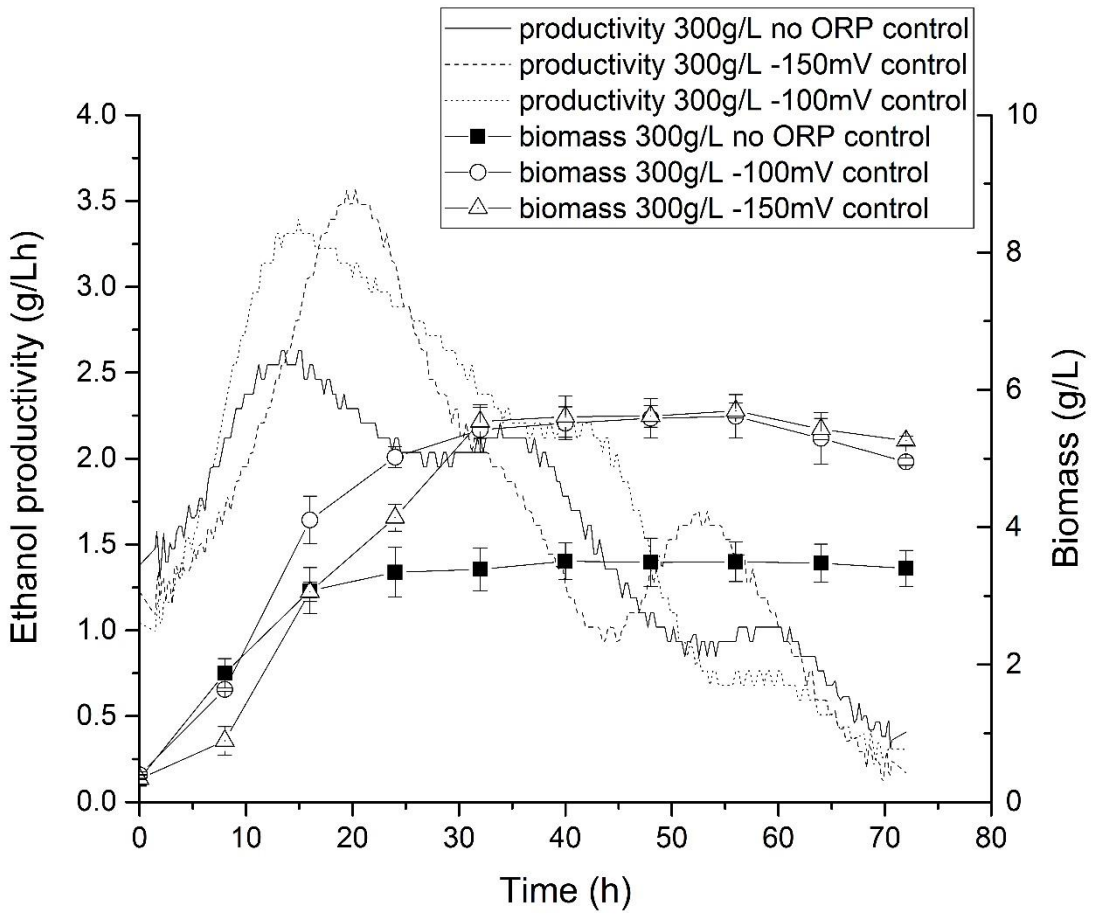

Figure 4.10 Productivity and biomass profiles of 300g/L glucose fermentation under varied ORP control conditions

The most susceptible parameter to ORP control was productivity in this study. From Fig 4.10 it can be observed that albeit started at similar position, productivity in ORP control groups tended to increase rapidly and maintain a vigorous production between 10 to 36 hours during fermentations. Combined with the analysis of biomass could reveal that this highly vigorous happened during the vigorous growth of yeast cells in media. It was likely that the higher productivity in ORP control fermentation was a result of prolonged active growth of microorganism during fermentation. Later in fermentations, although biomass concentration was still high enough, productivity would decrease as the activity of yeast cells were inhibited due to

severe stress brought by high ethanol concentration. Not quite consistent as we expected, ORP control could not provide yeast cells with improved tolerance to high ethanol concentration. It mainly improved fermentation productivity at early phase which corresponded to the rapid proliferation of yeast. Thus it was more reasonable to propose that ORP control could enhance the average fermentation productivity through improving yeast biomass concentration and maintain better activity during yeast growth phase. It was discovered from Fig 4.10 that from 0 to 48 hours was the most productive period for ethanol production with productivity remained above 1 g/Lh. The difference in productivity among different ORP control conditions occur in this period as well. Thus improving ethanol productivity during this period was critical in enhancing overall ethanol productivity in VHG fermentation.

4.4 The Alteration of Fatty Acid Composition on Yeast Plasma Membrane

4.4.1 The influence of media's gravity on membrane fatty acid composition

To examine the alteration of fatty acid composition on yeast plasma membrane, besides looking into each fatty acid component in detail, a saturation index was established to describe the tendency for saturation in overall fatty acid composition. The saturation index was defined in the equation (2) shown as below:

$$SI = \frac{FA_{C16:0} + FA_{C18:0}}{FA_{C16:1} + FA_{C18:1}} \quad [4.1]$$

Fig 4.11 presented the alteration of fatty acid composition under different starting glucose concentration without ORP control. It showed that without ORP regulation, yeast plasma membrane fatty acids in general tended to evolve more into saturated forms such as palmitic and stearic acids while unsaturated fatty acids' content decreased as fermentation went on. Palmitic acid content increased rapidly at the early stage of fermentation and maintained dominant share of total fatty acid composition throughout whole fermentation process. Fig 4.11 showed that palmitic acid could take up to 50 % of total fatty acid content on yeast plasma membrane. Stearic acid accumulated gradually during the fermentation and reached its highest share at the end. The content of palmitoleic acid on the other hand decreased at early stage of fermentation and maintained at the lowest level of shares among all four components. Meanwhile oleic acid concentration percentage also decreased but in a more smoothly trend. It should be mentioned that higher glucose concentration tended to induce higher ethanol concentration. In this study, the final ethanol concentration under 300 g glucose/l condition was 23.97% higher than 200 g glucose/L condition. Such difference offered a chance to observe the detrimental effect This observation corresponded with some researches including result reported by Dinh et al. (Thai Nho Dinh et al.,

2008) which indicated that palmitic acid content would decrease while oleic acid content remained constant during adaptive cultivation of yeast. However, this observation at the same time contradicted with the result discovered by Chen et al. (Chen & Xu, 2014) which reported that oleic acid content played an influential role in enhancing yeast ethanol tolerance during adaptive evolution process. All in all, the ratio between stearic acid and oleic acid might best reflect the influence of ethanol inhibition while the alteration in palmitic and palmitoleic acid content correlated more with the yeast adaption to osmotic stress and activity during exponential growth phase.

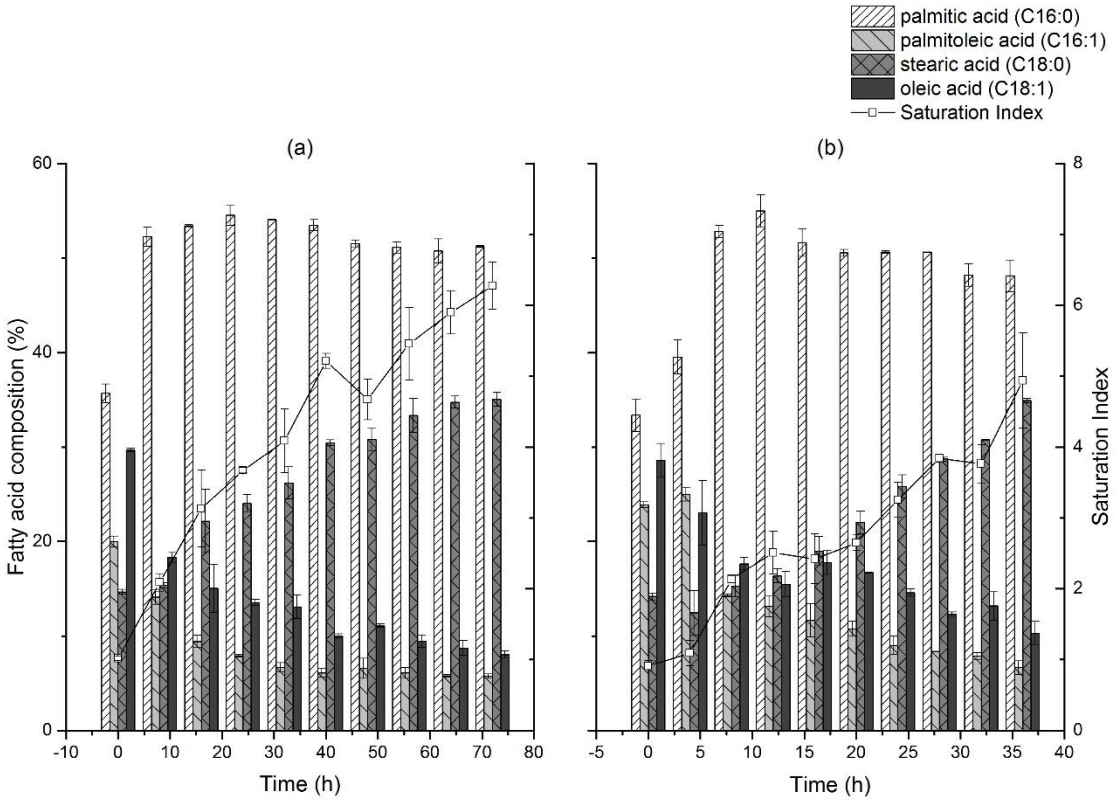

Figure 4.11 Fatty acid compositions of different glucose concentrations. (a) Fatty acid composition alteration during 300 g/L glucose fermentation; (b) Fatty acid composition alteration during 200 g/L glucose fermentation.

Fig 4.12 compared the trend of saturation index change under different glucose concentration. It should be noticed that there was no significant difference on trend of saturation index increase between two glucose concentrations till the end of 200 g glucose/L fermentation. The saturation index of 300 g glucose/L fermentation was higher than that under 200 g/L condition because the trend of increase continue under 300 g/L condition for longer period due to the difference in fermentation durations.

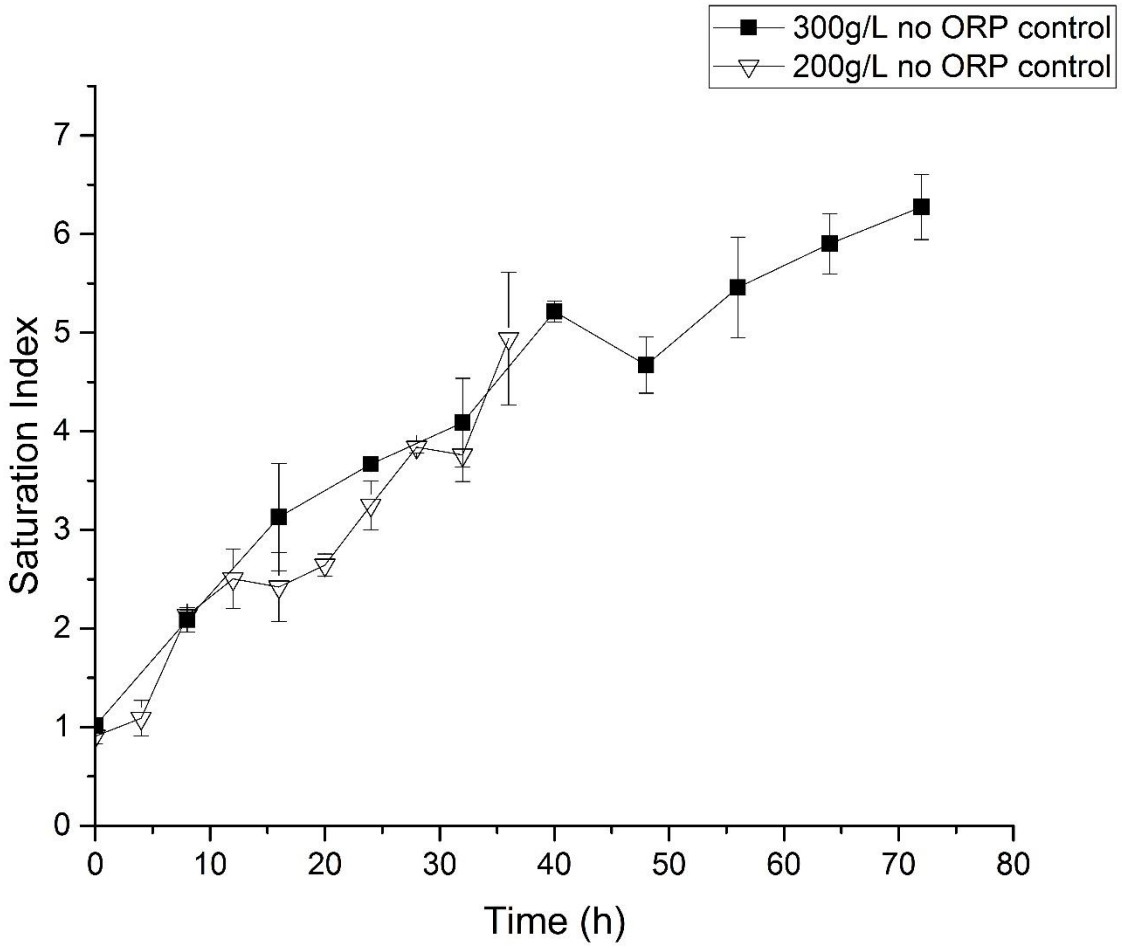

Figure 4.12 comparison between different concentrations without ORP control

In comparison of -100 mV ORP control fermentation of two glucose concentration, it was discovered that before ORP regulation was imposed both saturation indexes increase at a similar rate. After ORP regulation was imposed, saturation index would decrease instantly. It should be noticed that in spite of similar alteration trend at both gravities, saturation index of 300 g glucose/L maintained a higher value compared with 200 g/L situation. As fermentation proceeded, the saturation index of 300 g glucose/L would finally decrease to the same level as 200 g/L condition as was shown in Fig 4.13.

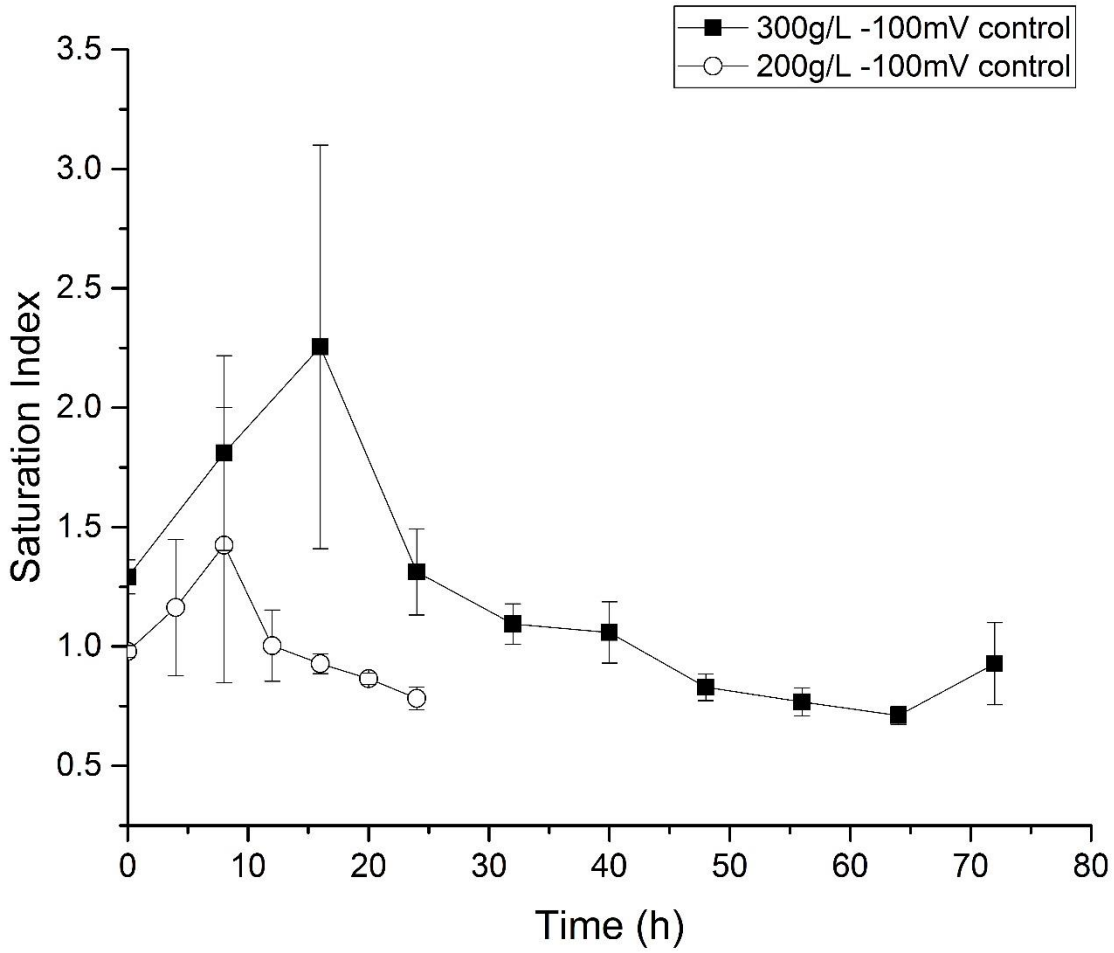

Figure 4.13 Saturation index during -100 mV ORP control fermentation of different glucose concentration

4.4.2 The influence of redox potential control on membrane fatty acid composition

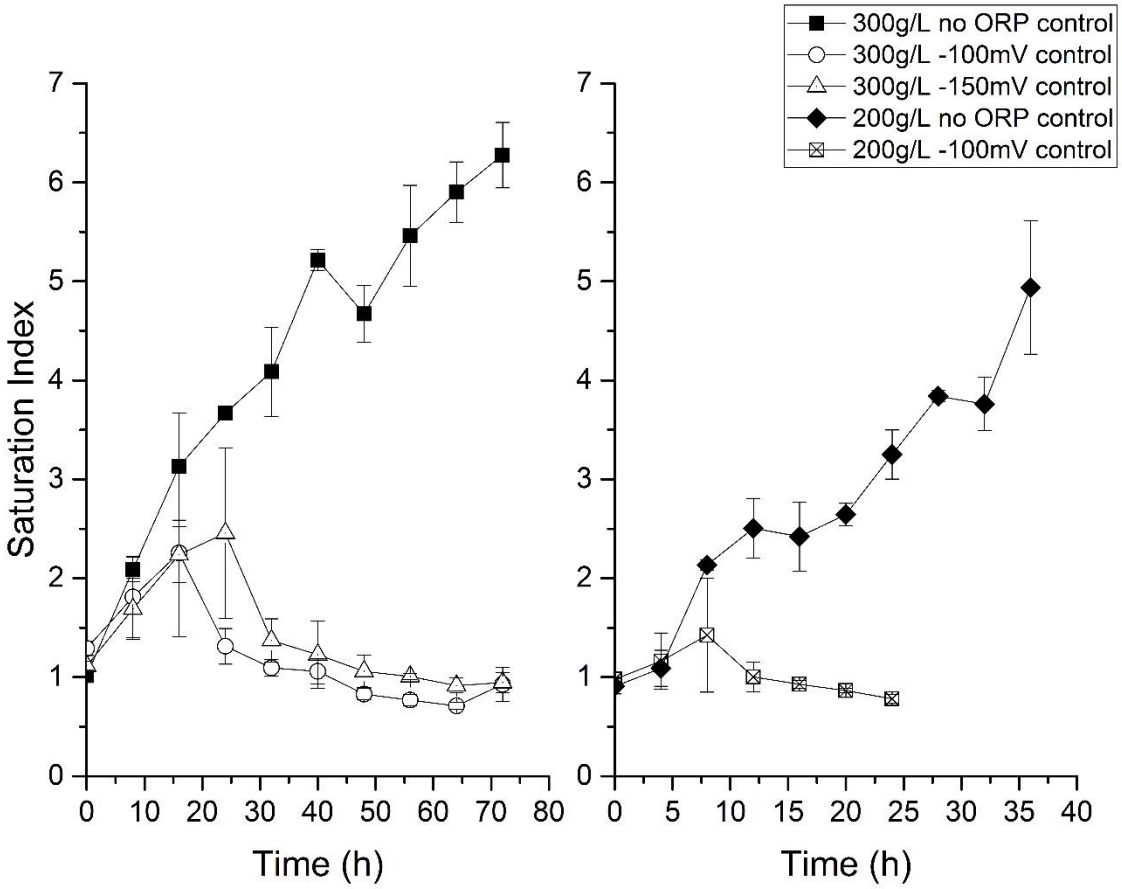

Figure 4.14 Saturation index comparison between with and without ORP control at different glucose concentration

Fig 4.14 illustrated clearly the alteration brought by ORP control on yeast plasma membrane fatty acid composition. It can be observed that when ORP control started, saturation index would decrease instantly under both glucose concentrations. It was worth noticing that the decrease was most significant during first 4 or 8 hours after sterilized air was sparged to regulate ORP level, albeit saturation index continued the decrease throughout the whole fermentation. It was likely that the availability of dissolved oxygen brought by ORP control helped induce such change in membrane fatty acid composition. The synthesis of unsaturated fatty acid, as it was illustrated in

Fig 2.3, required the involvement of oxygen which could be very scarce because it was the primary acceptor of electrons and was easily consumed during micro- or anaerobic fermentation. Another possible and more direct beneficial factor brought by ORP control was to regulate the balance of reducing/oxidizing power in media. In bioethanol fermentations this meant to restore the extracellular redox from over-reducing environment. Since both NAD^+/NADH and NADP/NADPH redox cofactors involved in the synthesis of membrane fatty acids, it was reasonable to raise the hypothesis that the ORP control could adjust intracellular redox balance through manipulating extracellular redox value and thus influence the fatty acid synthesis pathway.

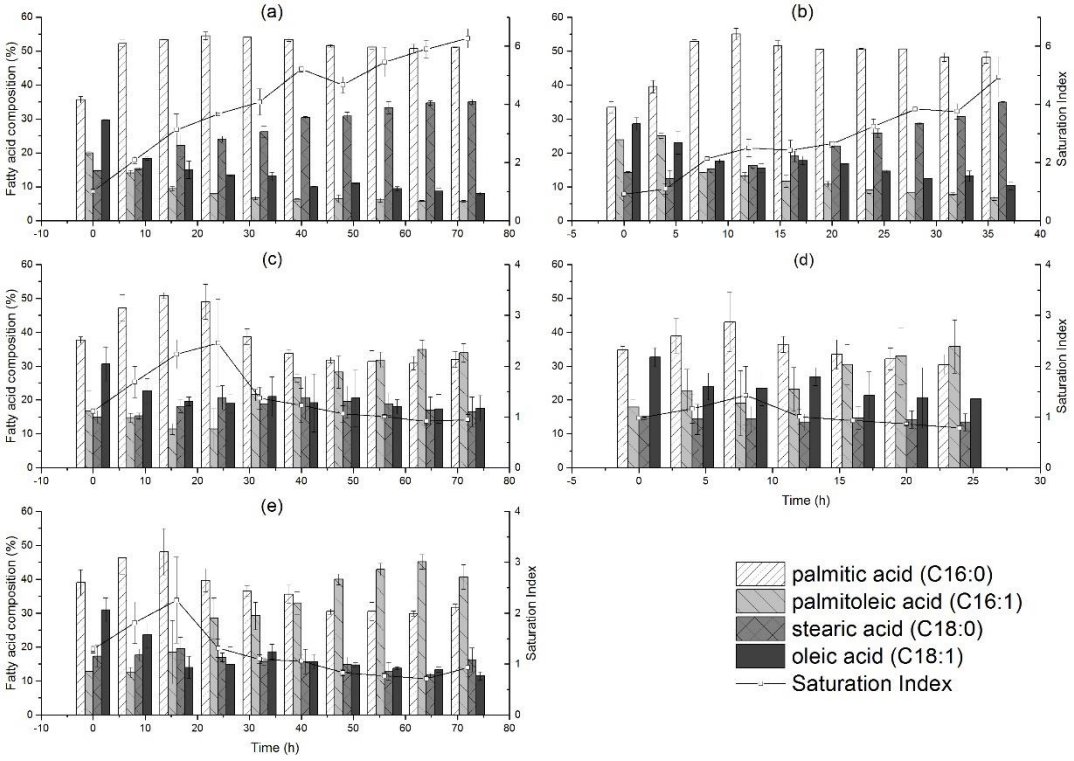

Figure 4.15 Plasma fatty acid profiles under 5 different fermentation conditions (a) 300 g/L glucose no ORP control (b) 200 g/L glucose no ORP control (c) 300 g/L glucose with -150 mV ORP control (d) 200 g/L glucose with -100 mV ORP control (e) 300 g/L glucose with -100 mV ORP control

Besides a general grasp of saturation index, a more detail study of each fatty acid component was necessary. As it was illustrated in Fig 4.15, palmitoleic acid accumulated more in yeast plasma membrane when ORP control was implemented on 300 g glucose/L fermentation while relatively palmitic acid content decreased. Different ORP control level also imposed certain influence on such change. Palmitoleic acid content in -150 mV control group increased by almost 6 times as much as it was in no ORP control group. This increase was further enhanced to 8-fold in comparison between -100 mV control group and no ORP control one. Oleic acid content doubled in -150 mV control group when compared with no ORP control group. The same trend was also observed in -100 mV control group. It was worth noticing that after ORP control, fatty acid composition on yeast plasma membrane was totally altered. The dominant share of palmitic acid and stearic acid was replaced by palmitoleic acid and oleic acid. Such observation matched the presumption we made based on the literature review we had done in Section 2. Another discovery was that palmitoleic acid content was more dominant during -100 mV controlled fermentation compared with -150 mV one. This indicated that palmitic and palmitoleic acid might be more sensitive to the change dissolved oxygen in media since they reacted more adaptively during ORP control condition. Plot (b) and (d) in Fig 4.15 showed the effect of ORP control in 200 g glucose/L fermentation. It should be noticed that when ORP control was implemented on 200 g glucose/L case, oleic acid tended to accumulate more in plasma membrane compared with -100 mV ORP control group under 300 g glucose/L condition.

5 Conclusions

In this study, a comprehensive analysis of the alteration in yeast plasma membrane fatty acid composition during ORP controlled bioethanol fermentation was performed. The analysis of membrane fatty acid composition combined with other analysis including: ORP trends during fermentation, growth profiles and profiles of major metabolites provided us a profound knowledge of the influence of ORP control strategy on yeast physiology during VHG fermentation. The comparison of fatty acid composition between two gravities revealed the effect imposed by VHG condition as well as yeast cells' adaption to these environmental stresses. The comparison of fatty acid composition among different ORP control levels illustrated the influence of ORP control strategy on regulating yeast membrane composition and function to facilitate yeast to overcome harsh survival environment while maintain their proper productivity in VHG fermentation.

In addition to previous researches regarding adaptive behaviors of yeast to ethanol inhibition, osmotic shock and dissolved oxygen depletion, we innovatively combined these studies and transfer them from flask to tank. Besides, we tried to trace the alteration of several parameters ranging from metabolic profiles to physiological constitutions. Such way of thinking offered us a more complete insight into what exactly happened during VHG fermentation in tank.

By comparing data we acquired at two gravities with or without ORP control, it can be concluded that high-glucose induced osmotic stress did impact yeast cells' growth. Both biomass accumulation rate and biomass concentration was lower under 300 g glucose/L condition compared with 200 g glucose/L. ORP control could partially relieve such repression by improving biomass concentration but not totally de-bottleneck this limitation since a difference in growth rate was observed even with the presence of ORP control. Correspondingly fatty acid composition on yeast plasma membrane tended to switch to more saturated contents like palmitic acid and stearic

acid. It was very likely the depletion of dissolved oxygen and intracellular imbalance of $NADP^+$/NADPH and NAD^+/NADH pairs resulted in the inability of desaturation of fatty acid, thus the increase in saturated fatty acid components. Based on previously established studies claiming that over-saturated fatty acid tended to become more rigid in structure and lost partial fluidity, it was reasonable to assume same issue occurred to the yeast cell in this study. Although whether more or less fluidity was beneficial to yeast under intense environmental stresses was not quite conclusive, it turned out that a saturated membrane fatty acid composition induced by VHG condition hold certain disadvantages over a more balanced fatty acid composition in our study. The saturated membrane fatty acid mainly undermined yeast proliferation function during the critical growth phase. We presumed it was the rigidity in saturated membrane that hampered flow and uptake of necessary nutrients across membrane which limited the intracellular availability of these nutrients for a robust growth of yeast in growth phase.

By comparing data we acquired at different ORP control level between two gravities, we could reach the conclusion that ORP control strategy mainly improved fermentation productivity. Such improvement was achieved by maintaining yeast's vigorous growth and activity during fermentation as well as prolonging growth phase. By controlling ORP level of the media, it could help restore the over-reducing fermentation environment (indicated by very low ORP value) to a relatively less reducing situation. Through this way fermentation productivity could be kept at a relatively high level for a longer period during fermentation. However, ORP control could not enhance yeast's tolerance to ethanol toxicity since productivities were observed to drop rapidly at late stage of fermentation despite of the presence of ORP control strategy. There was no significant difference in productivity observed after 50 hours among three ORP conditions in 300 g glucose/L fermentation. ORP control in addition could influence the membrane fatty acid composition

significantly. Fatty acid composition in yeast plasma membrane tended to restore to a balanced saturation index between 0.7 to 1, meaning that saturated and unsaturated fatty acids distributed more evenly in total fatty acid composition. It was observed that such restoration occurred instantly after the ORP control started. Thus it was likely that the rebalance between saturated and unsaturated fatty acids was beneficial in maintaining yeast proper function and active growth when exposed to high osmotic stress. It was reasonable to assume that regulating ORP level in media helped yeast cell reorganize its plasma membrane to facilitate its survival and proliferation in VHG condition. A better growth activity resulted from reorganization in plasma membrane fatty acid composition provided yeast with an enhanced productivity during VHG fermentation compared with unregulated condition.

It should also be noted that more glycerol was synthesized in VHG condition compared with normal gravity condition. Such trend could be further enhanced when ORP control existed during fermentation. Instead of rapid accumulation responding to osmotic stress, glycerol inclined to increase its concentration in a continuous pattern throughout whole fermentation process. This indicated that it was more likely that synthesis of glycerol was a tool yeast employed to rebalance its intracellular redox metabolism or a protectant yeast used to antagonize the adverse effect brought by ethanol.

6 Recommendations and Future Work

Based on the analysis we had done in this study, there were several recommendations and potential expansion of current work I would like to mention in the last section of my thesis.

Most recent studies reported fatty acids or derived fatty alcohols produced from yeast as prospective biodiesel solutions with some metabolic manipulation researches needed. Such idea was very likely to be achieved on some typical yeast strains defined as oleaginous yeasts. Since it was observed that ORP control was able to manipulate the fatty acid composition on yeast plasma membrane and oleic contents were much more preferred in biodiesel production, it might be possible to apply such control in oleaginous yeast cultivation to achieve optimal lipid composition. Another interesting perspective was to determine whether adjusting ORP was able to improve the synthesis of fatty acids or even other forms of lipids in yeast. It was already proved in this study that ORP control was beneficial in increasing biomass concentration in media. If combined with the fact that lipid synthesis could also be enhanced by ORP control strategy, it could really shed light on the potential application of this technology on biodiesel production.

Although fatty acids were critical as well as oxygen dependent components on yeast plasma membrane, it was still unreasonable to rule out other potential targets in yeast cells could possibly be affected by ORP regulation. It should be recognized that even metabolic network in yeast, which was probably the simplest eukaryote in world, was still complicated that a single external perturbation could possible influence multiple targets simultaneously. Hence such correlation between fatty acid composition and ORP regulation still need systematically examination and causal connection in theory. If possible, genetic study tracing specific fatty acid synthesis genes using real-time PCR technique or carbon or oxygen metabolic flux analysis with the help of novel isotope technique could applied to solve this problem.

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Appendix

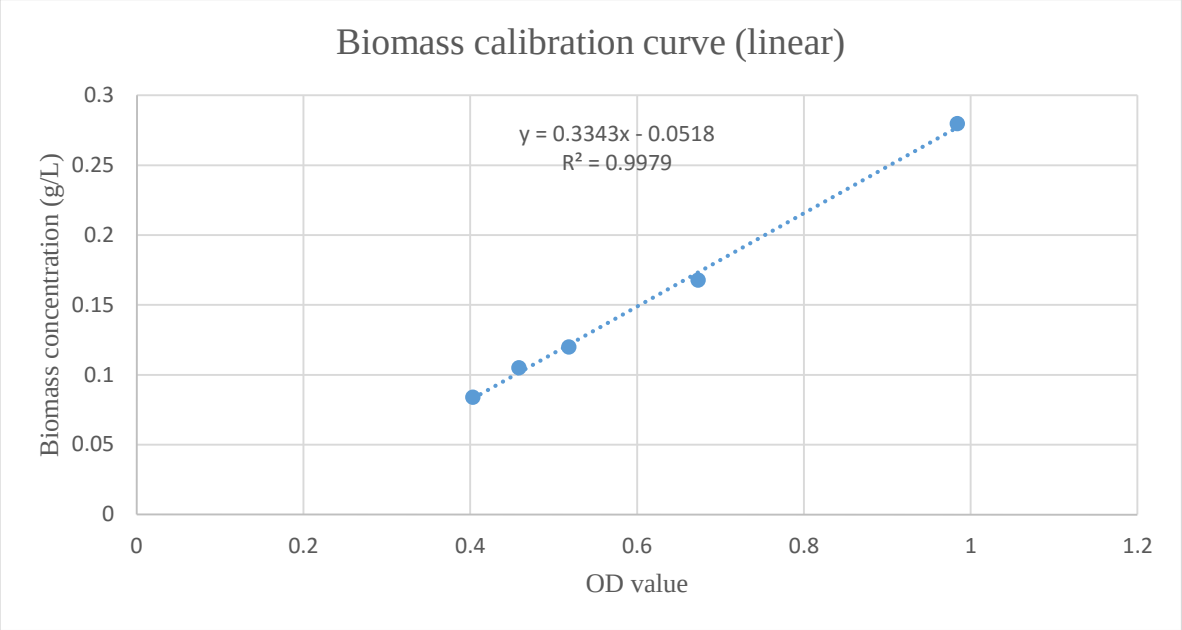

Figure A.1 Calibration table for biomass

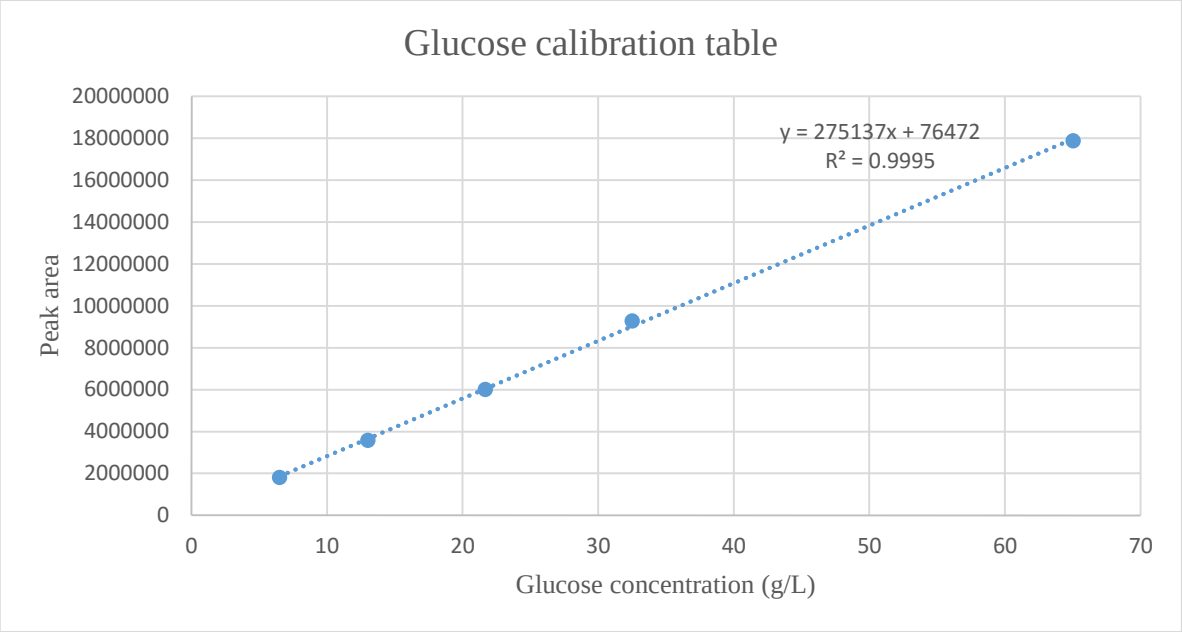

Figure A.2 HPLC calibration table for glucose

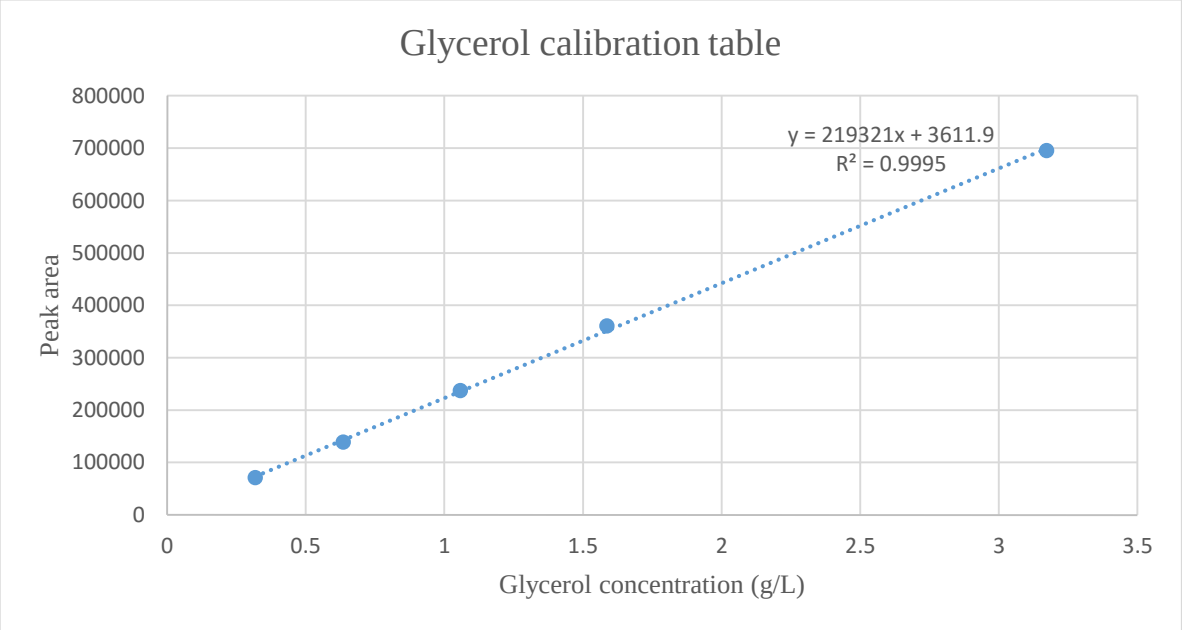

Figure A.3 HPLC calibration table for glycerol

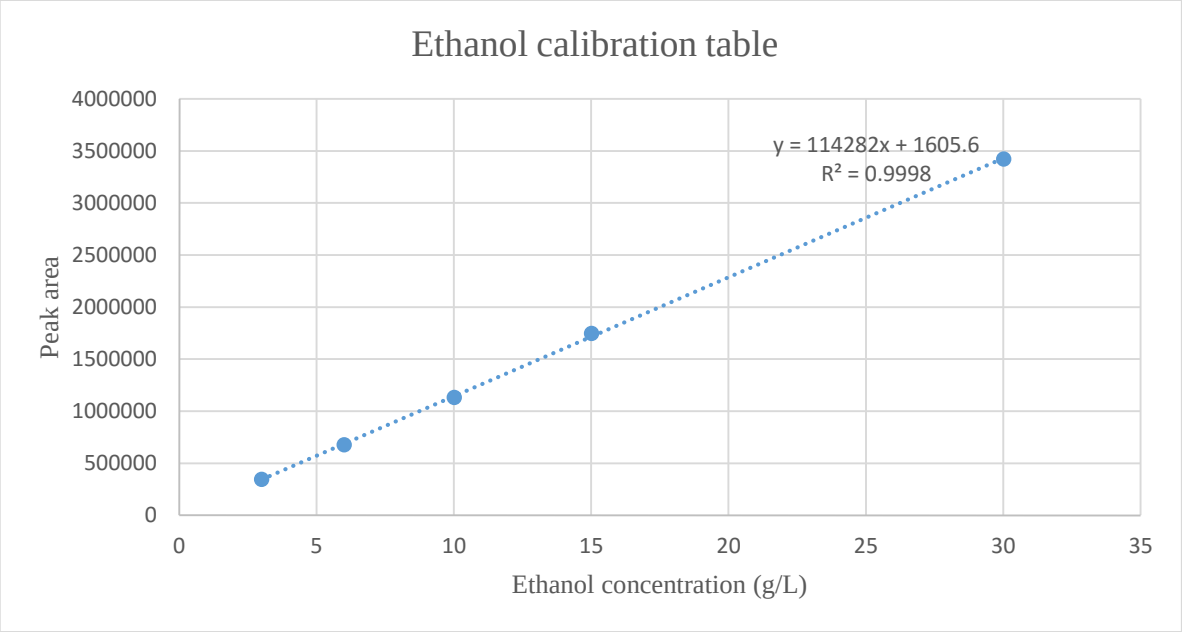

Figure A.4 HPLC calibration table for ethanol

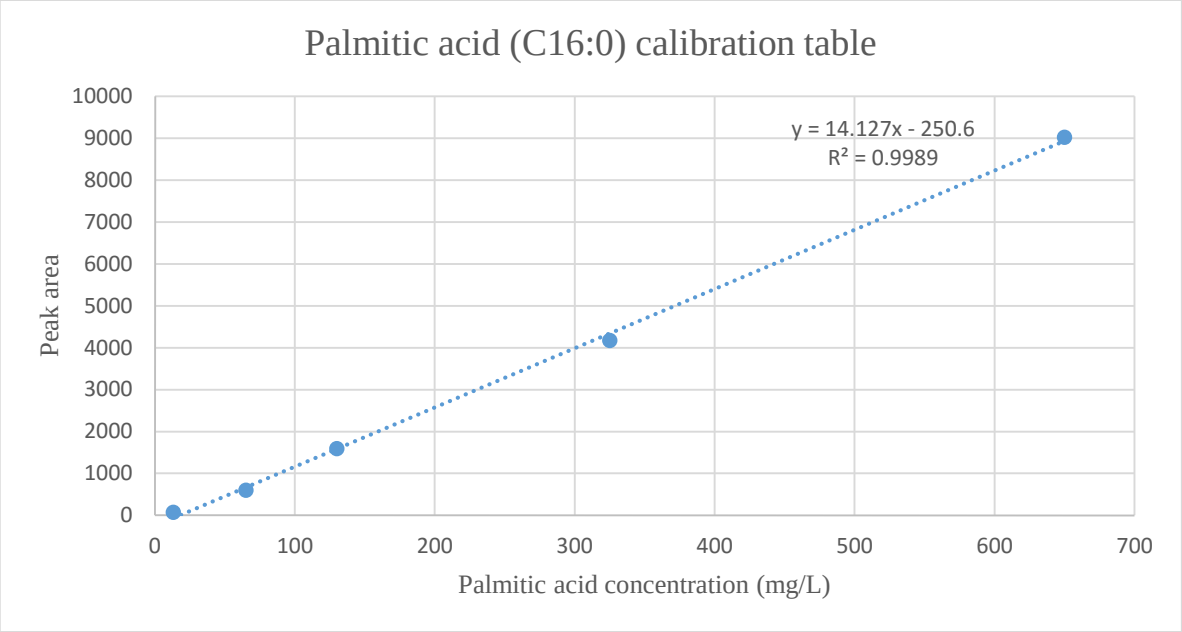

Figure A.5 GC calibration table for palmitic acid

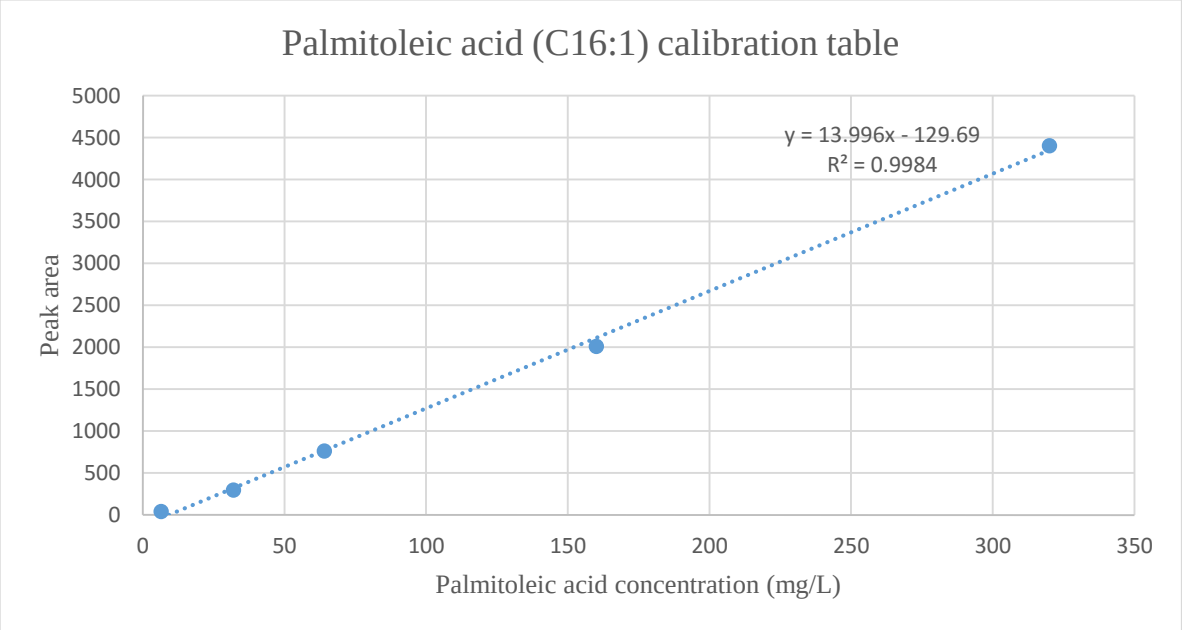

Figure A.6 GC calibration table for palmitoleic acid

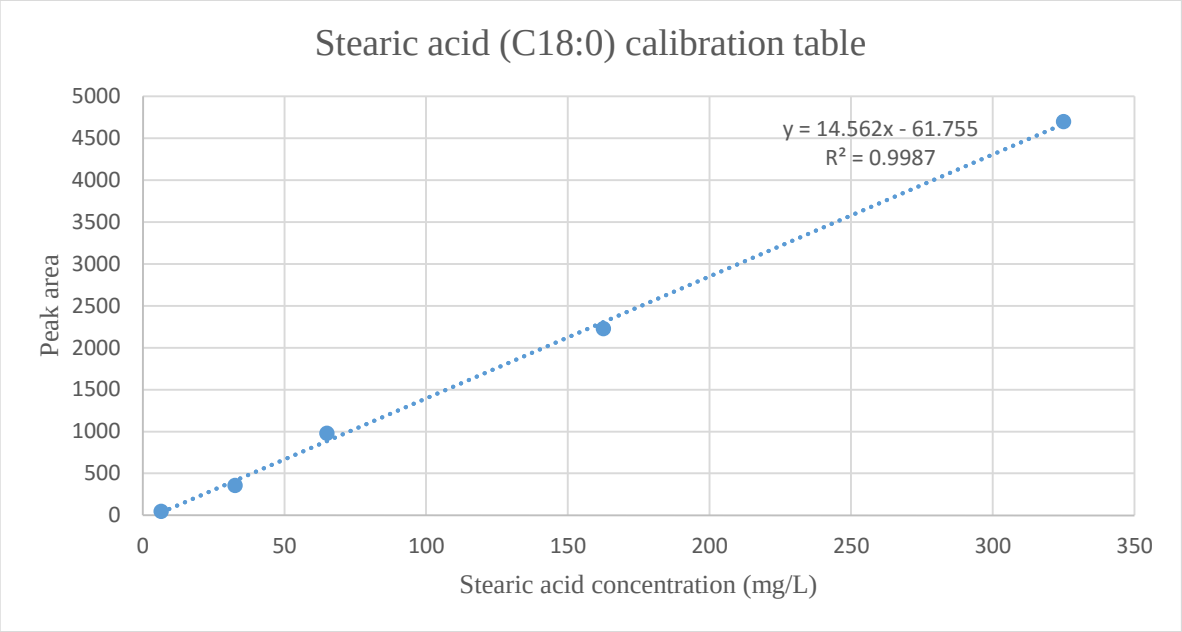

Figure A.7 GC calibration table for stearic acid

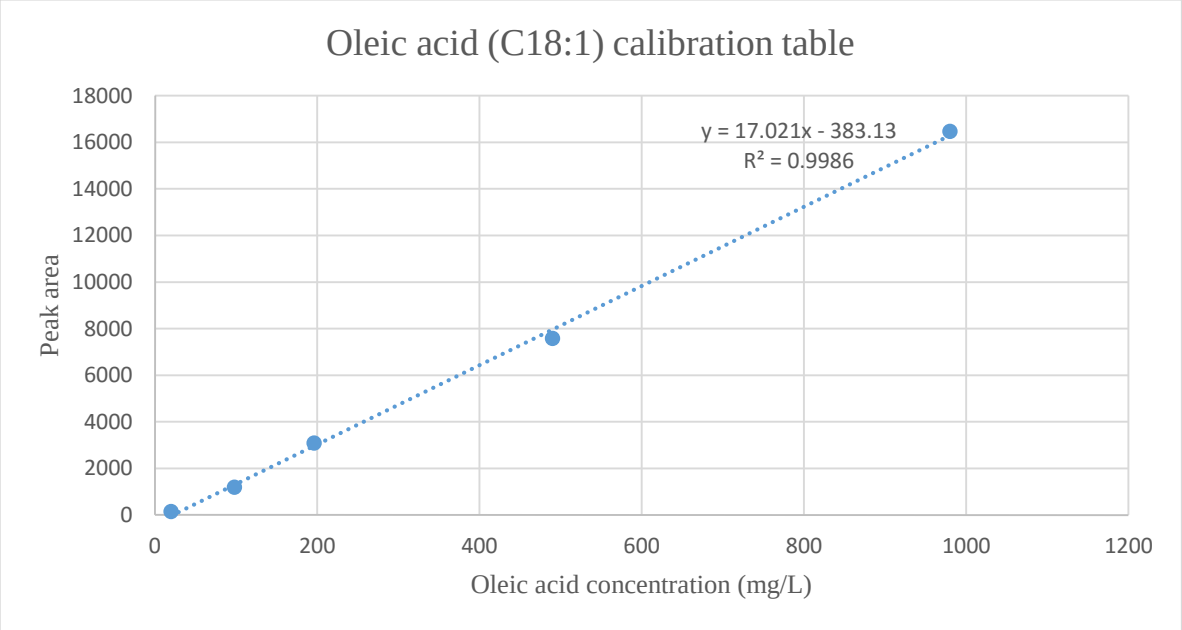

Figure A.8 GC calibration table for oleic acid