Gene Expression Profiling in *Saccharomyces cerevisiae* Grown at Different Specific Gravity Environments

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ABSTRACT

The global gene expression profiles of industrial strains of *Saccharomyces cerevisiae* responding to nitrogen deficiency and very high sugar concentrations stresses were determined by oligonucleotide microarray analysis of ~ 6200 yeast open reading frames. Genomics analysis showed that 400 genes in *S. cerevisiae* was differentially expressed by more than 1.5-fold compared with controls at late-logarithmic phase of fermentation, as the yeast adapted to changing nutritional, environmental and physiological conditions. The genes of many pathways are regulated in a highly coordinated manner. The repressed expression of GDH1 and up-regulation of ARO10 within the contrast of Q270/Q10 indicated high energy demanding of yeast cells under high sugar stress. Activities of G3P shuttle indicated that under very high gravity environment, sufficient assimilatory nitrogen enhances yeast's ability of redox balancing, and therefore higher stress-tolerance and higher fermentation efficiency of yeast. Under contrast W270/Q270, the up-regulation of DUR1,2 responsible for urea degradation induces the glutamate biosynthesis and the consumption of α-ketoglutarate. This may indicate that higher nitrogen level would enable higher activities in the TCA cycle, and therefore generate more energy for biosynthesis and yeast cell proliferation under very high gravity fermentation conditions. Nitrogen metabolism was also stimulated by high nitrogen level when yeast was grown in very high gravity environment.

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CHAPTER 1 INTRODUCTION

1.1 Background

The use of ethanol as an alternative transportation fuel provides tremendous environmental and economic advantageous and it enables countries to achieve energy security and independence (Duncan, 2003). The recent increases in petroleum prices and government legislation and regulations have stimulated the production of fuel ethanol. The demand of ethanol for producing reformulated gasoline and for use as an extender of the gasoline supplies is expected to accelerate the growth rate of the ethanol industry as long as petroleum prices remain high (Eidman, 2006).

Currently, the most significant barrier to wider use of fuel ethanol is its cost. However, fuel ethanol has the potential to be cost-competitive with petroleum fuels if there are government incentives and continued progress with both conventional and advanced ethanol production technologies (Zhang et al., 2003).

In fact, in the past decade, the conventional fermentation process has been improved through the application of very high gravity (VHG) technology capable of fermenting higher-density mashes with a higher initial sugar level (Thomas et al.,

1993). This exciting technology aims at increasing both the rate of fermentation and the final ethanol concentration and thereby reducing processing costs (Ingledew, 1993).

Nevertheless, the economic advantages of VHG technology are accompanied by a number of problems: as the sugar concentration increases, the yeast is exposed to severe conditions, such as the increase of both osmotic pressure and produced ethanol, nutrient deficiencies, especially dissolved oxygen and assimilable nitrogen. These may result in a significant delay in fermentation and drop in yeast viability (Pratt et al., 2003; Casey et al., 1984; Day et al., 1975; White, 1978).

In today's fuel market, every penny in cost savings makes a difference. Thus, a deeper understanding of stress-tolerance mechanisms of *Saccharomyces cerevisiae*, which may lead to new process design that may improve yield and performance in the conversion process are essential to making fuel ethanol competitive with gasoline.

1.2 Objectives

In this project, we aimed at investigating the influence of nutritional deficiency and VHG stresses on global gene expression profiling of yeast cells using oligonucleotide microarray technique. The objectives of this project include:

- Exploring global gene expression profiling of *S. cerevisiae* grown under two different specific gravity environments in the absence and presence of urea respectively.

- Comparing gene expression profiling patterns obtained from two different specific gravity environments in the absence and presence of urea respectively.
- Revealing the function of genes involved in cellular metabolism with respect to tested conditions.

1.3 Thesis organization

Chapter 1 is the introduction to this thesis, and provides a summary of project background, objectives, and a description of the thesis organization.

Chapter 2 examines the current literature in the field of fuel ethanol production. It is a review of VHG technology, the economic value it brings and the trouble it causes, the techniques we use to study it, and an overview of related work in the field. The features of other sustainable transportation fuels are also introduced.

Chapter 3 covers experimental design, the materials and methodology we followed in this work. Modified protocols are described.

Chapter 4 provides the results of fermentation and gene expression work, and discussions of the major discoveries.

Chapter 5 states conclusions and suggests possible directions for future research.

CHAPTER 2 LITERATURE REVIEW

2.1 Sustainable Transportation Fuels

It is an inescapable fact that, within our lifetime, the fossil fuels will run out no matter how many more oil wells are drilled, since most of the fossil fuels are used for transportation - cars, buses, ships, trains and planes, and these vehicles have been consuming fossil fuels at a rate far greater than the fuels can be replenished. As fossil fuel supplies diminish, concern is growing over what will happen after these supplies run out.

Sustainable transport fuels are of key interest as they can be reproduced by nature indefinitely, therefore reducing the dependency on fossil fuel as well as helping reduce fuel price and pollution. There are four kinds of sustainable fuels that vehicles can run on that are not made from petroleum. Some of them are now used as transportation fuels and will likely be playing an increasingly important role in the future.

Hydrogen is a perfect ecological fuel, producing only water when used. Hydrogen is renewable and abundant. It can be produced from domestic resources including natural gas, coal, biomass, and even water (Dicks et al., 2004). The main

problem with it is the costly extraction process, since it requires a huge amount of energy to break apart the incredibly strong bond between hydrogen atoms no matter what method is used, and its storage is also a serious problem (Dresselhaus et al., 1999; Alimova et al., 1987).

Methane, the primary component in natural gas, is an important fuel, but it is also the second-most important greenhouse gas (Spokas et al., 2006). Methane occurs naturally, but human-related activities such as fossil fuel production, biomass burning and waste management release significant quantities of methane into the atmosphere (Hindrichsen et al., 2005; Khalil, 1993). These emissions would be reduced if methane could be captured and used as a major energy source, while, if improperly used, methane may cause devastating damage to the environment as it decompose into gas and water when they enter the atmosphere, which may contribute to greenhouse gas accumulations (Spokas et al., 2006).

Other than the alternatives described above, biodiesel is one of the most common gasoline alternatives. Biodiesel is a clean burning fuel produced from any material that contains fatty acids, such as vegetable fats and oils, animal fats, waste greases and so on (Haas et al., 2006). The environmental benefits of this sustainable fuel are tremendous. For example, it has been shown to have lower emissions of particulate matter, unburned hydrocarbons, and carbon monoxide than petroleum-based fuels (Graboski et al., 1998). It is non-toxic as well as biodegradable, and it can be blended at any level with petroleum diesel to create a biodiesel blend without any engine modifications (Crabbe et al., 2001).

Biodiesel has been gaining popularity as an alternative fuel to the traditional fossil fuels in Europe, especially in Germany and France, but it is not yet in widespread use in North America due to the limitation in supplying the feedstock fats and oils (Körbitz**,** 1999).

Alcohol fuels such as ethanol, produced by infinite nature resources like locally agricultural crops, has increasingly becoming the answer to the ever-growing oil deficiency that the world is facing. Ethanol can be used either as fuel for automobiles alone or as an additive to gasoline (Bayraktar, 2005). Due to the fact that the low energy density of ethanol makes it heavier and takes up 1.5 times as much tank space as gasoline, ethanol is usually blended with gasoline (Sagar, 1995). Two common mixtures are E10 and E85 which contain 10% (v/v) and 85% (v/v) ethanol, respectively (Niven, R.K. 2005).

In North America, the E10 fuel is becoming common practice because blends that contain up to 10 percent ethanol does not require any engine modification for all the automobiles sold in North America (Jarvis, 1992; Hsieh et al., 2002). E85 fuel requires special engine design, such as corrosion-resistant materials and a higher engine compression ratio, which is one of the factors preventing its widespread use in North America (Canadian Renewable Fuels Association, a).

Ethanol not only reduces the consumption of petroleum fuels, but also addresses the problems of air pollution from vehicles and global warming at the same time. Ethanol molecules contain 35% of oxygen by weight, which allows more complete fuel combustion and thus reduces harmful tailpipe emissions and particulate emissions that pose a health hazard (Niven, 2005). It has been reported

that gasoline containing a 10% (v/v) ethanol blend reduces smog generating emissions like carbon monoxide by 25-30% (v/v), particulate matter by 50% (v/v) and volatile organic compounds by up to 7% (v/v) (Canadian Renewable Fuels Association, b). The process is carbon neutral, because the carbon dioxide released during ethanol production and combustion is recaptured as a nutrient to the crops as they grew. Therefore, the increased use of fuel ethanol will partially offset the global warming effect of burning gasoline.

Methanol is another alcohol fuel which can be made from renewable resources. With a far higher cumulative toxicity rating and lower energy density than that of ethanol, methanol is a less attractive alternative transport fuel (Sagar, 1995).

As discussed above, ethanol as a gasoline alternative is the most clean burning and sustainable fuel for our vehicles. Much attention has been placed on the prospects of using ethanol as a transportation fuel due to the recent increases in gasoline prices. However, current fuel ethanol production methods make the energy value of the produced fuel not significant compared to the energy put into production. For this reason, it is not feasible to replace all current fossil fuel consumption entirely by ethanol unless the yield efficiency of fuel ethanol can be steadily improved and more efficient alternate feedstock crops are developed.

2.2 Fuel Alcohol Production

Ethanol can be made from grain-based sources, which are rich in starch, such as wheat, barley, and corn, or cellulose-based sources, from just about any

agriculture waste, forestry byproducts and even municipal solid waste, such as grasses, crop residues, wood, waste paper, and yard waste (Wyman, 1994).

Ethanol is traditionally produced from the fermentation of starchy materials. Basically the process requires the conversion of starch to sugars by the use of enzymes and then fermenting those sugars by adding yeast. During fermentation the yeast converts the sugars to ethanol and carbon dioxide. Corn is the predominant feedstock in the North American ethanol industry, because of its high starch content, low prices and wide availability. Corn is converted to ethanol in either a dry or wet milling process. The main difference between the two is in the initial treatment of the grain. In dry milling operations, liquefied corn starch is produced by heating corn meal with water and alpha-amylase enzyme. A second enzyme, glucoamylase, converts the liquefied starch to sugars, which are fermented by yeast into ethanol and carbon dioxide. Wet milling operations separate the fiber, germ (oil), and protein from the starch before it is fermented into ethanol (Kim et al., 2005).

While chemically identical to the grain-based ethanol, cellulose ethanol utilizing a cheaper substrate such as crop residues could make fuel ethanol more competitive with fossil fuel. Differing from grain-based ethanol production, using cellulose is more complicated, as cellulose, the main component of plant cell walls, are protected by various layers of other material that makes the plant cellulose difficult to degrade, in order to render the sugars in the cellulose fraction accessible to conversion, it is necessary to treat the plant cellulose with a combination of chemical and enzymatic processes, and these technical challenges has been extensively researched in the last two decades (Dale et al., 1984; Azzam, 1989;

Bjerre et al., 1996; Wright, 1998; Reshamwala et al., 1995; Duff and Murray, 1996). Efficient pretreatment processing, optimized cellulose enzymes and the enzyme loading are considered to be essential to the efficient and economical production of cellulose ethanol (Sun et al., 2002; Mosier et al., 2005). Until very recently, the enzyme cost for cellulose conversion has been the main challenge for commercial production of cellulose ethanol (IEA Bioenergy - update 23, 2006).

Cellulose is much more difficult to convert to ethanol than starch, but the reward for a successful outcome could huge, and cellulose is predicted to be the future of fuel alcohol production due to the fact that we will eventually run out of feed corn and small grains. Application of cellulose-based technologies could significantly reduce the level of greenhouse gases emissions (Wyman, 1999), as well as the amount of waste entering the landfills and agricultural land needed for ethanol production (Kenney et al., 1983), and offer additional revenue streams to farmers for the collection and sale of currently unused crop waste (Lockeretz, 1981).

The fuel alcohol industry in North America truly has experienced a dynamic emergence from the 1980s to the present, and the use and production of fuel ethanol are expected to continue to grow vigorously alongside the rising energy prices and environmental problems. A look into this evolution is shown in Figure 2.1.

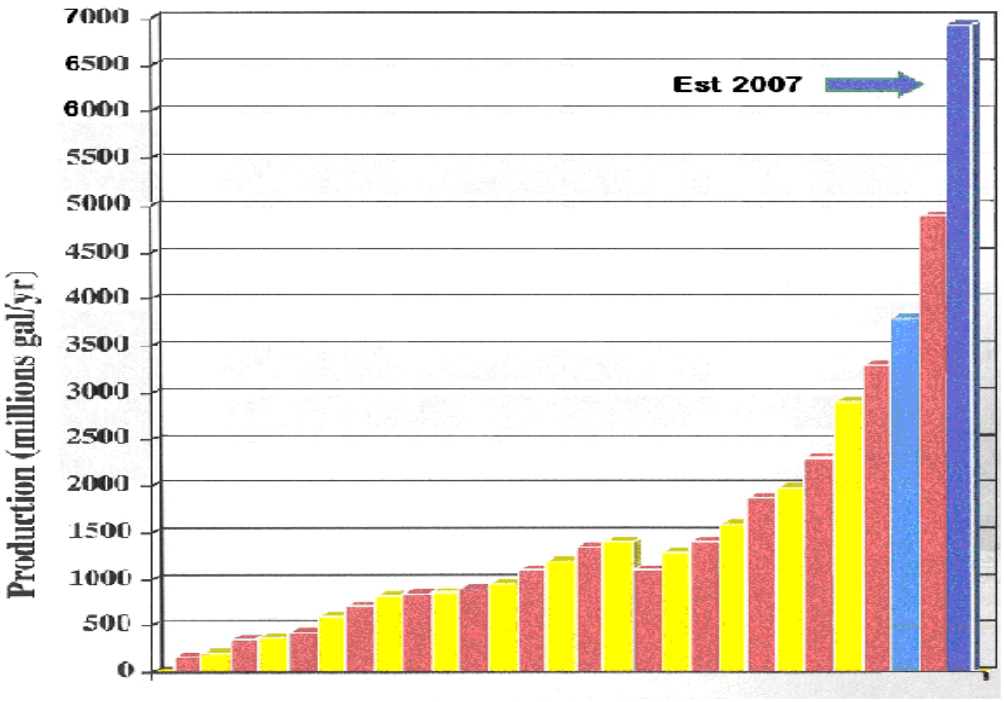

Figure 2.1 Historical chart of ethanol production
Source of data: http://www.ethanolrfa.org/industry/statistics/#A

The figure shows that the industrial alcohol capacity has been rising exponentially in the past decade. According to the Renewable Fuels Association (RFA), the United States manufactured 3.85 billion gallons in 2005 and 4.86 million gallons in 2006; The RFA also estimated that more than 6.9 billion gallons of ethanol is going to be produced in 2007.

Aside from conventional ethanol production, after a long time of research, Canada's Iogen Corporation, the world's only company operating a demonstration facility that converts biomass to ethanol is now negotiating with the federal government to locate its long-promised cellulosic ethanol plant in Saskatchewan (Bioproducts, 2007). Iogen plans to break ground on a commercial-scale biorefinery in the summer of 2007, and plans to be supplying ethanol to commercial markets by 2009, which promises to greatly increase the volume of fuel ethanol that can be

produced in Canada (Ethanol producer magazine, 2006). The cost of cellulose ethanol is expected to be competitive with the price of rack (pre tax) gasoline as more cellulose ethanol plants are constructed (Iogen in the News, 2005).

The Government of Canada and some provincial governments have also supported the development and use of ethanol fuel and co-products from starch and cellulose-based feedstock through research and development programs (Natural Resources Canada, accessed in July, 2006). The federal government has announced that all fuel in Canada must contain five per cent ethanol by 2010. This mandate would require a production of approximately two billion liters of ethanol, which is a very big jump but can be done if current and announced biofuel programs are implemented (Lepage-Monette, 2006).

2.2.1 Very High Gravity Fermentation Technology

Ethanol is being widely investigated as a substitute for gasoline as a transportation fuel (Lynd et al., 1991). However, based on current technologies, no process can produce ethanol at a selling cost competitive with gasoline or petroleum derivatives of fossil fuels. This situation therefore necessitates improving and optimizing the fermentation process for a quicker and cheaper ethanol product. Very-high-gravity (VHG) fermentation is one such process improvement that has proven successful in increasing fermentation rate and the final ethanol concentration and thereby reducing processing costs (Ingledew, 1993).

VHG technology refers to the use of mashes containing 270 or more grams of dissolved solids per liter (Bayrock et al., 2001). This technology has significantly increased industrial ethanol levels all over the world in the past decade. Since less

water must be heated, cooled, and evaporated, an increase in fermentation ethanol levels brings concomitant energy reductions and environmental benefits (Thomas et al., 1996). The conditions necessary to increase ethanol levels have been tested in pilot plants and led to production of 23.8% v/v ethanol from wheat mash containing 38% w/v dissolved solids (Thomas et al., 1993), and some of these changes are now being incorporated into process designs which target higher ethanol concentrations to lower costs.

2.2.2 VHG Fermentation and Its Affect on Yeast Viability

With the growing awareness in VHG fermentation for economic advantages, there has been prevailing interest in understanding the mechanisms and regulation of stress tolerance in yeast, as maintaining yeast viability is one of the most important parameters in order to reach high ethanol concentrations in fermentation process.

When VHG fermentation process is applied, different and increased demands are placed on the yeast. Exposure of yeast cells to VHG environment implies both exposure to very high osmotic stress and high level of toxicity of ethanol (Pratt et al., 2003) which can cause a loss of cell viability and consequently slow and stuck fermentations (Day et al., 1975; White, 1978).

Osmotic pressure is the force that develops between two solutions of differing concentrations separated by a semipermeable membrane (Heggart et al., 1999). In order for yeast to be able to reproduce, the osmotic pressure inside the cell must exceed that outside the cell (Owades et al., 1981). This is not likely to be the case at very high gravities. In VHG fermentation environment, yeast cells decrease their volume and cytoplasmic water content in response to hypertonic stress

(Marechal et al., 1994), which directly causes the loss of viability of *S. cerevisiae* (Morris et al., 1986).

S. cerevisiae accumulates glycerol as an osmotic regulatory solute in response to hypo-osmotic shock (MacKenzie et al., 1988). In addition, a strong relationship between intracellular trehalose levels and resistance to osmotic stress was also observed (Albertyn et al., 1994; Thomas et al., 1994). Both glycerol and trehalose have been demonstrated to be involved in protection against severe osmotic stress (Heggart et al., 1999). Proline is also believed to be the explanation of the yeast being able to ferment high sugar mashes with little apparent osmotic stress or alcohol intolerance (Ingledew et al., 1985; Thomas et al., 1992).

Ethanol is a primary metabolic product of yeast fermentation. Unfortunately, as the concentration increases, ethanol itself inhibits cell growth and viability and the yeast has a tendency to quit fermentation (Rose, 1980). This is particularly important in industrial ethanol production and has direct relevance in VHG fermentation (Heggart et al., 1999).

Although the mode-of-action of ethanol has not been fully understood, the primary target of ethanol is thought to be plasma membrane. Yeast plasma membrane fluidity increases with ethanol concentration and becomes especially permeable for protons, resulting in an intracellular acidification (Jones et. al., 1987; d'Amore et. al., 1987). This alteration in membrane permeability leads to changes in fatty acid and sterol composition. Supplementation of membrane unsaturated fatty acids were suggested to increase yeast ethanol tolerance (Rose, 1980; d'Amore et. al., 1987). It was also observed that yeast cells adapt to ethanol stress by

synthesizing trehalose, which has a protective effect on membranes (Sharma, 1997; Hallsworth, 1998).

Moreover, Mg^{2+} as a supplement of high sugar media has been observed to aid in the production of more ethanol, indicating that Mg^{2+} has protective effect on yeast growth from ethanol stress (d'Amore et. al., 1988; Walker et. al., 1996). Physiological factors such as mode of substrate feeding, intracellular ethanol accumulation, temperature and osmotic pressure all contribute to the ethanol tolerance of yeast (d'Amore et. al., 1987).

Other studies show that growth inhibition results, in part, from ethanol-induced water stress (Jones et al., 1986; Guerzoni et al., 1994). Ethanol can reduce water availability to below the level at which enzymes, membranes and cells remain functional and structurally stable. As a response, yeast cells synthesize compatible solutes such as glycerol and trehalose to protect against water stress (Hallsworth, 1998).

Although the stuck fermentations and poor yeast viability were ascribed to ethanol toxicity and high osmotic pressure, later studies indicated that nutritional deficiency was also a factor limiting the production of high levels of ethanol (Casey et al., 1984). It has been shown that supplementation of assimilable nitrogen could help eliminate most stuck fermentations (Ingledew et al., 1985), leading to prolonged and increased production of yeast cell mass, and results in higher ethanol yield as well as the survival of yeast to 15% ethanol (Casey et al., 1984; Kalmokoff et al., 1985). The usable forms of nitrogen include urea, individual amino acids, small peptides and ammonium ion, and they are known collectively as Free Amino

Nitrogen (FAN) (Pugh et al., 1997). In addition, low levels of oxygen (Andreasen et al., 1953, 1954) for the synthesis of sterols and unsaturated fatty acids are also potential for yeast cells reproduction and therefore the rate of fermentation (Casey et al., 1984).

The optimized production condition has increased plant productivity and resulted in increased profit margins for the fuel alcohol industries. This necessitates a fair amount of research for deeper understanding the mechanisms governing the regulation of stress tolerance in yeast, which would bring up strategies on improving cell viability, and designing biotechnological processes for producing fuel ethanol more economically.

2.3 DNA Microarray Technology

The genomic expression program required for maintenance of the optimal internal milieu in one environment may be far from optimal in a different environment. Thus, when environmental conditions change abruptly, yeast cells respond to environmental stress by altering the expression of thousands of genes, creating a genomic expression program that is specific to certain stress (Gasch et al., 2000). A thorough census of transcripts before and after administration of a stimulus therefore would reveal alterations in gene expression between the two situations. DNA microarray technology is one such approach that comparatively analyzes genome-wide patterns of RNA expression.

Microarrays are microscopic arrays of immobilized nucleic acids. One common use of microarrays is to determine which genes are activated and which

genes are repressed when two populations of cells are compared. In microarray technology, an experiment with a single DNA chip provides researchers information on the interactions between thousands of genes simultaneously, which has therefore dramatically accelerated many types of investigations, and the technology has become a standard tool for the analysis of transcription profiles among other high-throughput analytical methods (Duggan et al., 1999; van Berkum et al., 2001; Case-Green et al., 1998 and Young, 2000). One of the key challenges is to interpret the data: to identify key genes or patterns of expression associated with some condition and so to gain valuable clues about the biological processes related to that condition.

S. cerevisiae has been the model organism for higher eukaryotes for the development of microarray technology since the sequencing of its genome was completed (Miklos and Rubin, 1996; Goffeau et al., 1997). Many comprehensive studies have been designed to demonstrate how gene messages vary in response to different stimuli, such as high saline concentration (Posas et al., 2000), carbon and nitrogen starvation (Kao, 1999), alkylating treatment (Jelinsky et al., 1999), as well as to identify genes whose expression depends on a cell state, such as cell-cycle progression (Spellman et al., 1998), diauxic shift (DeRisi et al., 1997) and sporulation (Chu et al., 1998). Most of these studies are of some practical application to the biology of industrial yeasts for the improvement of the ethanol production processes. There are, however, many other applied fields for DNA microarray technology where advances are rapid, such as drug targets research, neurobiology studies and so on (Shoemaker et al., 2002). The applications for this technique are considerable and will continue to gain momentum.

DNA microarrays come in two main types of technical platforms. The first is based on standard microscopic glass slides on which cDNAs or long oligonucleotides (typically 70–80 mers) that correspond to transcripts of many different genes have been spotted (DeRisi et al., 1997). The second is based on photolithographic techniques to synthesize 25-mer oligonucleotides on a silicon wafer and constitutes the patented technology of Affymetrix Inc (Lipshutz et al., 1999). Among these methods, the spotted oligonucleotide microarrays are relatively inexpensive and less labour involvement as compared to the others (Stanton, 2001).

The use of microarrays to monitor gene expression is a rapidly evolving technology and has brought about a rethinking of the biology and disease at a global ('systems biology') level (Kitano et al., 2002; Hood et al., 2004). The technology is still being improved because the value of array experiments depends on the quality of the array and different technical solutions are emerging (Wodicka et al., 1997; Hauser et al., 1998; Cox et al., 1999; Nau et al., 2000 and Hughes et al., 2001).

2.4 Integrative approaches for gene function assignment

With the genome sequences of many organisms in hands, post-genomic studies have shown a prevailing trend in gene function assignment and comprehensive investigations of biological systems in response to external stimuli. DNA microarrays have provided scientists with a first step towards uncovering gene function on a global-scale at expression levels. Further understanding of functional genomics is addressed by integrative studies that include analyses at multiple levels, including the level of gene expression (transcriptomics), protein translation

(proteomics) and more recently the metabolite network (metabolomics) (Delneri et al., 2001).

Metabolites are the end products of cellular processes, and their levels can be regarded as the ultimate response of biological systems to genetic or environmental changes and so reflect more closely the activities of the cell at a functional level (Fiehn, 2000). Metabolomic analysis aims to provide a comprehensive insight into the metabolic state of a system – profiles of a number of predefined target metabolites of an organism under a given set of conditions (Daviss, 2005; Fiehn, 2001). The target can be a set of metabolites shared among different pathways or all metabolites of a specific pathway (Fiehn, 2001).

The metabolomics-based approach is expected to explore and define the function of genes involved in metabolic processes and gene-to-metabolite networks (Fiehn et al., 2000; Oksman-Caldentey et al., 2005). In particular, an innovative integrative approach that links comprehensive gene expression profile and targeted metabolite analysis can bring us deeper understanding of the links between different levels of biological systems (Goossens et al., 2003; Askenazi et al., 2003; Oksman-Caldentey et al., 2005). Bioinformatics are then being used to relate the data to the genome. Such a network not only will assist biologists in testing the treatment effects at the metabolic pathway level and extracting a comprehensive overview of experimental effects from microarray data, but also will help identify gene function and subsequent metabolic engineering targets for future biotechnological applications (Patil et al., 2004; Nielsen et al., 2002).

2.5 Overview of metabolism and gene regulation during fermentation

Metabolism is a large circular process of energy conversion involved in maintaining the living state of the cells, and thus the organism. In general metabolism may be divided into two categories: catabolism (dissimilation), where the energy is released from the oxidative degradation of complex organic compounds; and anabolism (assimilation), where energy is used for reductive synthesis of new molecules to maintain the structure and function of an organism. The oxidative and reductive processes of anabolism and catabolism are mediated by dehydrogenases, which predominantly use NAD^+ or NADH and $NADP^+$ or NADPH, respectively, as redox cofactors (Walker, 1998). The coenzyme NAD(H) system is primarily involved in energy-producing (catabolic) metabolism, normally maintaining their NAD^+/NADH ratio near 1000 that favours metabolite oxidation, while the coenzyme NADPH system is primarily involved in biosynthesis (anabolism) metabolism, keeping their $NADP^+$/NADPH ratio near 0.01 that favours metabolite reduction (Voet et al.,1995).

Glucose metabolism, the citric acid cycle and oxidative phosphorylation are central biochemical pathways in cellular energy metabolism (Oexle et al., 1999). *S. cerevisiae* exhibits different modes of metabolism to gain energy depending on the availability of oxygen and the carbon sources. Under aerobic conditions, pyruvate, the output of the glucose metabolism is oxidized by the citric acid cycle completely to CO_2 with oxygen (O_2) as the terminal electron acceptor in the electron transport chain (respiration) (Otterstedt et al, 2004), while, under anaerobic conditions,

pyruvate is channeled into the production of ethanol to cancel the debt of NAD^+ originating in the glycolytic pathway (fermentation) (Rose et al., 1987). As a facultative anaerobic organism, *S. cerevisiae* has a strong tendency towards a mixed respiro-fermentative metabolism, in which ethanol is produced even in the presence of oxygen, as long as the external glucose concentration exceeds 0.8 mM (Verduyn et al, 1984).

When cells grow on nonfermentable C2 and C3 substrates or fatty acids, energy has to be provided by the electron transport chain, and in addition to this, gluconeogenesis and the glyoxylate cycle are necessary for the biosynthesis of sugar phosphates (Neeff et al., 1977), therefore, one may consider genes involved in glyoxylate cycle as gluconeogenic genes. In the presence of glucose or other easily fermentable sugars, glycolysis is the major energy-yielding pathway, and ethanol is produced. The syntheses of all gluconeogenic and glycoxylate cycle enzymes are strongly repressed under such conditions (Polakis et al., 1965), so are the enzymes of the respiratory chain and most citric acid cycle enzymes (Zimmermann et al., 1997).

The primary pathways of yeast during respiro-fermentative metabolism include glycolysis and gluconeogenesis pathways, pentose phosphate pathway, glycerol metabolic pathway and citric acid cycle as shown in Figure 2.2, nitrogen metabolism and urea cycle as shown in Figure 2.6, and proline and arginine metabolism as shown in Figure 2.7.

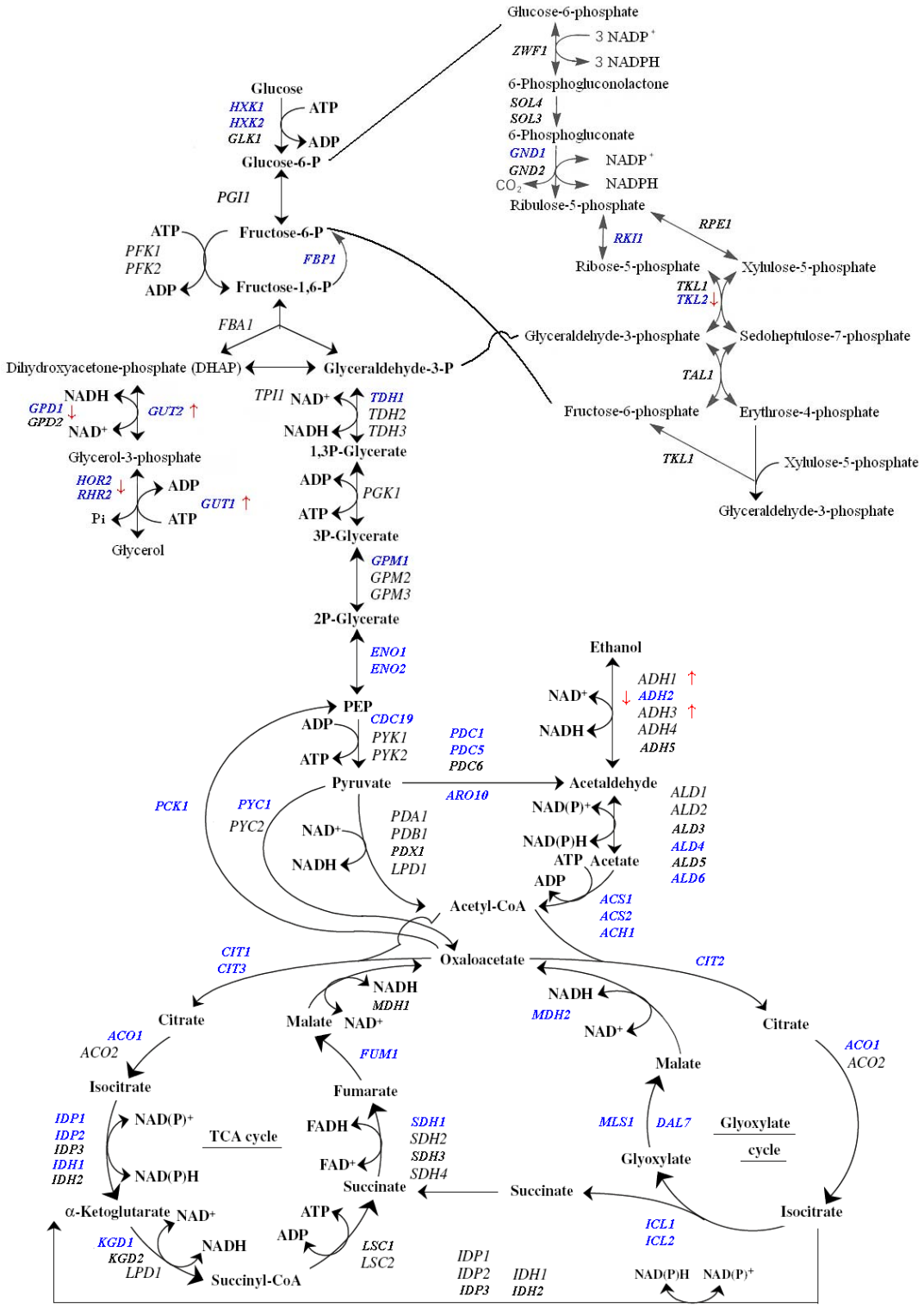

Figure 2.2 Scheme of primary pathways and energy metabolism in yeast.
The genes involved in the pathways are listed. The genes with significant fold change are shown in blue. Red arrow shows the flux direction of the regulating genes. Scheme was modified from Kresnowati et al. (2006).

2.5.1 Glycolysis and gluconeogenesis

Glycolysis is the sequence of reactions that converts glucose into pyruvate with the concomitant production of two ATP and two NADH. Glycolysis can be carried out anerobically and thus an important pathway for organisms that can ferment sugars. Gluconeogenesis proceeds in the opposite direction, which enables yeast cells to grow on non-sugar carbon sources like ethanol, glycerol, or peptone (Schuller, 2003), and expends two NADH, four ATP and two GTP (the equivalent of ATP). Most of the enzymes in glycolysis and gluconeogenesis catalyze reversible reactions. Whether glycolysis or gluconeogenesis occurs is influenced by the activity levels of a few non-reversible reactions. The irreversible steps in glycolysis are catalysed by hexokinase, phosphofructokinase-1 (PFK) and pyruvate kinase (PK). Those of gluconeogenesis are catalysed by phosphoenolpyruvate carboxykinase (PEPCK), pyruvate carboxylase, fructose 1,6-bisphosphatase (F-1,6-bisPase) and glucose 6-phosphatase (McElwee et al., 2006). A futile cycle consisting of both pathways would waste four ATP equivalents per cycle. In order to prevent this, glycolysis and gluconeogenesis pathways are reciprocally regulated (Polakis et al., 1965).

2.5.2 Pentose phosphate pathway (PPP)

Some glucose-6-phosphate is diverted from the common glycolytic pathway into the pentose phosphate pathway. The main role of the PPP is to provide the cell with a source of NADPH as reducing power for biosynthetic reactions and to supply pentose phosphate for the synthesis of nucleotides (Zimmermann et al., 1997). This pathway is also important for protecting yeast from oxidative stress, since NADPH

is an essential cofactor for glutathione- and thioredoxin-dependent enzymes that defend cells against oxidative damage (Miosga et al., 1996; Slekar et al., 1996).

The pathway can be divided into two parts (Rose et al., 1987). One is oxidative and irreversible, which converts glucose-6-phosphate into ribulose-5-phosphate and CO_2, generating NADPH with two dehydrogenate enzymes involved. Since no transhydrogenase activity could be detected in *S. cerevisiae* (Bruinenberg et al., 1985), no direct interconversion of NADH and NADPH can occur. The oxidative part of the PPP was therefore thought to be the major source of NADPH in *S. cerevisiae* (Zimmermann et al., 1997). The other is non-oxidative and reversible and is important for interconversion of ribulose-5-phosphate, fructose-6-phosphate and glyceraldehyde-3-phosphate, which are required for many biosynthetic pathways. Since glucose-6-phosphate, fructose-6-phosphate and glyceraldehyde-3-phosphate are also glycolytic intermediates, so they can be shunted to glycolysis and oxidized to pyruvate, or utilized by the gluconeogenic enzymes to generate more 6 carbon sugars (fructose-6-phosphate or glucose-6-phosphate).

2.5.3 Glycerol metabolism

Glycerol is a by-product of yeast ethanol fermentations and its formation is essential in the maintenance of the redox balance (van Dijken et al., 1986). Glycerol is produced by reduction of dihydroxyacetone phosphate to glycerol-3-phosphate by a cytosolic NAD^+-dependent glycerol-3-phosphate dehydrogenase (GPD), followed by dephosphorylation to glycerol by glycerol-3-phosphatase (Gancedo et al., 1968).

As stated above, excess NADH produced in the cytoplasm due to the formation of biomass and different by-products must be reoxidized to allow an

enduring cellular metabolism. Glycerol formation is the only way of restoring the cytoplasmic redox balance with a net result of NADH consumption under anaerobic conditions since ethanol production is a redox neutral process (Van Dijken et al., 1986). The oxidation of NADH in glycerol assimilation is catalyzed by cytosolic GPD that encoded by GPD1 and GPD2 (Albertyn et al., 1994; Eriksson et al., 1995).

Respiratory oxidation of cytosolic NADH can also play a role in maintaining cytosolic redox balance. However, NADH is unable to cross the mitochondrial inner membrane and mechanisms are required for conveying cytosolic NADH to the mitochondrial electron transport chain, such as the glycerol-3-phosphate (G3P) shuttle (Larsson et al., 1998). G3P shuttle comprising DHAP and L-G3P carries electrons from cytosolic NADH to the respiratory chain with the help of the cytosolic GPD encoded by GPD1 and GPD2, and mitochondrial GPD encoded by GUT2.

Beside G3P shuttle, yeast cells can also indirectly exchange the NADH between mitochondria and cytosol by some other metabolite shuttles, such as the ethanol-acetaldehyde shuttle (Nissen et al., 1997). The mechanisms of the G3P shuttle and the ethanol-acetaldehyde shuttle are shown in Figures 2.3 and 2.4.

Glycerol also acts as a compatible solute when yeast cells are exposed to osmotic stress (Brown, 1978). The prominent physiological response of yeast cells to osmotic stress is the enhanced production and intracellular accumulation of glycerol and the glycerol production under this situation is solely controlled by the level of cytosolic NAD^+-dependent glycerol-3-phosphate dehydrogenase activity, the key enzyme of glycerol synthesis (Andre et al., 1991; Albertyn et al., 1994).

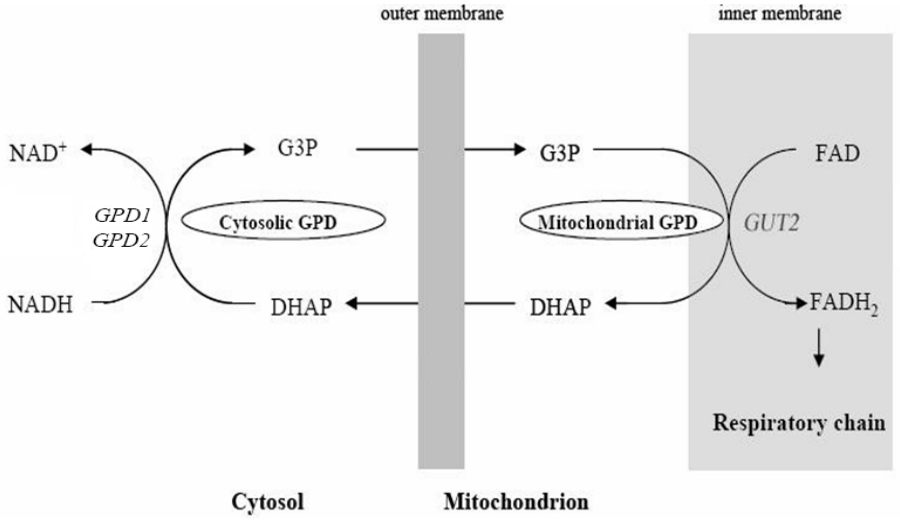

Figure 2.3 Illustration of glycerol-3-phosphate shuttle. Modified from Nguyen (2004)

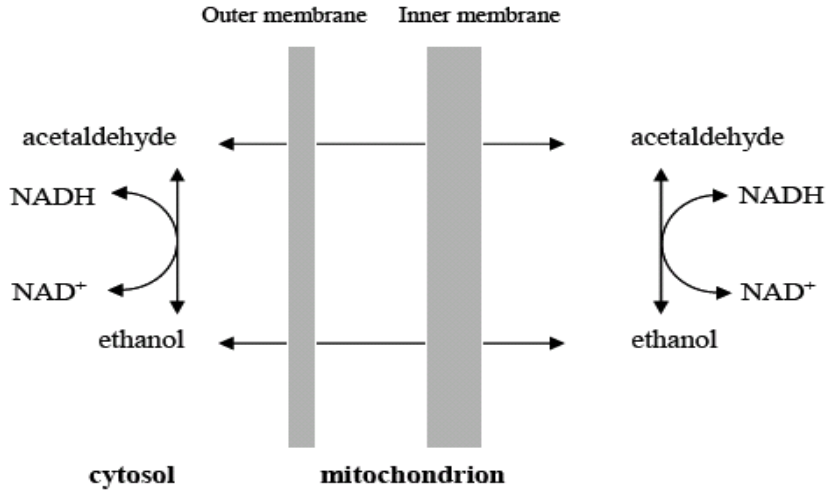

Figure 2.4 A scheme presentation of ethanol-acetaldehyde shuttles. Modified from Nguyen (2004)

2.5.4 Citric acid cycle (TCA cycle)

The citric acid cycle (also known as the tricarboxylic acid cycle, the TCA cycle, or the Krebs cycle) is oxidative, generating NADH, which drives the synthesis of large amount of ATP for the cell metabolism. As well, this cycle provides the

carbon skeletons used in many biosynthetic reactions, such as the synthesis of glutamate (Stryer, 1988).

Pyruvate dehydrogenase (PDH) links glycolysis to the TCA cycle via conversion of pyruvate to acetyl-CoA, which is the initiator of the TCA cycle (Ullrich et al., 1975). The reactions of TCA cycle serve to completely degrade the two-carbon unit acetyl-CoA (which is derived primarily from three major food groups: carbohydrate, lipids, and proteins) in to CO_2, while transforming the energy of the acetyl-CoA into one high-energy phosphate bond in the form of GTP and four reducing equivalents (three NADH + H^+, and one $FADH_2$). The NADH and $FADH_2$ are then oxidized by the electron transport chain that coupled with the citric acid cycle, and ultimately yielding fifteen ATP equivalents per pyruvate molecule under aerobic metabolism. The conversion of pyruvate to acetyl CoA and all reactions of the TCA cycle take place in the mitochondria which is also the location of the electron transport chain (Rose et al., 1987).

2.5.5 Nitrogen metabolism, urea cycle and metabolism of amino groups

Nitrogen is a critical chemical element in both proteins and DNA, and thus every living organism must metabolize nitrogen to survive. Yeasts are capable of utilizing a range of different inorganic and organic sources of nitrogen (Cooper, 1982). However, *S. cerevisiae* selects the preferred nitrogen sources that yield relatively higher growth (such as ammonia, glutamine, and asparagine) or stimulates metabolisms of alternative nitrogen sources (such as proline and urea) when the preferred ones have been consumed (ter Schure et al., 2000; Marzluf, 1997).

In order to use the nitrogen sources, yeast cells have to convert them into glutamate and glutamine − the predominant nitrogen donors for all other nitrogen containing compounds in the cell such as amino acid (ter Schure et al., 2000). Both glutamate and glutamine can be synthesized directly using ammonia as the amino group donor. In *S. cerevisiae*, two genes (GDH1 and GDH3) encoded $NADP^+$-dependent glutamate dehydrogenase isoenzymes (Avendaño et al., 1997) are required to catalyze glutamate synthesized from ammonia and α-ketoglutarate (Figure 2.5). Glutamine is synthesized from ammonia and glutamate in a reaction that requires ATP, catalyzed by glutamine synthetase. Therefore nitrogen catabolism is centered on degrading nitrogen sources to finally yield either ammonia or glutamate as end products. Several systems, such as those degrading allantion, urea, or asparagine, generate ammonia as the final product. The remaining systems, such as those participating in proline and arginine metabolism, generate glutamate (Cooper, 1982).

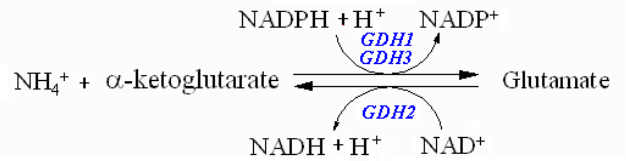

Figure 2.5 The glutamate synthesis reaction

In the degradative pathway of every amino acid, its carbon skeleton enters the TCA cycle or is channeled into gluconeogenesis, part of the ammonia is reused for biosynthetic purpose, and the rest of it must be excreted and is mainly in the form of urea. The series of reactions that operate to eliminate excess nitrogen and form urea is known as the Urea Cycle or the Krebs-Henseleit Cycle. As a nitrogen

source for *S. cerevisiae*, urea is carboxylated by urea amidolyase (DUR1,2) to produce two molecules of ammonia via allophanate (Cooper et al., 1980). The summary of the links between the urea cycle and the citric acid cycle is shown in Figure 2.6.

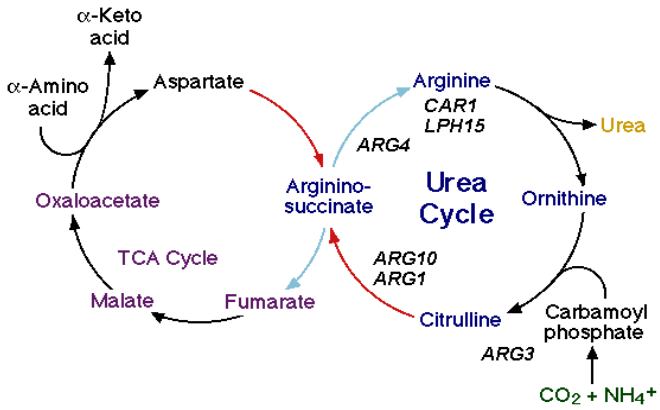

Figure 2.6 The relationship of the urea cycle to the TCA cycle
Source: http://138.192.68.68/bio/Courses/biochem2/AminoAcids/UreaCycle.html

2.5.6 Proline and arginine metabolism

Proline is an amino acid that is not only required for protein synthesis but can also serve as a nitrogen source, the least-preferred nitrogen source. As stated above in nitrogen metabolism, when other sources of nitrogen like ammonia, asparagine or glutamine are unavailabe, *S. cerevisiae* cells degrade proline into glutamate via the proline utilization pathway. The conversion is assisted by proline oxidase and delta-1-pyrroline-5-carboxylate dehydrogenase encoded by gene PUT1 and PUT2, respectively (Brandriss et al., 1979).

Arginine is another nitrogen source that *S. cerevisiae* can utilize when optimal sources of nitrogen are unavailable. Three enzymes, arginase (CAR1) and ornithine transaminase (CAR2), delta 1-pyrroline-5-carboxylate reductase (PRO3)

are responsible for arginine catabolism (Middelhoven, 1964) (Figure 2.7) which occurs in the cytosol with the hydrolysis of arginine to proline, releasing three nitrogen atoms. The proline ring is able to be further degraded to glutamate via the proline utilization pathway under aerobic condition, and therefore entering TCA cycle through α-ketoglutarate (Brandriss et al., 1980).

The metabolic pathway of arginine biosynthesis and the genes involved in its metabolism is presented in Figure 2.8. The first five steps of arginine biosynthesis in *S. cerevisiae* take place in the mitochondrion and result in the formation of ornithine which is then exported to the cytoplasm. In the cytoplasm, L-ornithine is converted to L-arginine in three reactions mediated by ornithine carbamoyltransferase, arginosuccinate synthase, and argininosuccinate lyase. Transcription of genes of the arginine biosynthetic pathway, as well as of other amino acid biosynthetic pathways, is activated upon amino acid starvation.

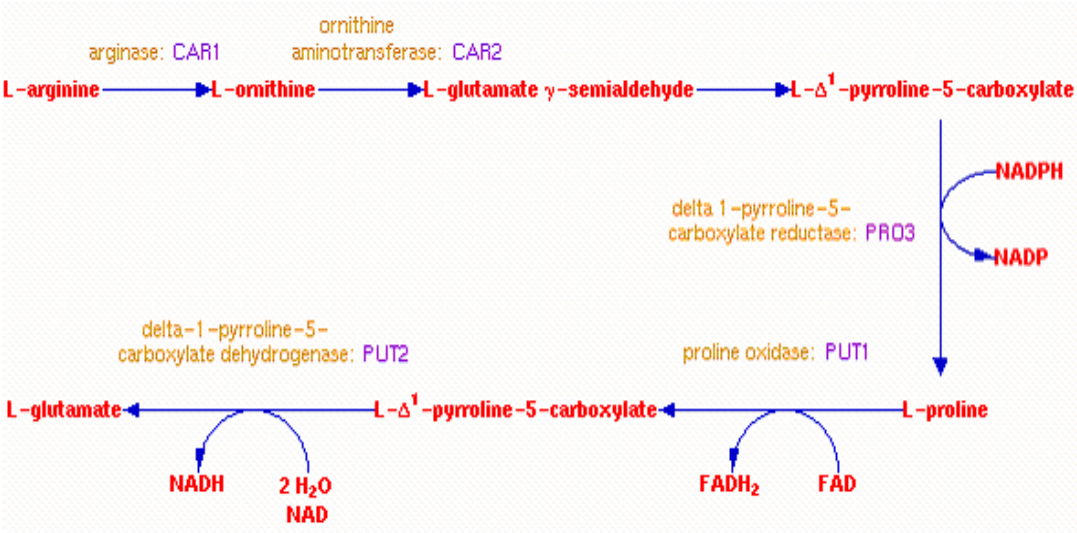

Figure 2.7 Arginine and proline degradation pathway.
Source: http://pathway.yeastgenome.org:8555/YEAST/new-image?type=PATHWAY&object=ARGDEG-YEAST-PWY&detail-level=2

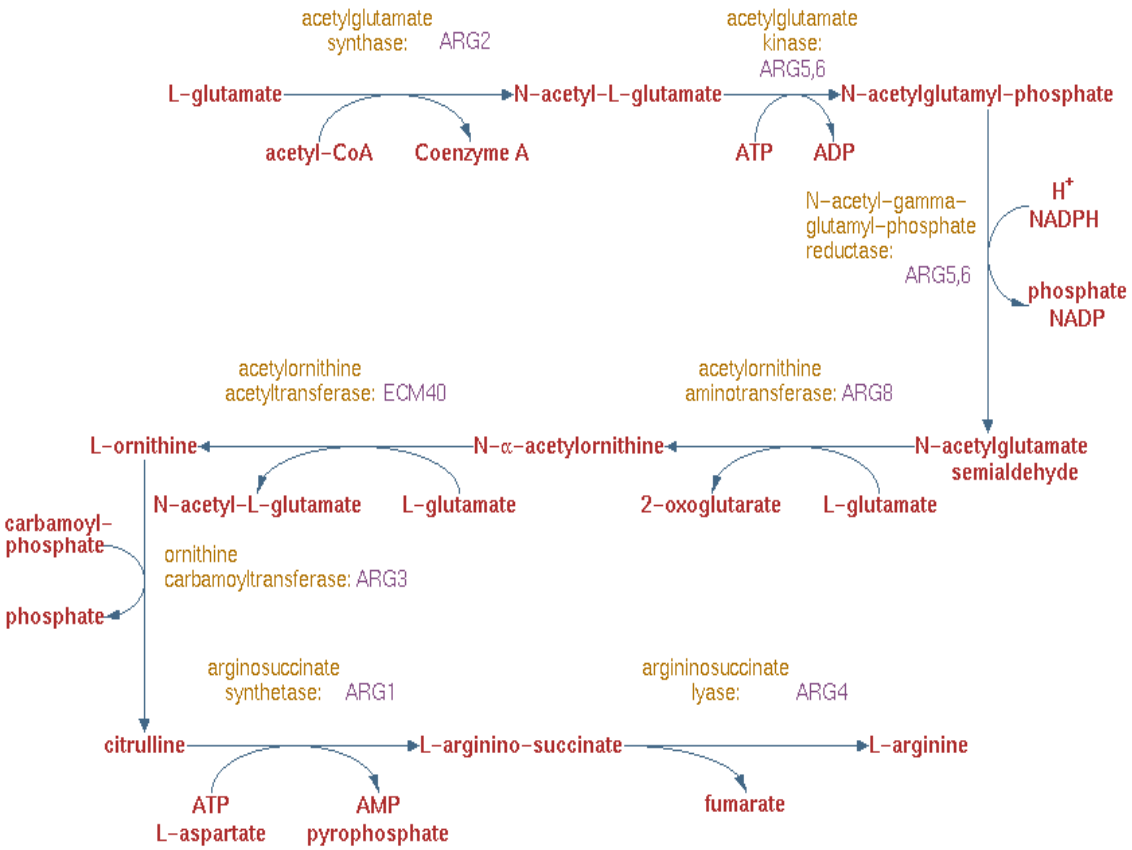

Figure 2.8 The metabolic pathway for the arginine synthesis from glutamate in yeast Source: http://pathway.yeastgenome.org:8555/YEAST/new-image?type=PATHWAY&object=YEAST-ARG-SYN-PWY&detail-level=2

2.6 Knowledge gap

The transcriptional responses of yeast cells to osmotic, ethanol stress and nitrogen limitation have been investigated by several studies using DNA microarray technique (Posas et al., 2000; Alexandre et al., 2001; Backhus et al., 2001). These works have successfully determined the molecular mechanisms involved in protection against individual stresses. However, industrial fermentations are dynamic in nature, with multiple stresses or biological changes interacting simultaneously to affect the physiological traits of the yeast or fermentation

parameters. Thus, studying the effect of individual stresses on yeast does not give the full picture of the important environmental parameters in fermentation. Although a further study focusing on the influence of VHG fermentation on the transcriptional profile of *S. cerevisiae* has been carried out (Devantier et al., 2005), it didn't consider the effects of assimilable nitrogen level which are important for the efficiency of the VHG fermentation (Casey et al., 1984).

The work described in this thesis contributes to the understanding of transcriptional gene expression profile in *S. cerevisiae* grown in a defined medium mimicking the fermentation conditions in the fuel ethanol industry with regard to supplementation of assimilable nitrogen, carbohydrate source and concentration, which has not been studied so far.

CHAPTER 3 MATERIALS AND METHODS

3.1 Experimental design

In order to understand the cell behaviour in response to different environments at a global level, the overall gene expression profiles of the yeast were examined using Agilent in situ synthesized 60-mer oligonucleotide microarrays by harvesting the yeast at the late logarithmic phase of fermentations under different conditions. The chosen time for sample collection ensures both high expression levels of stress genes and high activities of the yeast cells (Rossignol, 2003).

For comparison, chemically defined medium with various combination of glucose levels (10 g glucose/l vs. 270 g glucose/l) and free amino nitrogen levels (no supplementation of urea vs. 16 mM urea) were used for fermentation. Thus, a 2^2 experimental plan was designed and implemented. Hybridizations between contrasts were conducted to monitor expression difference of interesting genes triggered by certain stress.

In a microarray experiment, in order to distinguish true differences between conditions, several replicates per condition are needed to obtain sufficiently reliable enough estimates of variation among samples within condition. Dye-swap technique is used to offset the bias caused by dye effect. In this comparative study of four conditions, the experiments were designed as shown in Figure 3.1 where circles

represent samples, and arrows represent chips; the red and green ends of the arrows represent the dyes used for the samples at either end.

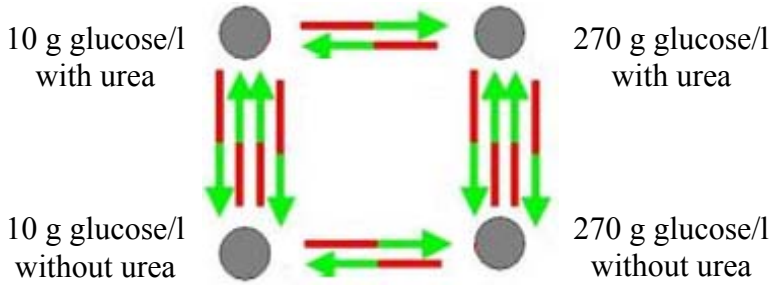

Figure 3.1 An experimental design for a comparative study of the effect of nitrogen deficiency on yeast grown at two gravity environments.
Source: http://discover.nci.nih.gov/microarrayAnalysis/Experimental.Design.jsp

To validate the microarray results, targeted metabolites and organic acids were profiled using High Performance Liquid Chromatography (HPLC) in combination with Ion Chromatography (IC), which facilitate the interpretation of expression data as well.

3.2 Yeast cultivation

S. cerevisiae, originally supplied by Alltech Co. (Nicholasville, KY) and held in pure culture at Dr. W. M. Ingledew's laboratory at University of Saskatchewan, was cultivated in batch. The chemically defined medium was used to cultivate yeast (Narendranath et al., 2001; Stephanopoulos et al., 1998).

The final concentrations of ingredients in the chemically defined medium were: (mmol/l) $(NH_4)_2SO_4$, 2; Urea (if added), 16; K_2HPO_4, 0.86; KH_2PO_4, 6.83; $MgSO_4$, 2.03; NaCl, 2.05; and (μmol/l) H_3BO_3, 24; $MnSO_4$, 20; $NaMoO_4$, 1.5;

$CuSO_4$, 10; $CoCl_2$, 1.5; $ZnSO_4$,100; KI, 1.8; $FeCl_3$,100; $CaCl_2$, 82; and (μg/l) biotin, 300; calcium pantothenate, 3000; folic acid, 30; myoinositol ,15000; niacin, 600; pyridoxine HCl, 600; riboflavin, 300; and thiamine HCl, 300. The medium contained either 10 or 270 g glucose/l as the sole carbon source. The vitamin solution (with the unit of "μg/l") was prepared as a 1000-fold concentrated stock and kept frozen at -20° C. When needed, an aliquot was thawed and filter-sterilized (0.2-μm pore size membrane filter) and the required amount was added to medium.

Working cultures of this organism were maintained at 4° C in YPD (a medium containing 1% yeast extract, 1% peptone, and 2% glucose) slant tubes with an addition of 2% agar. The cultures were started from a fresh single colony and incubated with 100 ml YPD (complete) liquid medium at 30° C. The flasks used were 250-ml screw-capped, side-arm Erlenmeyer flasks. The growth of yeast cells was measured turbidometrically using a Klett Summerson colorimeter (Clinical model, Klett Manufacturing, NY) equipped with a No. 59 filter (560-630nm). Yeast cells were stained with methylene blue, and the viable cells (cells that were not stained blue) were counted directly using light microscopy at 200 × magnification and a hemocytometer (Bright Line Counting Chamber, Hausser Scientific, PA). After cell number reached 3×10^7 cells/ml (the corresponding Klett unit is 105), a 10-ml culture was withdrawn and used to subculture to 90 ml of chemically defined medium with 270 g glucose /l; for the condition of 10 g glucose/l, a 1-ml culture was inoculated into 99ml chemically defined medium.

3.3 Batch growth of yeast cells grown under four different conditions

In order to study the influence of nitrogen deficiency and very high sugar concentration on global gene expression profiling of yeast cells, chemically defined medium with various combination of glucose levels (10 g glucose/l vs. 270 g glucose/l) and free amino nitrogen levels (no supplementation of urea vs. 16 mM urea) were used for fermentation. They are the medium with 10 g glucose/l in the presence of urea (10U); the medium with 10 g glucose/l without urea supplementation (10Q); the medium with 270 g glucose/l in the presence of urea (270U); and the medium with 270 g glucose/l without urea supplementation (270Q).

3.4 Glucose consumption and ethanol production

Aliquots of cell suspension at different points during batch fermentation were centrifuged and 1 ml of supernatant was analyzed for glucose and ethanol concentration using Biochemistry Analyzer (YSI 2700 Select, YSI Incorporated, Yellow Springs, OH) and Gas Chromatograph (GC) (5890 series II, Hewlett-Packard, Palo Alto, CA), respectively.

3.5 RNA extraction

Total RNA was isolated from cell culture using the Agilent Total RNA Isolation Mini Kit (Agilent Technologies, Inc., USA). The manufacturer's protocol, "Isolation of High-Purity Total Cellular RNA from Yeast Using the Agilent Total RNA Isolation Mini Kit" was used. The only deviation from the stated procedure

was that glass beads were used instead of rotor-stator homogenizer in disruption of yeast cells. One volume of glass beads (0.45-0.55-mm diameter) was added to three volumes of the lysis buffer in the 1.5 mL Eppendorf tubes. The tubes were first placed on ice for two minutes and vortexed vigorously for 1 minute, and then placed on ice for one minute (This process was repeated for two more times to attain thorough disruption and homogenization). Purified RNA was stored at -80° C until use.

To assure the success of microarray experiments, the quantity and quality of the RNA were always checked before use through a combination of UV absorbance with gel electrophoresis detection.

3.5.1 Quantification of RNA

The concentration of total RNA was determined by measuring the optical absorbance at 260nm with UV-VIS spectrophotometer (UV mini 1240, SHIMADZU, JAPAN) using the following formula:

Total RNA (μg) = A_{260} reading × 40 μg/ml ×dilution factor × volume of RNA sample (ml)

10 μl of the RNA samples was diluted in 990 μl 10 mM Tris-HCl (pH 7.0) and transferred to a quartz cuvette (1 cm path length). The reading at 260 nm was read using a UV spectrophotometer against 10 mM Tris-HCl (pH 7.0) as blank solution.

3.5.2 Purity of RNA

The ratio of absorbance at 260 nm to absorbance at other wavelengths is a good indicator of the purity of the preparation, so a full UV spectrum was taken in 10 mM Tris-HCl, pH 7.5. Pure RNA should be observed with an A_{260}/A_{280} ratio of approximately 2.0, and an A_{260}/A_{230} ratio above 1.8. In addition, an A_{260}/A_{270} ratio greater than or equal to 1.2 indicates the RNA is free of phenol, and an A_{260}/A_{330} ratio of approximately 0 indicates the RNA is free of particle.

3.5.3 Integrity of RNA

RNA integrity was detected by denaturing agarose gel electrophoresis and ethidium bromide staining following the protocol "QIAGEN Guide to Analytical Gels" available on QIAGEN website.

3.6 Fluorescent Direct Labeling of cDNA

RNA isolated from two samples was converted to fluorescently labeled cDNA — either cyanine 3-cDNA (pink dye), or cyanine 5-cDNA (blue dye) using the Agilent Fluorescent Direct Label Kit (Agilent Technologies, Inc., USA) as per the manufacturer's instructions. The resulting labeled cDNA samples were combined for the hybridization to the same microarray. Figure 3.2 provides an overview of the procedure and shows a sample Microarray image.

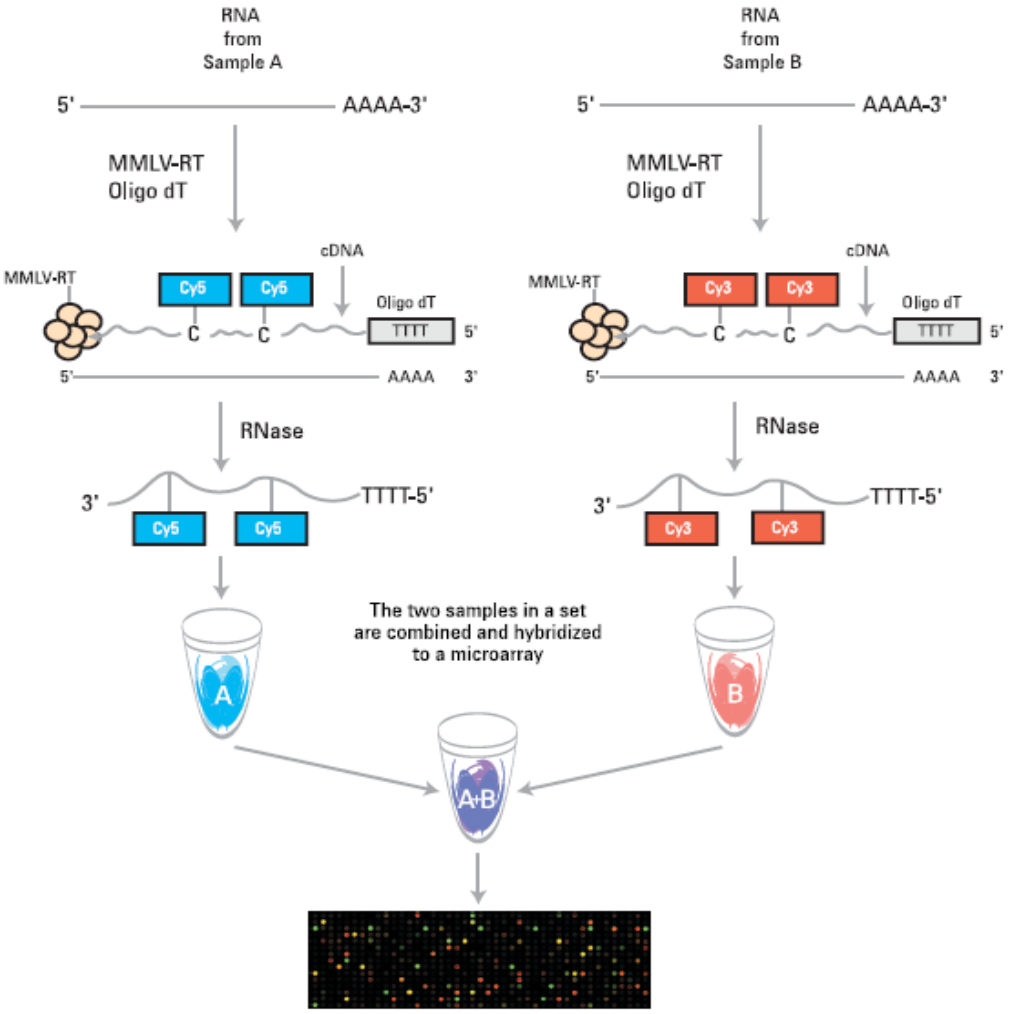

Figure 3.2 Agilent Fluorescent Direct label Kit Protocol
Source: Agilent Fluorescent Direct label Kit Protocol

3.7 Hybridization to an oligonucleotide-based Microarray

Hybridization was performed using hybridization kit (Agilent Technologies, Inc., USA) as per the manufacturer's protocol. After incubation at 60° C for 17 hours in the hybridization oven, unhybridized probe was removed by washing the microarray slide with several changes of buffer. A modified washing procedure

specially designed for cDNA labeled targets developed by Agilent Technologies, Inc. was used. The slides were then stored in dark until scanning.

3.8 Microarray image processing

Briefly, the microarray slides were scanned by a dual-beam laser scanner (Axon Instruments, Inc., Union City, CA), the obtained images were then subject to background correction, followed by normalization and scaling steps in order to bring all microarrays to a same scale. The scaled microarray data were linearized using marray and limma packges (retrieved from R statistics web site) to infer the expression profiles. An R script code was written in order to use the feature provided by the packages.

3.8.1 Image scanning

The hybridized microarrays were scanned with a GenePix 4000B dual laser scanner at 10-µm resolution complete with its own GenePix Pro software for microarray image analysis.

Cyanine 3-cDNA was excited at a wavelength of 532 nm producing a green color and cyanine 5-cDNA was excited at a wavelength of 632 nm producing a red color. Data from each fluorescence channel were collected and stored in TIFF image formats. The images were overlaid to generate the final microarray image consisting of red, yellow and green spots of varying intensities. The background intensity calculated locally, rather than globally was subtracted from the feature intensity resulting in the true spot intensity for calculating meaningful gene expression values.

3.8.2 Image pre-normalization

Before the gene expression profiles of the RNA samples can be analyzed and interpreted, the red and green intensities must be normalized relative to one another so that the red/green ratios are as far as possible an unbiased representation of relative expression. Each microarray was pre-normalized with the GenePix normalization factor so that the median of ratios of all of the features was equal to 1. The pre-nomalized microarray images can be found in Appendix 1.

The intensities were then converted to gene expression ratios by dividing Cy5 intensity by Cy3 intensity in each spot, and the ratios were log-transformed with base 2 so that up-regulated and down-regulated genes changed by the same amplitudes. The intensity of the spot is proportional to the amount of gene expression within the sample. A yellow spot represents equal gene expression between both samples and the gene expression is said to be non-differential.

3.9 Microarray data analysis

A software package for analyzing designed experiments and the assessment of differential expression, LIMMA (Linear Models for Microarray Data), was used to process pre-normalized microarray data. An R Statistics script developed by Dr. Lin incorporates LIMMA tools to mine biological information buried in the pre-normalized datasets. In the R script, the microarray dataset is sequentially evaluated as following: background correction of datasets, normalization between datasets, linear fitting of normalized datasets and Bayesian inference of up-, down- and non-

regulating genes in the datasets. The MA plots were used to illustrate data extracted from microarray images. These plots are collected and presented in Appendix 2.

3.10 Analysis of targeted metabolites and organic acids

Succinic acid and glycerol were quantified by HPLC (Series 1100, Agilent Technologies, Mississauga, ON) equipped with a refractive index detector (HP 1047A, Hewlett Packard, Mississauga, ON). The metabolites were separated in an ORH-801 column (ICE-99-9754, Transgenomic Company, Omaha, NE) in which the 2.5 mM sulfuric acid (H_2SO_4) mobile phase was used as at 0.2 ml/min, and the temperature was maintained at 50° C. In order to determine the distribution of metabolites and organic acids, a series of standard samples were prepared at a concentration of 50.0 mM, filtered with a 0.2 μm cellulose nitrate membrane filter (Cat. 7182-002, Whatman International Ltd, Maidstone, England).

Lactic acid, acetic acid, pyruvic acid, α-ketoglutaric acid, d-glucuronic acid, fumaric acid, citric acid, succinic acid, malic acid were analyzed by ion exclusion on a Dionex DX500 IC unit equipped with an AS11HC column (Dionex Corp., Sunnyvale, CA). The Potassium Hydroxide was used as eluent at 1.5 ml/min. Conductivity detection with an ASRS-Ultra II 4-mm suppressor was used. A series of standard samples were prepared at a concentration of 20 ppm, filtered with a 0.2 μm cellulose nitrate membrane filter.

Based on the relationship between the peak area and concentration of each standard sample, a plot of concentration versus peak area was obtained, which serves

as the calibration curves (see Appendices 3 and 4) for quantifying metabolites and organic acids of interest.

CHAPTER 4 RESULTS AND DISCUSSION

4.1 Batch Growth of Yeast Cells Grown under Four Different Conditions

When yeast cells were inoculated into the liquid medium with adequate nutrients and incubated with shaking at 30°C as mentioned in Chapter 3, typical batch growth curves were resulted when the viable population and turbidity of cells were plotted against time (Figure 4.1 and Figure 4.2).

As depicted by both figures, the lag phase occurred immediately after inoculation was a period of adaptation of cells to a new environment. During this phase, cells experienced a change of nutritional status or alterations in physiological growth condition, and there was no measurable growth. After cells transited from lag phase and commenced active cell division, they entered an acceleration phase and gradually finished adjusting to the new environment. In the exponential phase, cells multiplied rapidly, and cell mass and cell number density increased exponentially with time. Following the exponential phase, cell growth was retarded in a deceleration phase due to depletion of essential nutrients, and accumulation of growth inhibitory metabolites, such as ethanol. Yeast cells enter the prolonged periods of stationary phase without added nutrients and finally died or autolyzed due to the exhaustion of essential nutrients or the buildup of inhibitors.

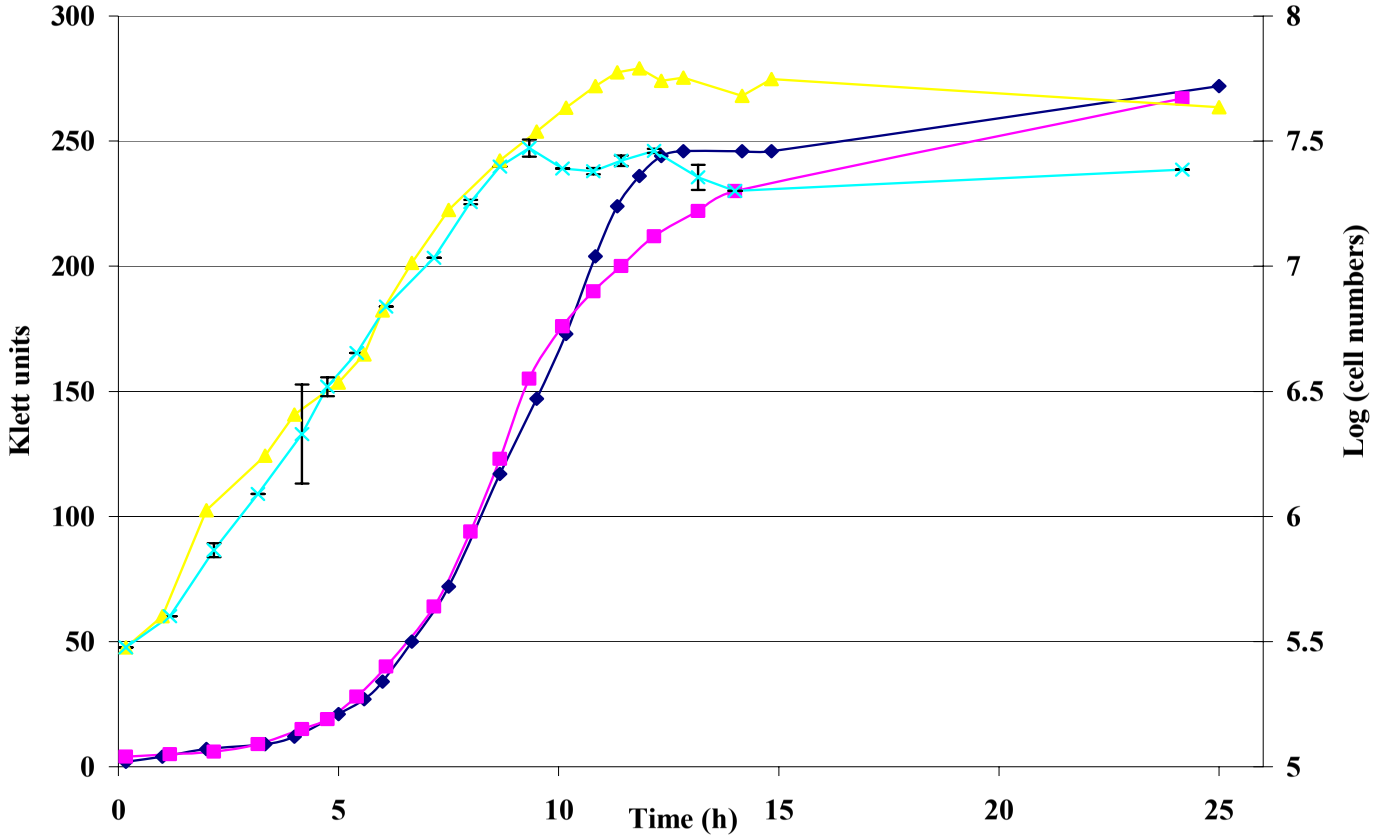

Figure 4.1 Growth curves of *S. cerevisiae* at 10 g glucose/l in the absence and presence of urea. Some of the cell number points do not have error bars due to the time constraint of the experiment

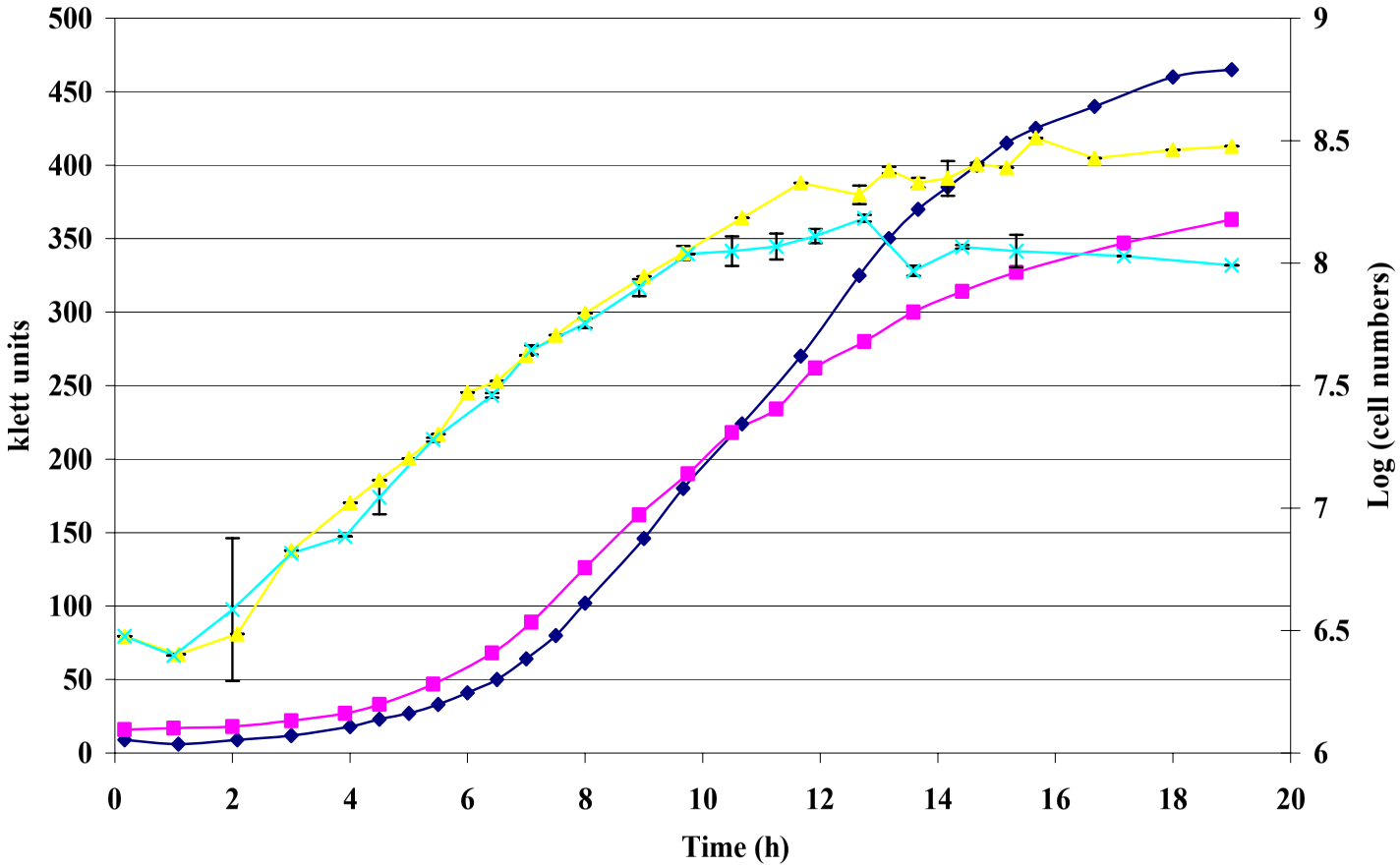

Figure 4.2 Growth curves of *S. cerevisiae* at 270 g glucose/l in the absence and presence of urea

The experiments under all conditions were conducted more than three times to ensure that reproducible results could be obtained, and each growth phase of the yeast cells in the three runs was statistically reproducible (within cell counting ±15%).

4.2 The Influence of FAN Levels on Yeast Growth and Fermentation Performance

In order to study the influence of FAN (Free Amino Nitrogen) levels on yeast growth, the viable yeast cell numbers were plotted against time in a semi-log format as shown in Figure 4.1 and Figure 4.2. Both figures shows that the yeast cells entered the deceleration phase sooner when grown in the absence of urea than in the presence of urea.

In order to understand the influence of FAN levels on fermentation performance, the curves illustrating the trends of glucose consumption and ethanol production during the course of fermentations with 10 g and 270 g glucose/l in the absence and presence of urea were generated and presented in Figures 4.3 and 4.4.

In the batch fermentation with 270 g glucose/l without urea in the medium (Figure 4.4), the glucose was taken up very slowly and the fermentation was not efficient due to the weak viability of yeast caused by the lack of nitrogen source. While, when adequate urea was present in the medium, the glucose concentration sharply decreased before the nutrients were depleted and then remained more or less

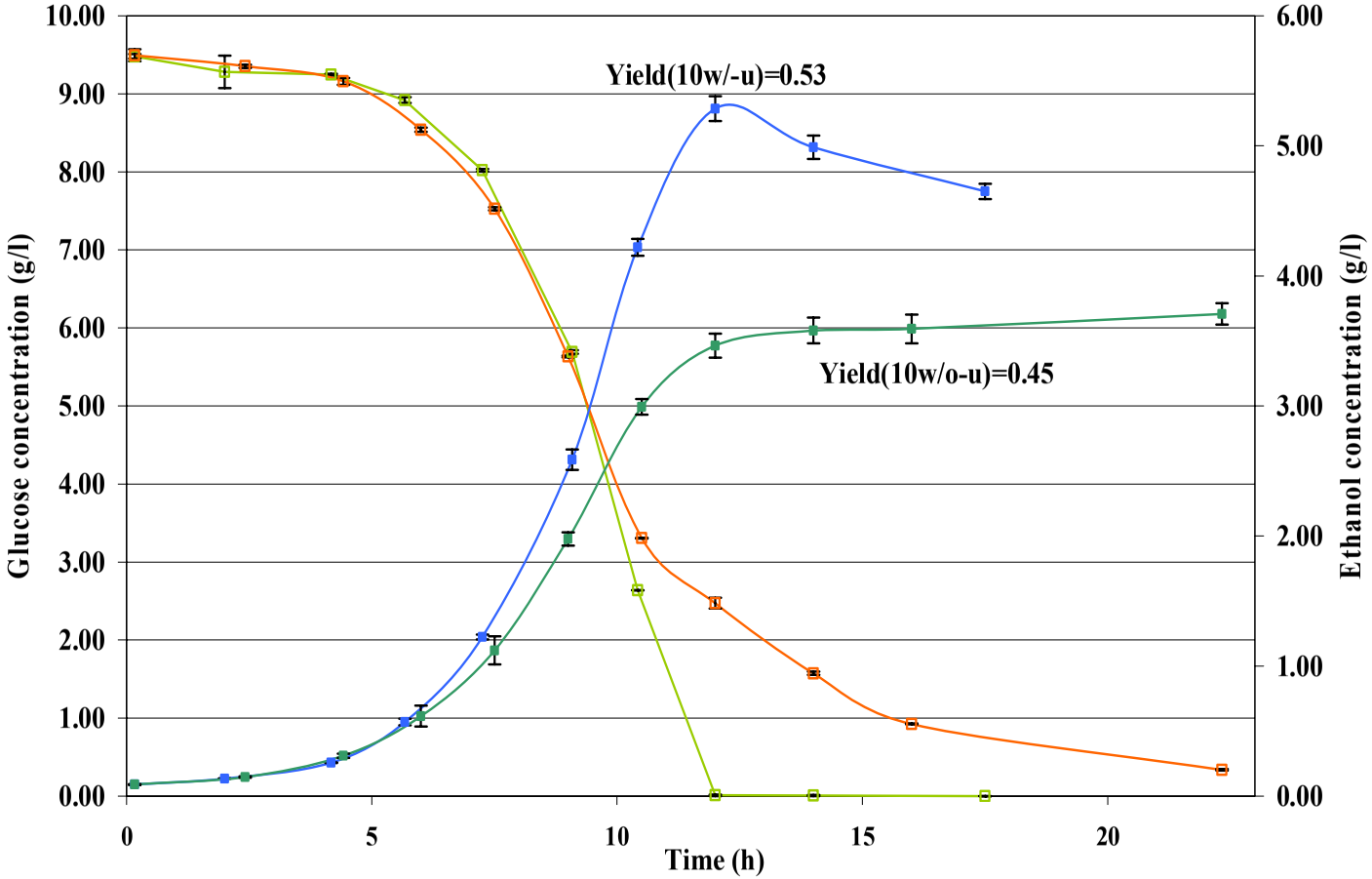

Figure 4.3 Glucose consumption and ethanol production during yeast growth at 10 g glucose/l in the absence and presence of urea

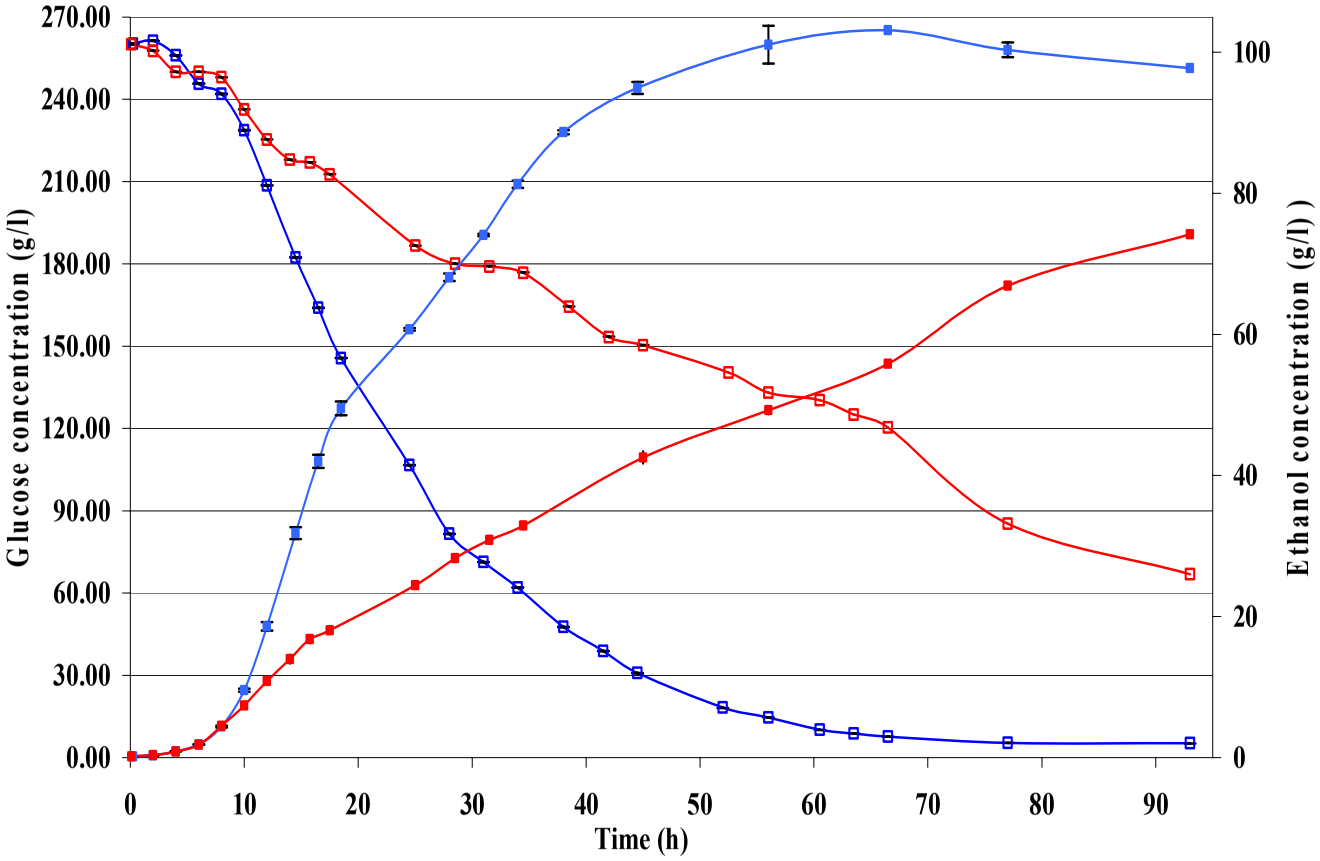

Figure 4.4 Glucose consumption and ethanol production during yeast growth at 270 g glucose/l in the absence and presence of urea. The error bars on some of the points are too small to visualize.

constant until the end of fermentation. The ethanol concentration increased in harmony and was considered to be related. However, as the growth continued, nutrient depletion occurred. Yeasts were dependent on glucose as a primary form of energy. As one can see in Figures 4.3 and 4.4, the diauxic shift appeared near the end of the fermentation, at approximately 12 h and 66 h respectively.

The results indicated that the lack of FAN prevents the yeast growth and imposes a limiting effect on consumption of glucose and ethanol formation. Addition of adequate levels of FAN in medium ensures efficient yeast growth and hence a desirable fermentation performance.

4.3 RNA Sample Collection

Culture samples were collected at late log phase considering the higher viability of yeast cells at log phase and the higher expression levels of stress genes upon entry into stationary phase. The integrity, purity and quantity of isolated RNA samples were detected by gel electrophoresis and UV spectrophotometer, and the resulted gel electrophoresis image was presented in Figure 4.5, where 28S ribosomal RNA bands were present with intensity approximately twice that of the 18S rRNA band, and all appeared as sharp bands which indicate there was no major RNA degradation.

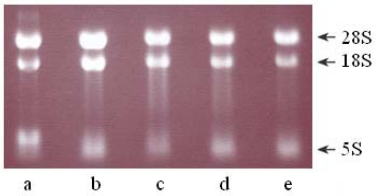

Figure 4.5 Gel electrophoresis image of RNA samples
From left to right: a, standards; b, 270 U; c, 270 Q; d, 10 Q; and e, 10 U.

4.4 Gene Expression Profiling

In this thesis, only genes involved in the primary pathways of yeast during respiro-fermentative metabolism with more than 1.5-fold transcriptional change are described and explained. They are the genes involved in glycolysis and gluconeogenesis pathways, pentose phosphate pathway, glycerol metabolic pathway and citric acid cycle (Figure 2.2), the genes involved in nitrogen metabolism and urea cycle (Figure 2.6), and the genes involved in proline and arginine metabolism (Figure 2.7). The concentrations of targeted metabolites and organic acids for microarray data validation are present in Table 4.1. The data of differentially expressed genes in the primary pathways are present in Table 4.2. Further details (microarray images, image processing, and data analysis etc.) can be found in the appendices.

Table 4.1 Average of extra-cellular metabolite concentrations measured at the late log phases of yeast growth under four different fermentation conditions;

Organic Acid (mM)	10W	10Q	270W	270Q
Acetic Acid	1.7648	0.6676	2.1653	1.9088
Lactic	0.0351	0.0123	1.6102	0.2810
Pyruvic Acid	0.0723	0.1195	1.0450	0.6509
α-Ketoglutaric Acid	0.0193	0.0216	0.1386	0.2244
Fumaric Acid	0.0011	0.0011	0.0039	0.0044
Citric Acid	0.0021	0.0017	0.0108	0.0063
Glycerol*	0.40	0.18	6.27	2.65

*g/l

Table 4.2 Genes in *S. cerevisiae* that experienced transcriptional change (>1.5-fold) when cells were grown in medium with different glucose and urea concentrations

1. Glycolysis and gluconeogenesis					
Gene name	**ORF**	**W10/Q10**	**W270/Q270**	**W270/W10**	**Q270/Q10**
ACS1	YAL054C	4.012	-0.911	-3.729	0.746
ACS2	YLR153C	1.365	0.36	-1.776	-1.12
ADH2	YMR303C	2.239	-0.194	-2.977	-0.111
ALD4	YOR374W	1.226	0.078	1.983	3.328
ALD6	YPL061W	2.2	0.176	1.602	3.663
ARO10	YDR380W	-0.482	-2.022	1.891	3.143
CDC19	YAL038W	-2.565	0.33	2.069	-0.72
ENO1	YGR254W	-1.731	0.707	1.958	-0.868
ENO2	YHR174W	-1.824	0.626	1.595	-1.006
FBP1	YLR377C	4.597	-0.174	-5.5	-0.033
GPM1	YKL152C	-1.893	0.467	1.788	-0.498
HXK1	YFR053C	0.073	-0.208	1.86	2.138
HXK2	YGL253W	-2.03	0.537	0.481	-2.153
PDC1	YLR044C	-2.461	0.138	2.218	0.041
PDC5	YLR134W	-2.475	0.196	2.521	-0.007
TDH1	YJL052W	-1.502	0.945	2.219	-0.452
2. Pentose phosphate pathway					
Gene name	**ORF**	**W10/Q10**	**W270/Q270**	**W270/W10**	**Q270/Q10**
ZWF1	YNL241C	-0.15	-0.727	0.578	1.209
GND1	YHR183W	-2.052	0.311	1.348	-0.969
GND2	YGR256W	-0.051	-1.119	-0.992	-0.327
RKI1	YOR095C	1.732	-0.432	-1.656	0.301
RPE1	YJL121C	0.061	0.136	-0.575	-0.676
TKL1	YPR074C	-0.365	0.026	-0.386	-0.642
TKL2	YBR117C	1.762	-1.206	-0.825	1.904
TAL1	YLR354C	0.1	-0.113	-0.12	0.001
FBP1	YLR377C	4.597	-0.174	-5.5	-0.033
3. Glycerol metabolism					
Gene name	**ORF**	**W10/Q10**	**W270/Q270**	**W270/W10**	**Q270/Q10**
HOR2	YER062C	1.441	-0.227	-3.347	-1.893
RHR2	YIL053W	3.181	-0.348	-3.913	-0.612
GUT1	YHL032C	2.328	-0.317	0.529	2.798
GUT2	YIL155C	1.291	-0.393	0.615	2.011
GPD1	YDL022W	1.681	-0.346	-2.69	-0.288
GPD2	YOL059W	0.398	-0.016	-1.256	-0.808
ADH2	YMR303C	2.239	-0.194	-2.977	-0.111
ALD4	YOR374W	1.226	0.078	1.983	3.328
ALD6	YPL061W	2.2	0.176	1.602	3.663
CHO1	YER026C	0.2	0.481	2.193	1.661

4. Citric acid cycle (TCA cycle)					
Gene name	**ORF**	**W10/Q10**	**W270/Q270**	**W270/W10**	**Q270/Q10**
ACO1	YLR304C	2.314	0.143	-2.992	-1.217
CIT1	YNR001C	2.541	-0.389	-1.842	0.692
CIT2	YCR005C	4.146	-2.342	-4.688	1.339
CIT3	YPR001W	2.731	0.095	-2.789	-0.025
FUM1	YPL262W	1.573	0.423	-2.554	-1.21
IDH1	YNL037C	1.639	-0.106	-2.303	-0.833
IDP1	YDL066W	1.309	0.371	-2.21	-1.727
IDP2	YLR174W	2.894	-0.371	-3.957	-0.511
MDH2	YOL126C	3.271	-0.645	-3.825	-0.002
KGD1	YIL125W	2.033	-0.096	-2.196	-0.357
PCK1	YKR097W	4.882	-0.472	-5.292	0.109
PYC1	YGL062W	2.25	-0.13	-1.481	0.442
SDH1	YKL148C	2.055	-0.345	-1.806	0.107
5. Urea cycle, amino groups and nitrogen metabolism					
Gene name	**ORF**	**W10/Q10**	**W270/Q270**	**W270/W10**	**Q270/Q10**
DUR1,2	YBR208C	-1.527	2.129	1.843	-1.758
ECM40	YMR062C	-0.025	0.39	-1.375	-1.68
ARG1	YOL058W	0.725	1.04	-1.666	-1.741
ARG3	YJL088W	1.426	-0.478	-2.497	-0.524
ARG4	YHR018C	0.049	0.718	-1.46	-2.02
CAR1	YPL111W	0.585	3.237	2.633	-0.134
CAR2	YLR438W	0.853	2.04	2.054	0.471
GDH1	YOR375C	-1.731	0.951	0.535	-2.053
GDH2	YDL215C	1.322	0.054	-0.681	0.122
GDH3	YAL062W	2.057	-0.029	-2.423	-0.106
6. Arginine and proline metabolism					
Gene name	**ORF**	**W10/Q10**	**W270/Q270**	**W270/W10**	**Q270/Q10**
PUT1	YLR142W	-2.835	0.167	3.645	0.876
PUT2	YHR037W	-0.049	0.176	0.373	-0.113
ALD4	YOR374W	1.226	0.078	1.983	3.328
ALD6	YPL061W	2.2	0.176	1.602	3.663
ARG1	YOL058W	0.725	1.04	-1.666	-1.741
ARG3	YJL088W	1.426	-0.478	-2.497	-0.524
ARG4	YHR018C	0.049	0.718	-1.46	-2.02
ARG5,6	YER069W	0.483	0.278	-1.253	-1.287
ARG8	YOL140W	-0.256	0.4	-0.348	-1.005
CAR1	YPL111W	0.585	3.237	2.633	-0.134
CAR2	YLR438W	0.853	2.04	2.054	0.471
PRO3	YER023W	-1.367	0.549	0.991	-1.016

4.4.1 The Effect of Urea at Low Glucose Condition (W10/Q10)

4.4.1.1 Glycolysis and gluconeogenesis

According to the growth curve (Figure 4.3), glucose was taken up very fast when adequate nitrogen was provided at low gravity condition. It was most likely that the only carbon source left at late log phase of W10 was ethanol. In conjunction to our data (Table 4.2), as compared to Q10, HXK2, TDH1, GPM1, ENO1, ENO2, PDC1, PDC5 and CDC19 involved in glycolysis were down-regulated nearly two folds, corresponding with low concentration of its end product, pyruvic acid (Table 4.1). Whereas, FBP1 (fold change= 4.597), responsible for the conversion of fructose-1,6-bisphosphate to fructose-6-phosphate during gluconeogenesis metabolism, ADH2 (fold change= 2.239), ALD6 (fold change= 2.2) and ACS1 (fold change= 4.012) catalyzing the utilization of ethanol were up-regulated. Transcription level of ALD4 and ACS2 were also increased but to a less extent than those above. HXK1 was not affected by the urea level. The regulations of ALD4 and ALD6 responsible for acetate production are in accordance with the extra-cellular acetic acid concentration (Table 4.1).

4.4.1.2 Pentose phosphate pathway (PPP)

The expression of the gene GND1, responsible for NADPH regenerating reaction in the PPP, was down-regulated, indicating the decreased flux of glucose-6-phosphate through the PPP when yeast was grown in an enriched media (Lagunas and Gancedo, 1973), such as W10. In addition, NADPH/NADH released from the oxidization of ethanol to acetyl-CoA makes the NADPH from PPP dispensable

(Zimmermann et al., 1997). The genes TKL2 and RKI1 were all up-regulated towards the direction of gluconeogenesis intermediate production to generate more 6 carbon sugars for the synthesis of cellular component.

4.4.1.3 Glycerol metabolism

Our data (Table 4.2) shows that genes involved in the biosynthetic and dissimilatory pathways for glycerol were all up-regulated. They are genes HOR2 (fold change= 1.441), RHR2 (fold change= 3.181), GUT1 (fold change= 2.328), GUT2 (fold change= 1.291), GPD1 (fold change= 1.681), and GPD2 (fold change= 0.398). The results shows that the G3P shuttle (Figure 2.3) and glycerol formation as to reoxidize the cytolic NADH was used more extensively with ethanol than with glucose as the substrate, which might be a reflection of an increased production rate of NADH when using a reduced substrate such as ethanol.

4.4.1.4 Citric acid cycle (TCA cycle)

In conjunction to Figure 4.3, ethanol was the only carbon source left. Energy for biosynthesis has to be provided through the respiratory chain (Neeff et al., 1977). As a result, the mitochondrial genes required for TCA cycle: ACO1, CIT1, CIT3, FUM1, IDH1, IDP1, IDP2, KGD1, SDH1, were all up-regulated in W10 as compared to Q10. Four genes involved in gluconeogenesis metabolism and glyoxylate cycle required for the metabolism of ethanol were also greatly induced. They are the genes encoding pyruvate carboxylase (PYC1, fold change= 2.25), phosphoenolpyruvate carboxykinase (PCK1, fold change= 4.882), citrate synthase (CIT2, fold change= 4.146), and cytosolic malate dehydrogenase (MDH2, fold

change= 3.271) (Kim et al., 1986; Walker et al., 1991; Proft et al., 1995; Minard et al., 1991). Extra-cellular α-ketoglutaric acid, fumaric acid and citric acid were detected at similar levels in both conditions, so no connection with the different expression levels could be shown.

4.4.1.5 Nitrogen metabolism, urea cycle and metabolism of amino groups

Two genes (GDH1 and GDH3) encoded $NADP^+$-dependent glutamate dehydrogenase were expressed oppositely, GDH1 was expressed to a lower extent (fold change= -1.731) in the presence of urea as compared to in the absence of urea, whereas, GDH3 was expressed to a greater extent (fold change= 2.057). This is because the expression of GDH1 and GDH3 is differentially regulated and depends on the nature of the carbon source (DeLuna et al., 2001). The NAD^+-dependent glutamate dehydrogenase GDH2 was up-regulated (fold change= 1.322). The expression of the gene for urea degradation (Cooper et al., 1980) was less induced (DUR1,2, fold change= -1.527) when urea is present in the medium. The urea effect on the regulation of ECM40 was insignificant.

4.4.1.6 Arginine and proline metabolism

PUT1, the gene encoding the yeast proline oxidase was down-regulated (fold change=-2.835) in W10 as compared to Q10, suggesting that the level of proline utilization as nitrogen source in the presence of urea is lower. The urea effect on the regulation of gene PUT2 was insignificant. ARG5,6, ARG4, ARG3 and ARG1 responsible for arginine biosynthesis, and CAR1 and CAR2 involved in arginine

utilization and urea cycle were all slightly increased when urea is present in the medium.

4.4.1.7 Summary

The influence of urea level on the low gravity fermentation was not significant due to the occurrence of diauxic shift at the time point of harvesting yeast. As compared to Q10, the up-regulation of genes involved in ethanol production, down-regulation of the genes involved in glycolysis, and up-regulation of mitochondrial genes all confirmed the hypothesis obtained from the growth curve of 10 g/l glucose with urea as nitrogen source condition (Figure 4.3) that the diauxic shift occurred at the time point of harvesting yeast.

4.4.2 The Effect of Urea at High Glucose Condition (W270/Q270)

4.4.2.1 Glycolysis and gluconeogenesis

At very high gravity conditions, the urea effect on the expression levels of all the glycolysis related genes was insignificant, except for the repressed regulation of gene ARO10 (fold change= -2.022), which catalyzes decarboxylation of phenylpyruvate to phenylacetaldehyde (Vuralhan et al., 2003).

4.4.2.2 Pentose phosphate pathway

Generally, the urea effect on the pentose phosphate pathway under very high gravity conditions was insignificant. Only genes ZWF1, TKL2, GND2, and RKI1 were slightly down-regulated. This may suggest that nitrogen level did not have significant effects on the NADPH/$NADP^+$ balance.

4.4.2.3 Glycerol metabolism

As compared to Q270, genes involved in the biosynthetic (HOR2, RHR2 and GPD1) and dissimilatory pathways (GUT1 and GUT2) for glycerol were slightly down-regulated in W270. The results showed that the G3P shuttle and glycerol formation as to reoxidize the cytolic NADH was used less extensively in the presence of urea, which might be a reflection of a more balanced environment if adequate nitrogen is provided.

4.4.2.4 Citric acid cycle (TCA cycle)

Under very high gravity conditions, the urea effect on the TCA cycle related genes was insignificant, only the gene involved in the glyoxylate cycle which serves for gluconeogenesis metabolism was down-regulated (CIT2, fold change= -2.342). Extra-cellular α-ketoglutaric acid, fumaric acid and citric acid were also detected at similar levels in both conditions (Table 4.1).

4.4.2.5 Nitrogen metabolism, urea cycle and metabolism of amino groups

DUR1,2 (fold change= 2.129) responsible for urea degradation had a higher transcription level at W270 as compared to Q270. The ammonia produced from urea degradation would induce the glutamate biosynthesis, which lead to the up-regulation of GDH1. The urea effect on the regulation of genes for glutamate catabolism or anabolism (GDH2 and GDH3) and urea cycle (ARG3, ARG4, and ECM40) were insignificant.

4.4.2.6 Arginine and proline metabolism

At very high gravity conditions, arginine utilization genes CAR1 (fold change= 3.237) and CAR2 (fold change= 2.04) were more active in the presence of urea, expression of gene ARG1 responsible for arginine biosynthesis was also slightly up-regulated. This result suggested that adequate nitrogen source could stimulate high activity of nitrogen metabolism when yeast was grown under very high gravity environment. The urea effect on the expression of proline utilization genes PUT1 and PUT2 was insignificant.

4.4.2.7 Summary

Overall, these data imply that the urea level did not affect the primary metabolic pathway, but it did stimulate the nitrogen metabolism. The enhanced amino acid degradation to glutamate could enable higher activities in the TCA cycle, which could therefore generate more energy for biosynthesis and yeast cell proliferation under very high gravity fermentation conditions. High urea level could also stimulate nitrogen metabolism when yeast was grown under very high gravity environment.

4.4.3 The Effect of Glucose in the Presence of Urea (W270/W10)

4.4.3.1 Glycolysis and gluconeogenesis

Given adequate nitrogen level, as compared to the glucose depleted environment in W10, the very high gravity stimulated the transcription of most of the genes involved in glycolysis metabolism and hence the accumulation of pyruvic acid (Table 4.1). Notable among these are the genes TDH1, PDC1, PDC5 and CDC19, each of which is increased over twofold (Table 4.2). Other genes whose transcription level showed modest increase are HXK1, GPM1, ENO1 and ENO2. Genes ALD4 and ALD6 responsible for a small amount of acetate production (Rose et al., 1987; Remize et al., 2000) during glucose fermentation were also up-regulated (fold change= 1.983 and 1.602), which is in accordance with the extra-cellular acetic acid concentration (Table 4.1). Accordingly, gene involved in ethanol utilization, such as ADH2 and genes involved in gluconeogenesis metabolism such as ACS1, ACS2 and FBP1 were subject to carbon catabolite repression. These data implies that the yeast has a strong tendency towards fermentation metabolism. ARO10 catalyzing decarboxylation of phenylpyruvate to phenylacetaldehyde was up-regulated (fold change= 1.891). The transcription of glucose-induced gene HXK2 was slightly increased.

4.4.3.2 Pentose phosphate pathway

Gene GND1 (fold change= 1.348) was up-regulated in W270 as compared to W10, as the rapidly proliferating cell needs large quantities of NADPH for the conversion of ribonucleotides (RNA) to deoxyribonucleotides (DNA). The gene

RKI1, responsible for the interconversion of ribose 5-phosphate and ribulose 5-phosphate, was down-regulated (fold change= -1.656).

4.4.3.3 Glycerol metabolism

Interestingly, as compared with W10, genes involved in the biosynthetic pathway for glycerol were all down-regulated in W270, even though the extracellular glycerol concentrations (Table 4.1) at sampling times were found to be 10-fold higher in VHG sample. They are HOR2 (fold change= -3.347), RHR2 (fold change= -3.913), GPD1 (fold change= -2.69). This is probably because sampling point was at the late exponential phase, whereas samples during VHG fermentations were measurable only in the stationary phase (Devantier et al., 2005). According to the comparison between W270 and W10, where glucose and ethanol were used as substrate, respectively, it seems likely that the glycerol formation played an important role in maintaining the cytosolic redox balance when NAD^+ was needed to oxidize the ethanol.

4.4.3.4 Citric acid cycle (TCA cycle)

As compared to the ethanol-grown environment in W10 where mitochondrial genes were more active, when yeast is growing in a glucose-grown environment in W270, all of the mitochondrial genes involving in the TCA cycle, such as ACO1, CIT1, CIT3, FUM1, IDH1, IDP1, IDP2, KGD1, SDH1, as well as several genes involved in gluconeogenic metabolism, such as CIT2, PYC1, PCK1, and MDH2 were subject to glucose repression (Polakis et al., 1965), because glycolysis is the major energy-yielding pathway in the presence of glucose. Extra-cellular α-

ketoglutaric acid, fumaric acid and citric acid detected in W270 were more than W10. Since the gene expression values are log-ratios of relative intensities, connection with the metabolite concentration may not be shown.

4.4.3.5 Nitrogen metabolism, urea cycle and metabolism of amino groups

In the presence of urea, as expected, GDH3 displayed a lower expression level (fold change=-2.423) under very high gravity condition as compared to W10 where glucose was depleted. The expression of glutamate dehydrogenase GDH1 and GDH2 did not very much. Genes ARG1, ARG3 and ARG4 displayed lower expression levels in W270 as compared to W10. The regulation of ECM40 was insignificant. The regulation of gene for urea degradation (DUR1,2) was elevated at W270 as compared to W10. This suggests that given adequate nitrogen, very high glucose concentration stimulates urea degradation.

4.4.3.6 Arginine and proline metabolism

In the presence of urea, genes responsible for arginine catabolism, CAR1 (fold change= 2.633) and CAR2 (fold change= 2.054) (Middelhoven, 1964), as well as the gene encoding the yeast proline oxidase, PUT1 (fold change= 3.645) all showed higher expression level at very high gravity conditions. The results suggested that at very high gravity environment due to the high urea uptake rate, at the point of yeast harvesting, arginine and proline might have been used as nitrogen sources when the primary nitrogen source was not available due to urea depletion.

4.4.3.7 Summary

The occurrence of diauxic shift in W10 shift the yeast metabolism from anaerobic fermentation of glucose to ethanol utilization, therefore different modes of metabolism to gain energy were exhibited. This mechanism affected the gene expression pattern in glycolysis and gluconeogenesis pathways and citric acid cycle. The yeast gene expression pattern in arginine and proline metabolism and nitrogen metabolism showed that very high gravity environment can stimulate urea uptake rate and therefore the activity of nitrogen metabolism of yeast.

4.4.4 The Effect of Glucose in the Absence of Urea (Q270/Q10)

4.4.4.1 Glycolysis and gluconeogenesis

In the absence of urea, with glucose as sole carbon source under both very high gravity and low gravity conditions, HXK1 (fold change= 2.138) had the opposite expression pattern to HXK2 (fold change= -2.153), in line with HXK2's known repression of HXK1 (Rodriguez et al. 2001). ALD4, ALD6 and ARO10 were up-regulated over threefold in response to very high gravity stress. The up-regulation of ARO10 that leads to ethanol production was triggered by the high energy demanding caused by ethanol stress (Piper, 1995), and the reason is that in the presence of high concentration of glucose, ethanol formation through glycolysis metabolism is the major energy-yielding pathway to meet the high energy demand. The increase in acetate-coding genes ALD4 and ALD6 is in accordance with the high acetic acid concentration in Q270 as compared with Q10. Other groups of genes involved in the glycolysis metabolism did not show significant transcription

changes. The concentration of extra-cellular pyruvic acid detected in Q270 was higher than Q10 (Table 4.1), so no connection with the gene expression levels could be shown.

4.4.4.2 Pentose phosphate pathway

When yeast was grown under conditions with nitrogen deficiency and very high gravity such as Q270, yeast stopped proliferating and the synthesis of nucleic acids, therefore, the gene TKL2 encoding transketolase for the generation of glyceraldehyde-3-phosphate was up-regulated and channeled the PPP intermediates into the glycolysis metabolism.

4.4.4.3 Glycerol metabolism

The genes involved in dissimilatory pathway of glycerol, GUT1 (fold change= 2.798) and GUT2 (fold change=2.011) were up-regulated, and genes involved in biosynthesis pathway of glycerol, GPD1, GPD2, RHR2, and especially the gene HOR2 (fold change=-1.893) were down-regulated. The up-regulation of gene GUT2 may indicate higher activity of G-3-P shuttle that deoxidizes excess NADH generated by higher biomass synthesis under very high glucose concentration. The down-regulation of glycerol biosynthesis genes suggested that in condition with urea deficiency, very high gravity caused less activity of glycerol formation and might have led to failure of maintaining cytosolic redox balance, which would otherwise keep the cells functioning.

4.4.4.4 Citric acid cycle (TCA cycle)

When nitrogen level was not adequate, the glucose effect on the expression of genes involved in the TCA cycle was insignificant, and only the gene IDP1 encoding isocitrate dehydrogenase which diverts α-ketoglutarate to biosynthetic processes (Haselbeck et al., 1993) was repressed. Extra-cellular fumaric acid and citric acid were also detected at similar levels in both conditions.

4.4.4.5 Nitrogen metabolism, urea cycle and metabolism of amino groups

The repressed expression of GDH1 in Q270 as compared to Q10 (fold change= -2.053) indicated that Gdh1p catalyzed the reaction in an opposite direction to produce α-ketoglutarate and energy, as well as to supplement to TCA cycle, as more ATP was required when yeast was grown in a very high gravity environment. The expression of genes GDH3 and GDH2 did not very much. Genes ARG1, ARG3, ARG4 and ECM40 involved in urea cycle (Jauniaux et al., 1978) displayed lower expression levels in Q270 as compared to Q10. The regulation of gene for urea degradation (DUR1,2) was repressed in Q270 as compared to Q10.

4.4.4.6 Arginine and proline metabolism

In the absence of urea, the glucose effect on the regulations of genes for arginine utilization, CAR1 and CAR2, and genes for proline utilization, PUT1 and PUT2 was insignificant. Genes ARG1, ARG4, ARG5,6 and ARG 8 were down-regulated, and the results indicated that the activities of genes encoding enzymes of the nitrogen metabolism were inhibited by very high gravity environment.

4.4.4.7 Summary

Under nitrogen deficiency condition, the glucose effect on the yeast reflected that high gravity environment triggered higher energy demand and higher activity of redox balancing metabolism and that yeast grown under low gravity environment was provided with a better opportunity for redox balancing which maintains the cell function. In low nitrogen level culture, the nitrogen metabolism was inactivated by very high gravity stress.

5. CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Our data provide insight into the regulatory mechanisms of *S. cerevisiae* in coping with VHG stresses and nutritional deficiency. A number of conclusions can be drawn from the metabolism and gene regulation analysis. Briefly these are:

1. The high nitrogen culture displayed higher mRNA levels for genes involved in nitrogen compound recycling, which would enhance the activities in TCA cycle and generate more energy for biosynthesis and yeast cell proliferation under very high gravity fermentation conditions, although the up-regulation of genes involved in TCA cycle was not shown.
2. According to the gene expression profile of contrasts Q270/Q10 and W270/Q270, respectively, we conclude that in nutrient deficient culture, the activities of genes encoding enzymes of the nitrogen metabolism were inhibited by very high gravity environment. In energy sufficient cultures, the activities of nitrogen metabolism were stimulated when yeast was grown in high nitrogen culture.

3. Activities of G3P shuttle under different conditions indicated difference in redox balancing, which might have suggested an explanation of higher stress-tolerance and higher fermentation efficiency of yeast under certain condition. Very high gravity with high nitrogen level culture provides a more redox balanced environment, while very high gravity with urea deficiency culture leads to failure of maintaining cytosolic redox balance, which eventually affects the cells function.

5.2 Recommendations

The following recommendations should be considered in future studies:

1. Time points for harvesting yeast must be carefully chosen to avoid the affection of any undesired environmental changes on gene expression pattern. Such as the fermentation under 10 g/l glucose case, diauxic shift occurs as the glucose is being depleted, therefore sampling before this point would allow greater confidences in explaining the effect of certain stress on yeast transcriptional profile.
2. In glass slide microarray studies, RNA from the target sample and from the 'control' sample are pairwise studied as an equivalent mixture in which the 'control' RNA is the reference for expressing the gene transcript levels in the target sample, thus the quantification of gene transcript levels in pairwise comparisons makes the results less straightforward. To overcome this, Affymetrix microarray platform is recommended, because it generates a gene expression profile of one sample and therefore quantifies gene transcript

levels in arbitrary (but well-defined) expression units and enhances the reliability of the comparisons between separately analyzed samples.

3. When using metabolite profiling approach to test the hypothesis that suggested by genomics, an combined measurement of both intracellular and extracellular metabolite concentrations would give a more direct and comprehensive analysis of the real outcome of the potential changes.
4. To get a complete picture of the yeast response to nutritional deficiency and VHG stresses, it will be interesting for further studies to integrate data from all levels of "-omics" which include metabolomic, proteomic and transcriptomic data to a single data matrix to get a holistic view on the changes associated with VHG fermentation. Such analyses would link metabolic changes in biochemical pathways to the enzymes involved, and subsequently to the genetic alterations, leading altogether to obtain the direction of strain improvement for enhanced ethanol production.
5. The data presented here are based on a 2^2 microarray experimental design (see Chapter 1, Experimental Design). Analysis of three pairs of replicate dye-swaps (6 chips) using the same contrast RNA samples may reveal further trends in gene expression not considered herein.

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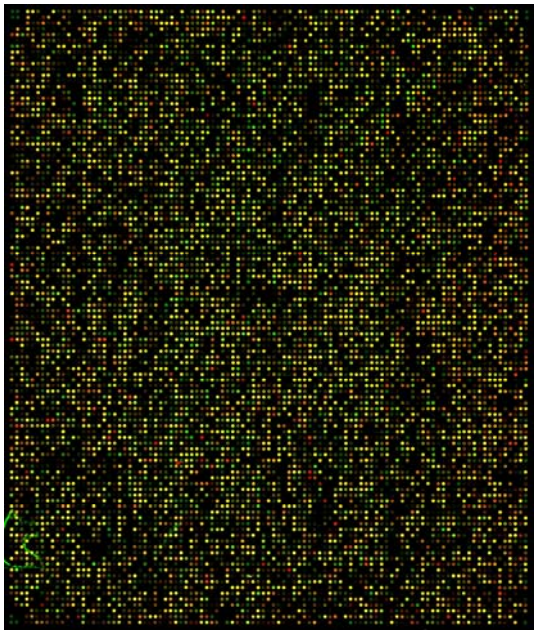
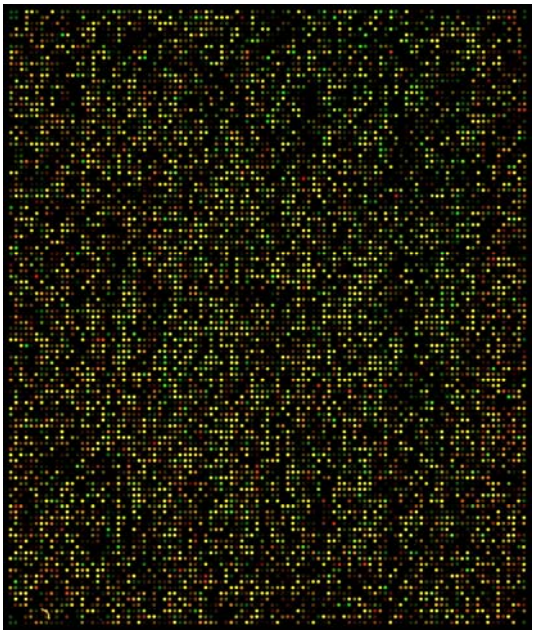
APPENDICES

A1-Oligonucleotide Microarray Images

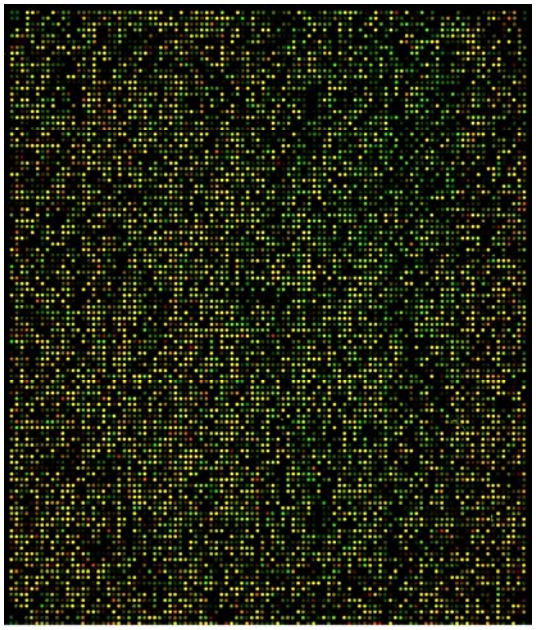
The mircroarray slides were scanned by a dual-beam laser scanner. The obtained pre-normalized images with the presence of non-biological differences (inherent noise and systematic variation) between two samples are shown in Figure A1.



a. 10Q-3 270Q-5



c. 10U-3 270U-5



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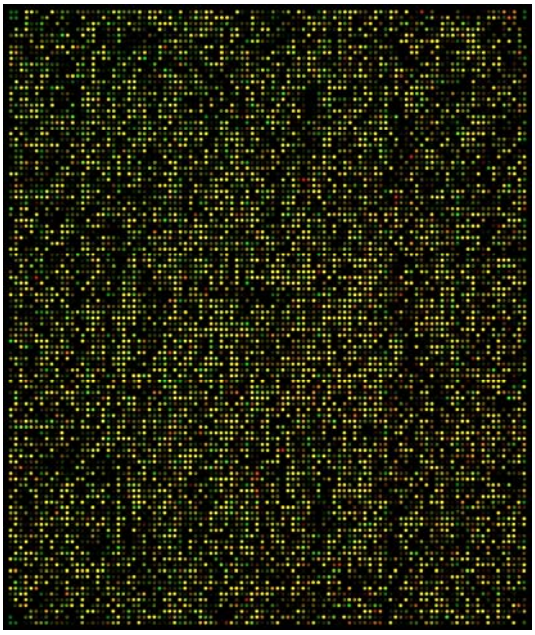

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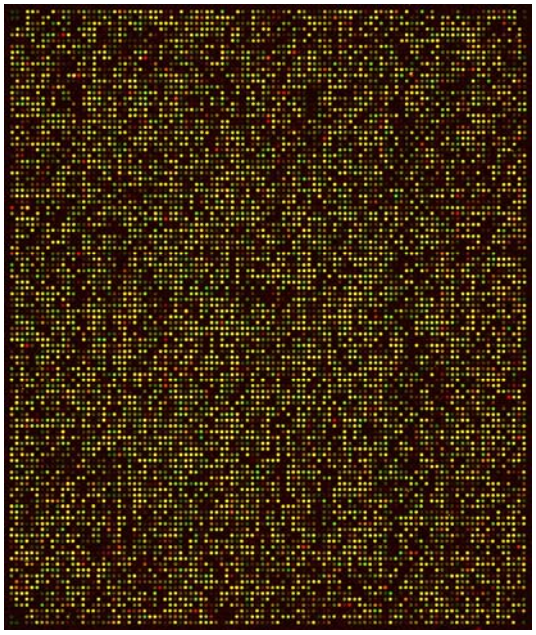
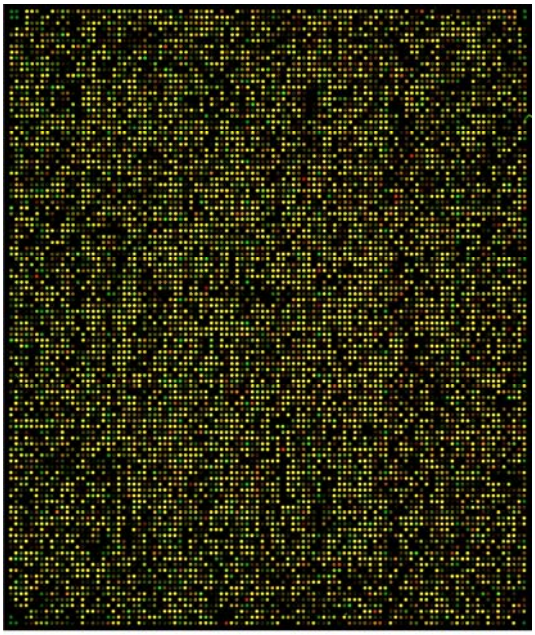


d. 10U-5 270U-3



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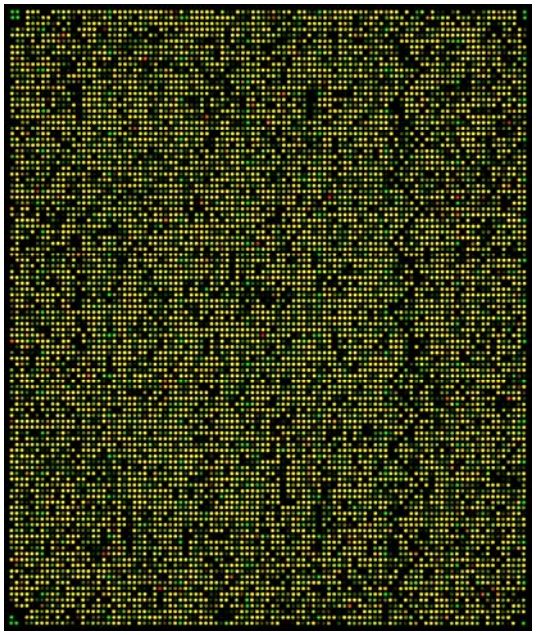
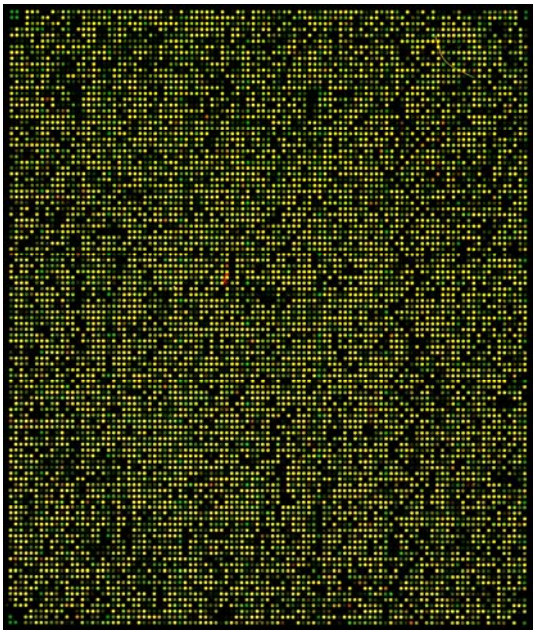
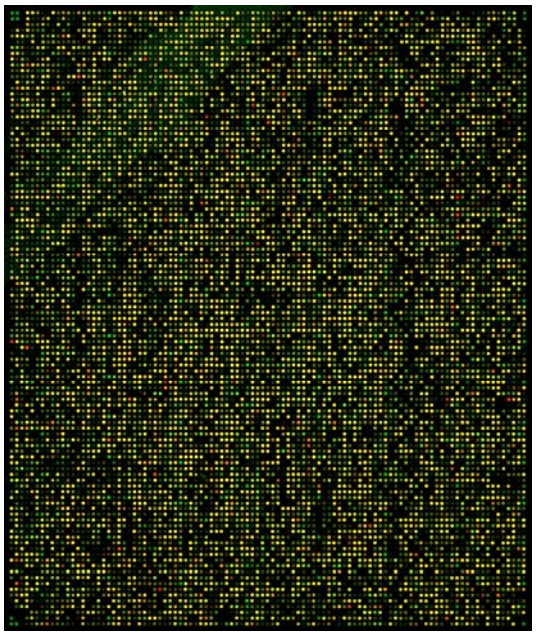


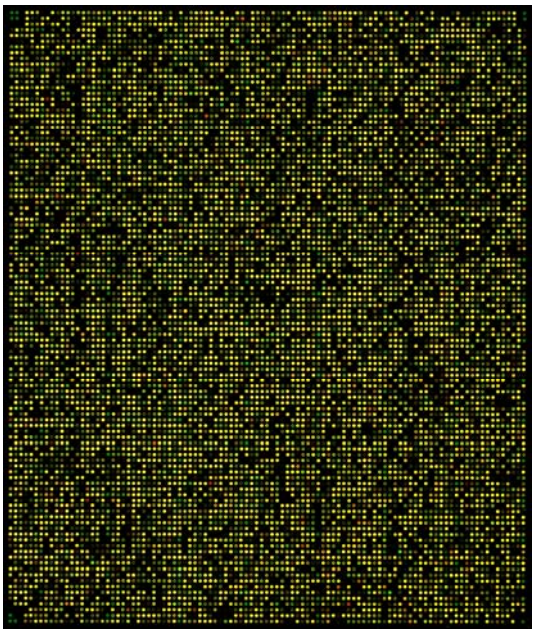
f. 10U-5 10Q-3



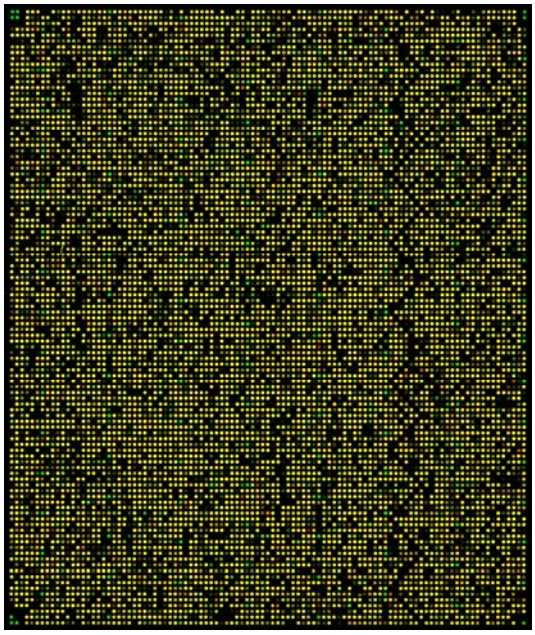
i. 270U-3 270Q-5



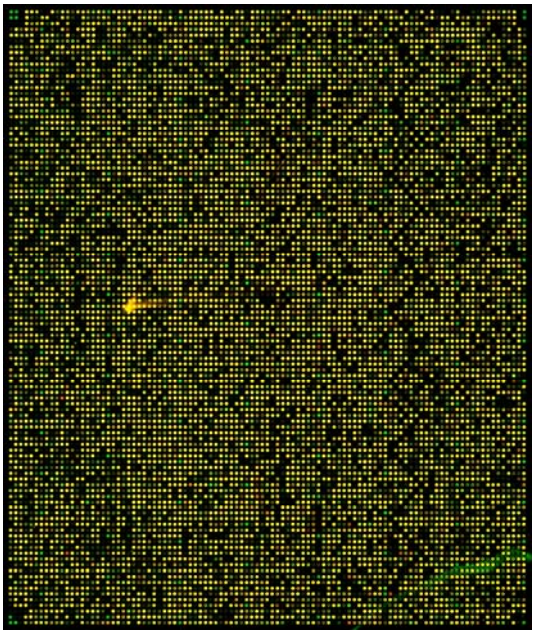
h. 10U-5 10Q-3



k. 270U-3 270Q-5



j. 270U-5 270Q-3



l. 270U-5 270Q-3

Figure A1: Oligonucleotide Microarray images prior to normalization
'3' and '5' represent Cy3 dye and Cy5 dye that were used to process RNA samples. 3 stands for Cyanine 3; 5 stands for Cyanine 5

A2-Microarray Data Processing

The microarray raw images (Figure A1) shown in Appendix 1 were re-represented using MA plots (Figure A2-1) and subject to the following normalization and inference steps:

1. Background correction (Figure A2-2)
2. Normalization and scaling among arrays (Figures A2-3 and A2-4)
3. Bayesian inference using limma package (Figures A2-5 and A2-6)

An MA plot (also called a RI plot) is a plot used to show the intensity-dependent microarray data in log-ratio scale. The MA plot is a scatter plot with transformed data; in which, x-axis represents the average log intensity from 2 channels ($A = \log_2 \sqrt{R \cdot G}$) while y-axis represents the log-ratios ($M = \log_2 R/G$). R is the intensity strength emitted by Cy5 dye (in red color), and G is the intensity strength emitted by Cy3 color (in green color).

Figure A2-1 provides an overview of all microarry data generated from different combinations of experimental conditions. For example, the condition 'cy5-cy3=Q10-W10' represents yeast samples harvested from 10 g glucose/l in the absence of urea (Q10) and in the presence of urea (W10) were colored with cy5 and cy3 dye, respectively. The 'marray' package, an R statistics package, was used to remove background noises embedded in the microarray images during scanning (Figure A2-2). These background-collected data were then normalized to the same scale among all microarray data, and presented in a format of MA plot (Figure A2-3) and box plot (Figure A2-4). Then, the 'limma' package, another R statistics package

designed for processing microarray data, was used to infer the normalized data. The inferred results are presented in a format of MA plot (Figure A2-5) and volcano plot (Figure A2-6).

The volcano plots provide an overall visualization of gene expression profiles. The upper right section of the plot indicates significant up-regulations, and upper left section indicates significant down-regulations.

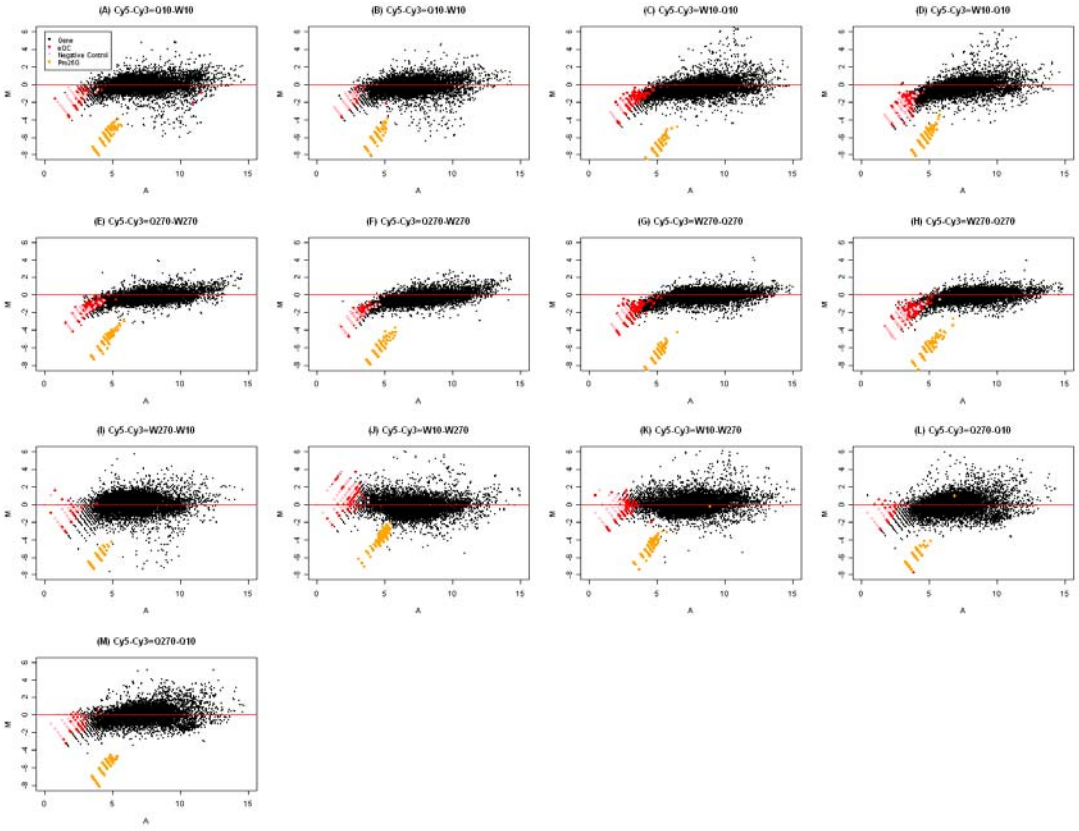


Figure A2-1 MA plots of raw data

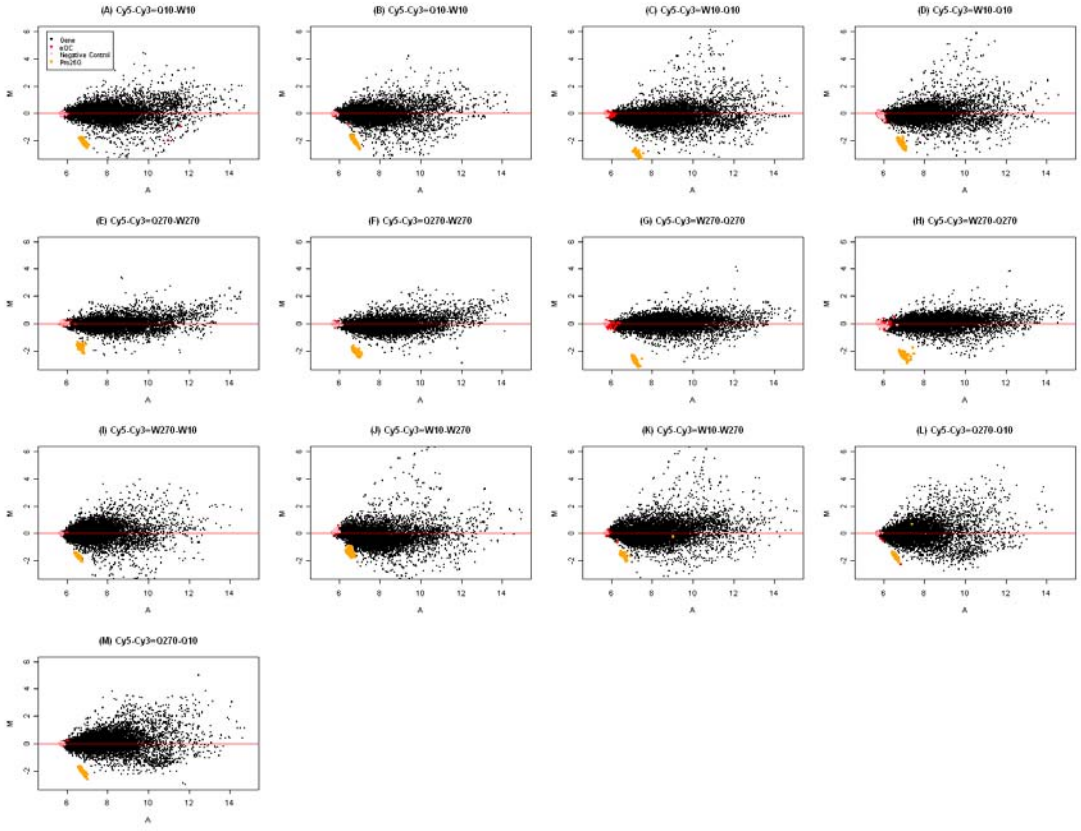


Figure A2-2 MA plots of background corrected array data

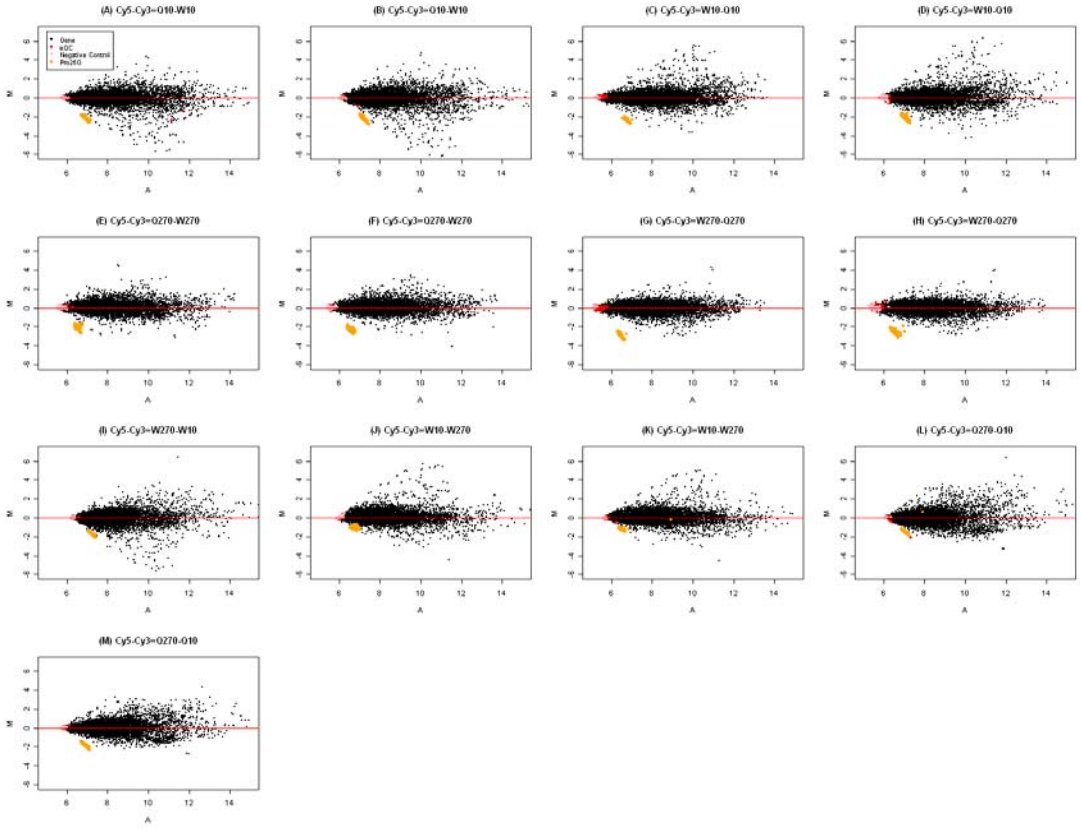


Figure A2-3 MA plots of normalized between-array data

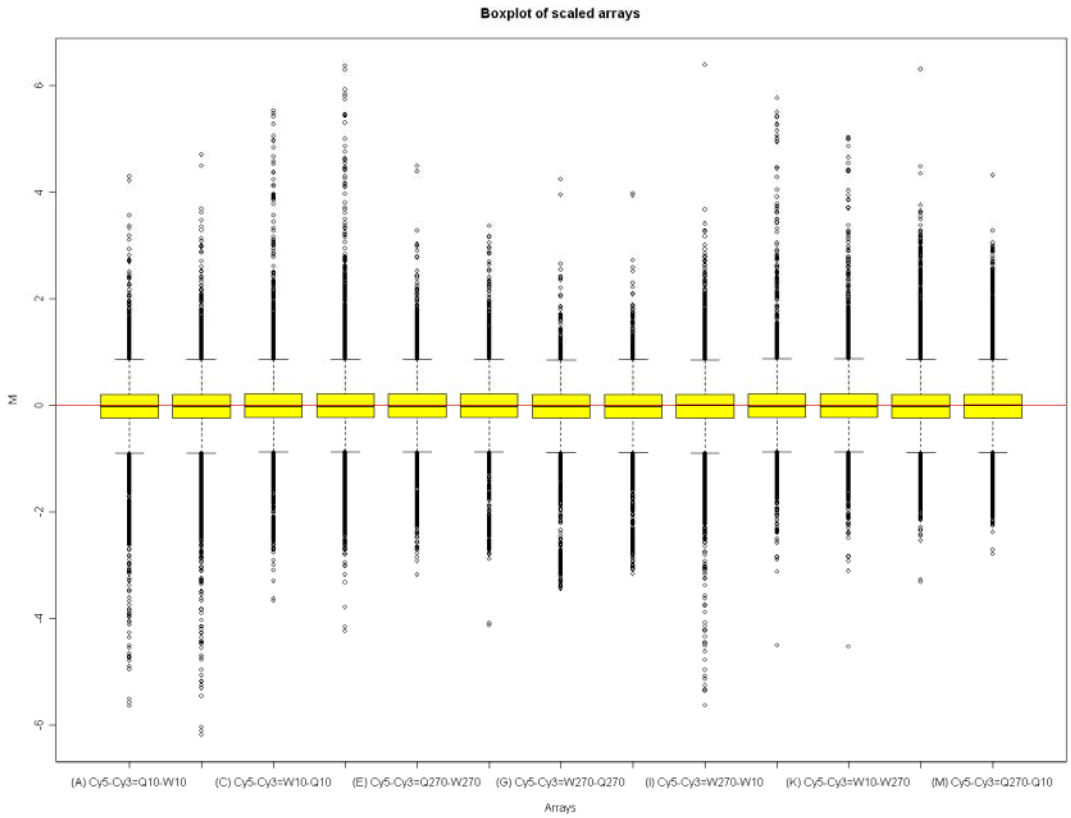


Figure A2-4 Box plot of normalized between-array data

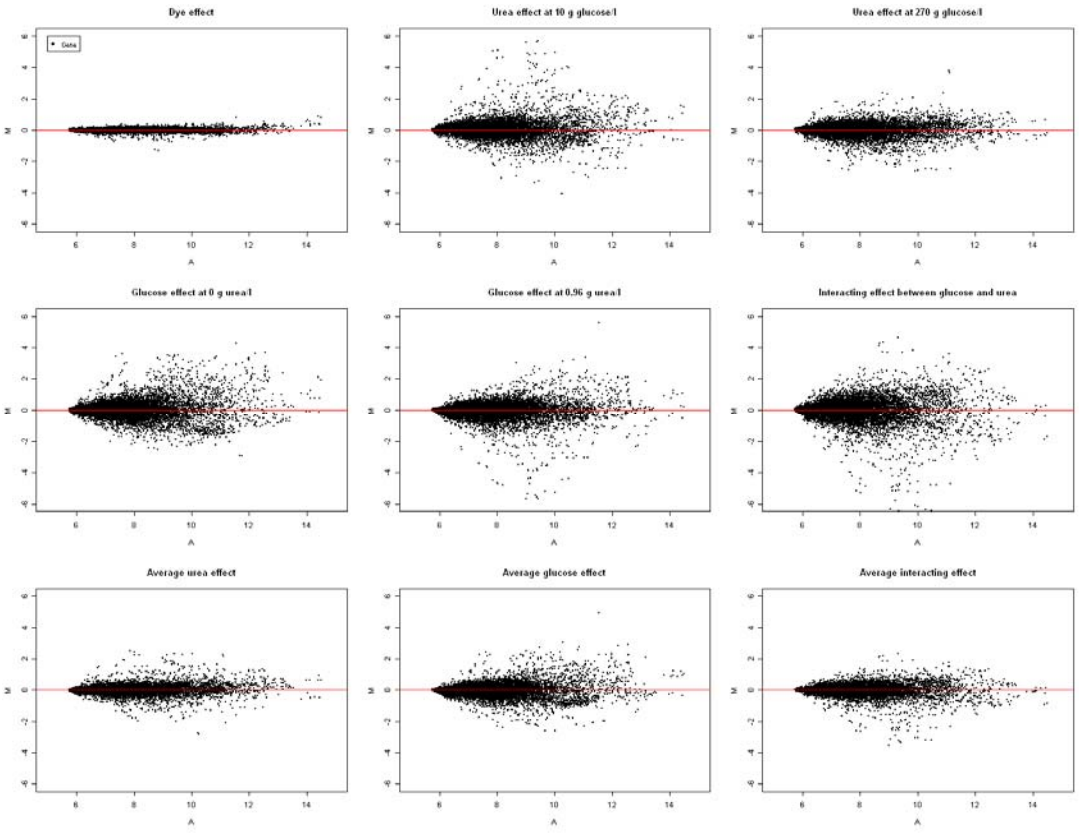


Figure A2-5 MA plots for different experimental conditions

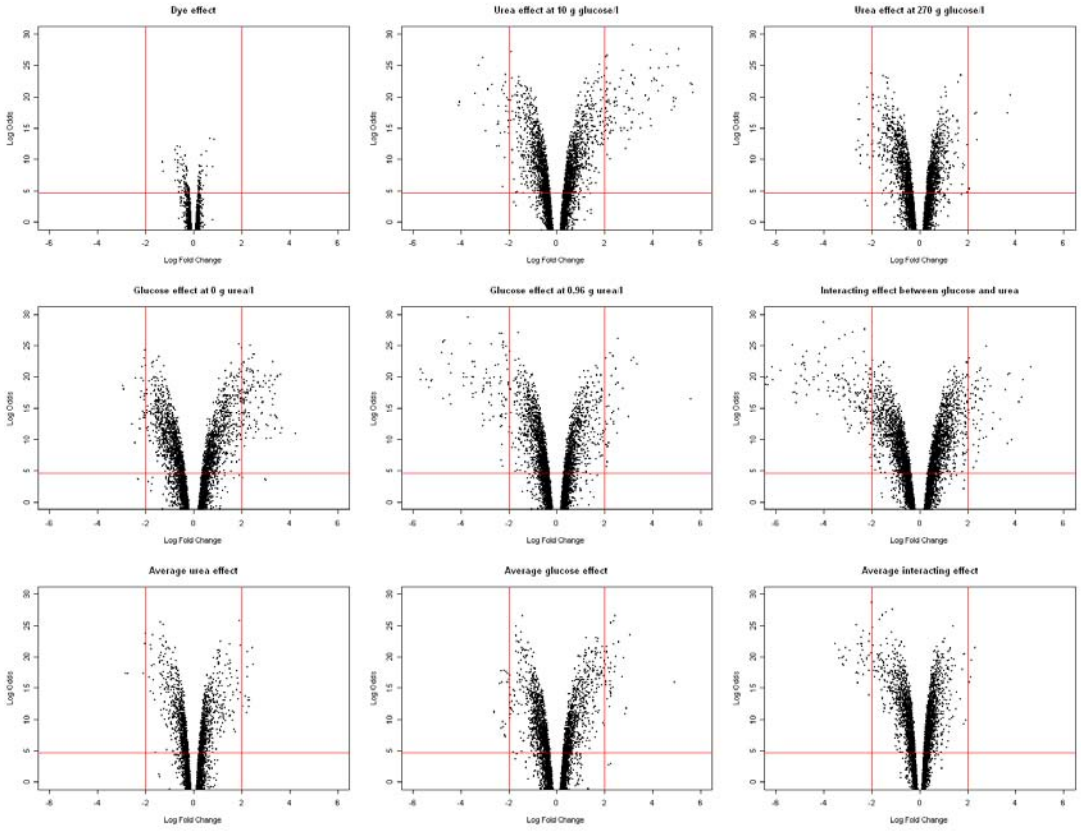


Figure A2-6 Volcano plots for different experimental conditions

A3-Calibration Curves for Organic Acids

The calibration curves of six organic acids were established using IC (see Chapter 3 Material and Methods). They are present in Figure A3. Included in these figures are the relationships between area counts and concentrations (ppm), as well as the equations and the regression coefficients for the calibration curves.

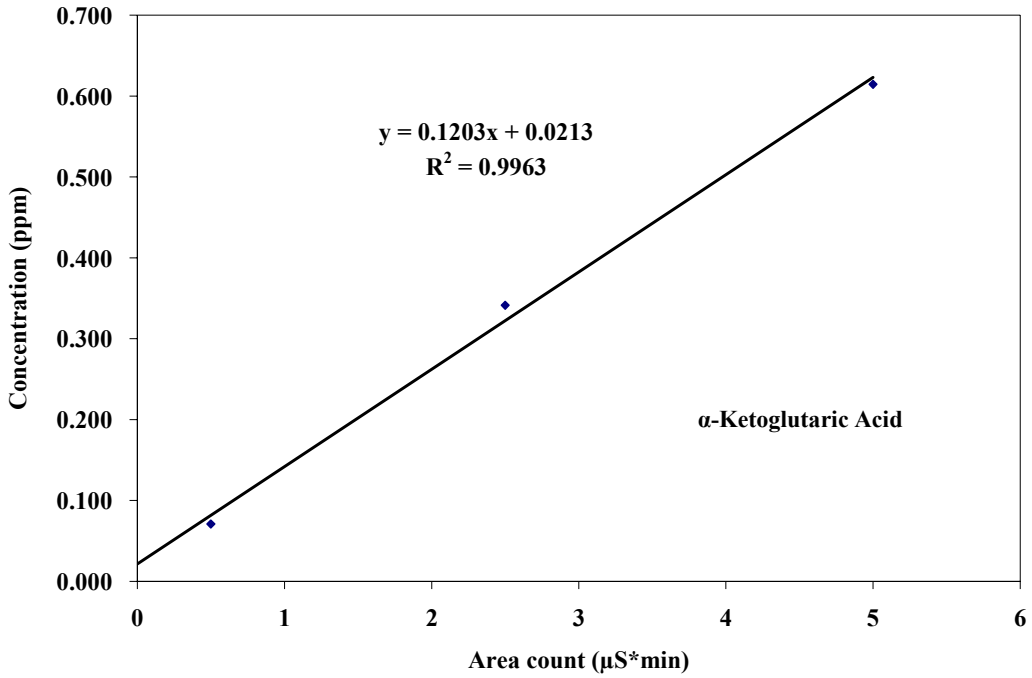


Figure A3-1 Calibration curve for α-ketoglutarate acid

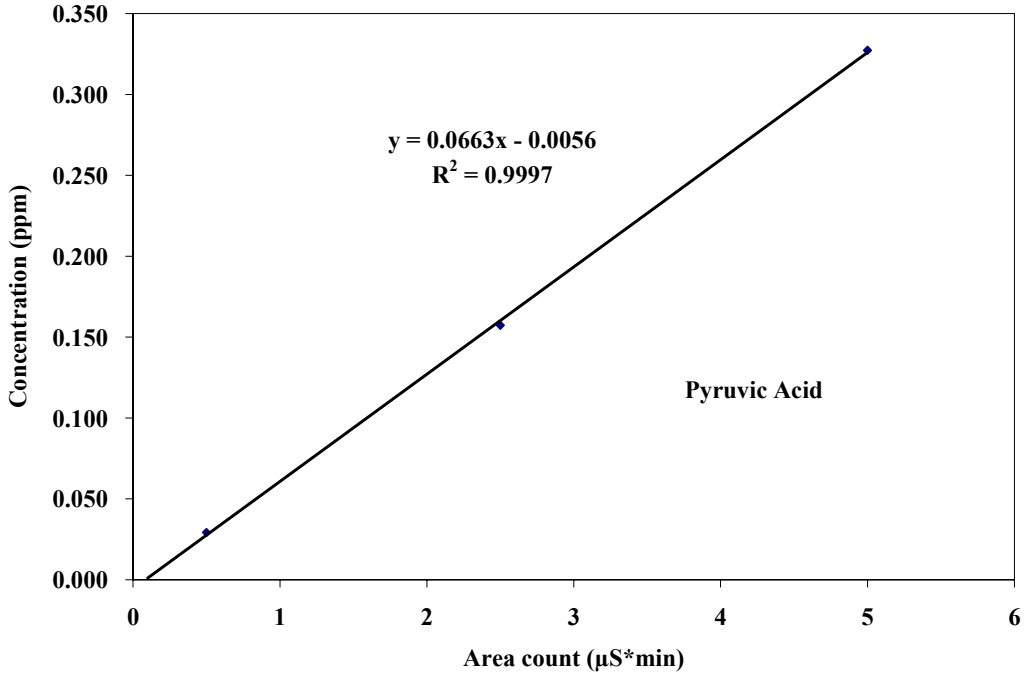


Figure A3-2 Calibration curve for pyruvic acid

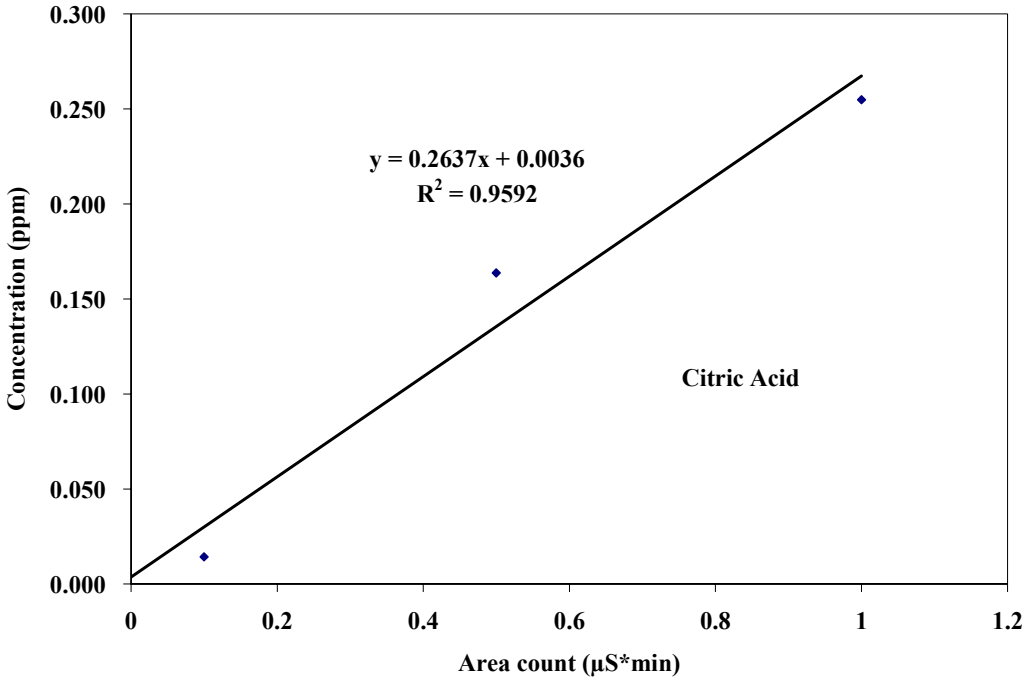


Figure A3-3 Calibration curve for citric acid

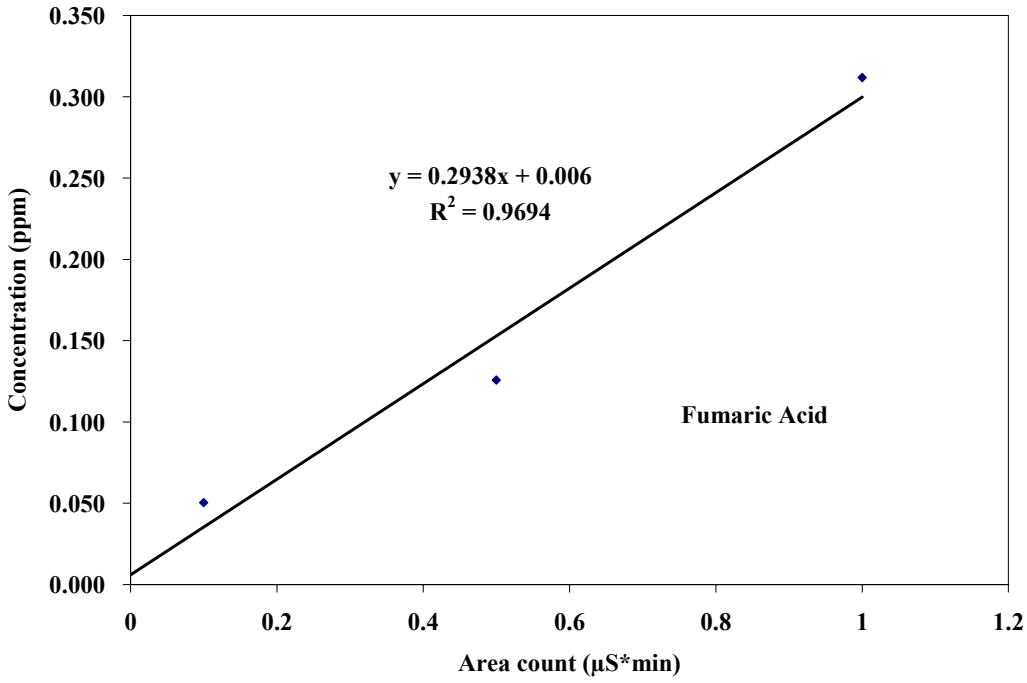


Figure A3-4 Calibration curve for fumaric acid

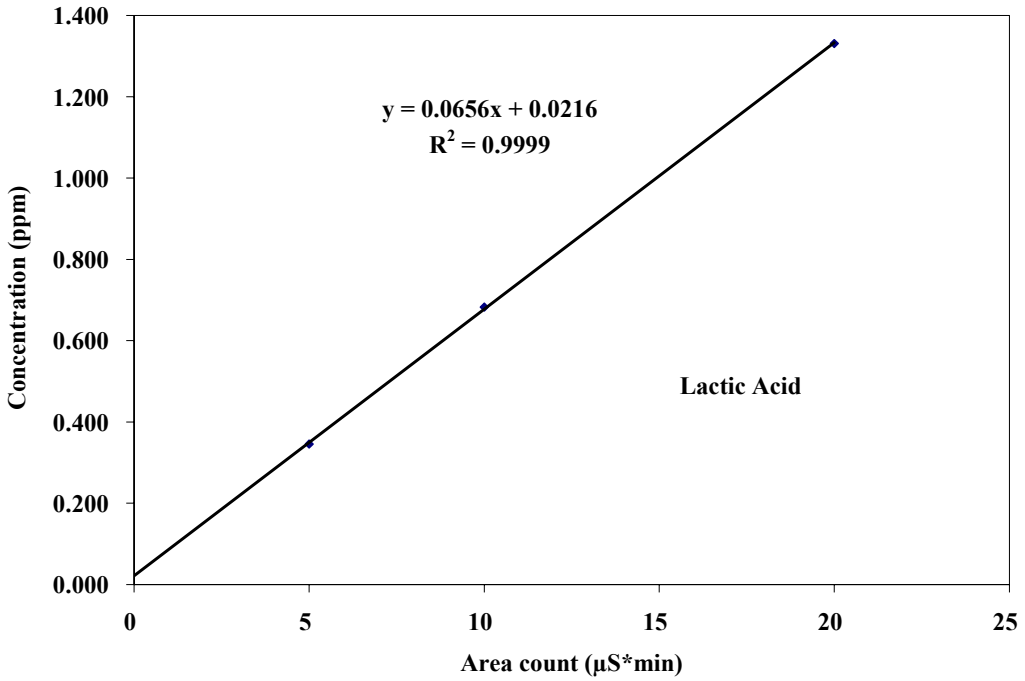


Figure A3-5 Calibration curve for lactic acid

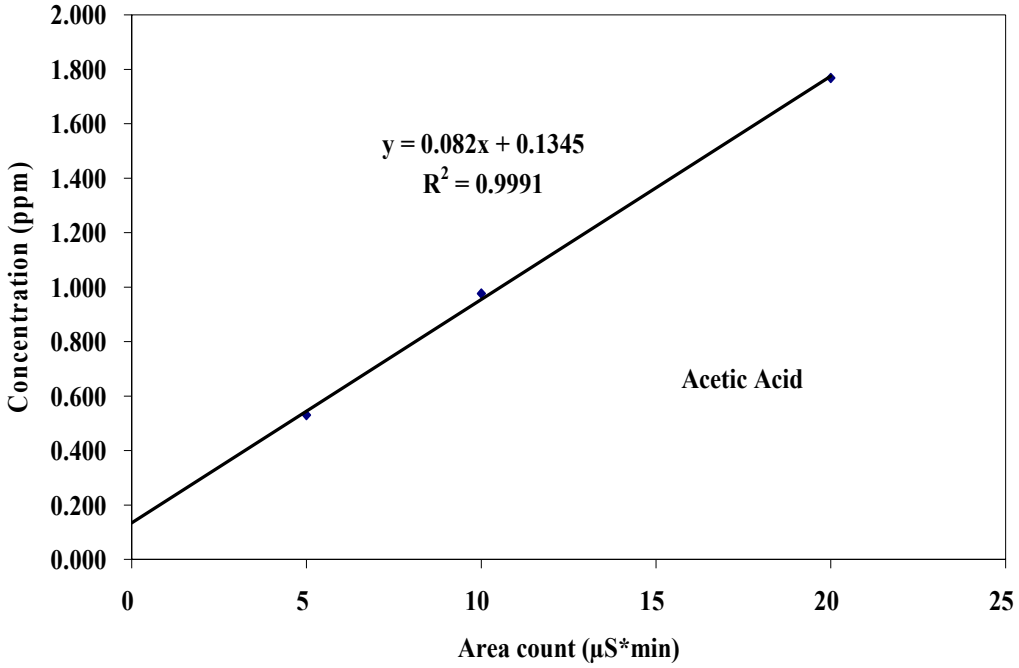


Figure A3-6: Calibration curves for acetic acids

A4-Calibration Curve for Glycerol

The calibration curve of glycerol was established using the HPLC (see Chapter 3 Material and Methods). The calibration curve is presented in Figure A4.

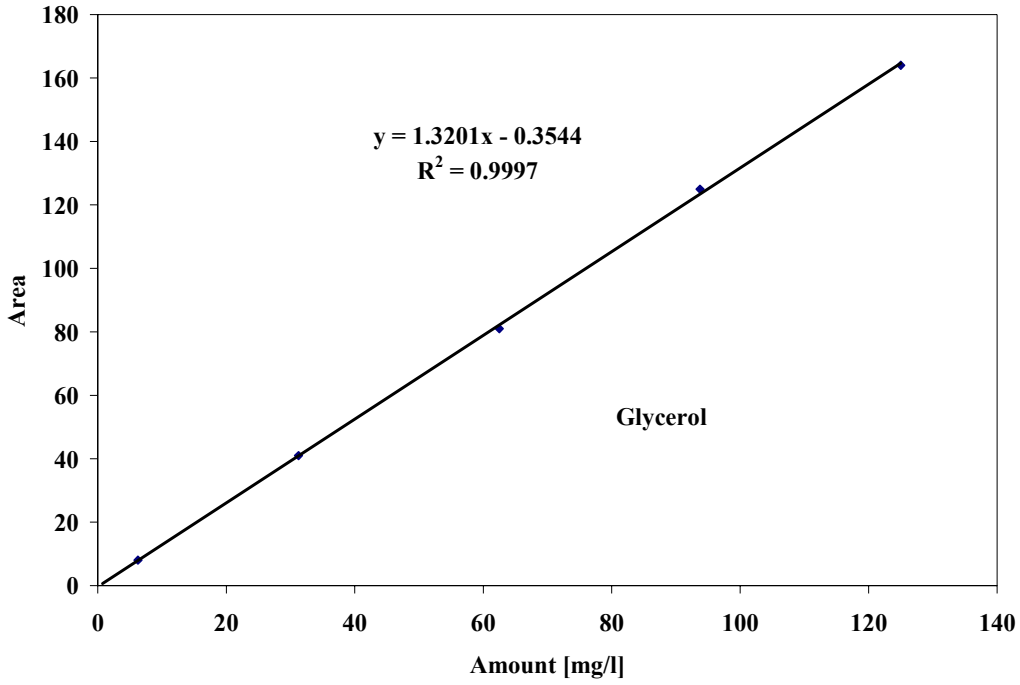


Figure A4: Calibration curve for glycerol