

İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

**INVESTIGATION OF GROWTH PHYSIOLOGY AND
METABOLISM OF MUTANT YEASTS OBTAINED
BY METABOLIC ENGINEERING**

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**METABOLİZMA MÜHENDİSLİĞİ KULLANILARAK
ELDE EDİLEN MUTANT MAYALARIN BÜYÜME
FİZYOLOJİLERİ VE METABOLİZMALARININ**

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ABBREVIATIONS

NAD	: Nicotinamide adenine dinucleotide
FAD	: Flavin adenine dinucleotide
PDC	: Pyruvate decarboxylase
PPP	: Pentose phosphate pathway
NADP	: Nicotinamide adenine dinucleotide phosphate
DHAP	: Dihydroxyacetone phosphate
GPD	: Glycerol 3-phosphate dehydrogenase
GPP	: Glycerol 3-phosphate phosphatase
G3P	: Glycerol 3-phosphate
HOG	: High osmolarity glycerol
HPLC	: High performance liquid chromatography
PGI	: Phosphoglucose isomerase
YMM	: Yeast minimal medium
YNB	: Yeast nitrogen base
CDW	: Cell dry weight
ToCh	: Total carbohydrate
RNA	: Ribonucleic acid

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METABOLİZMA MÜHENDİSLİĞİ KULLANILARAK ELDE EDİLEN MUTANT MAYALARIN BÜYÜME FİZYOLOJİLERİ VE METABOLİZMALARININ İNCELENMESİ

ÖZET

Alkol fermantasyonunun yan ürünü olan gliserol, günümüzde endüstrinin birçok alanında kullanılmaktadır. Substrat olarak kullanılan glikoz miktarının en yüksek miktarda gliserole dönüştürülmesine yönelik çalışmalar uzun yıllardır yapılmaktadır. Bu çalışmada, gliserol üretimini daha fazla artırmak amacıyla, metabolizma mühendisliği stratejisi ile daha önce elde edilmiş mutant mayalar kullanılmıştır. Bu mutant mayalarda, fosfoglikoz izomeraz enzim aktivitesi azaltılmıştır. Böylece asıl glikoz yıkımı, pentoz fosfat yol izine kaydırılarak fazla miktarda NADPH (Nikotin-Adenin-Dinükleotit-Fosfat) üretilmesi sağlanmıştır. Gliserol biyosentez yol izinde, dihidroksiaseton fosfatın'ın (DHAP) gliserol-3-fosfata (G3P) dönüşüm basamağında, fazla miktarda üretilen NADPH kofaktör olarak kullanılacaktır. Bu reaksiyonu katalizleyen, gliserol-3-fosfat dehidrogenaz (GPD), *Bacillus subtilis*'ten (*gspA*) *S. cerevisiae* (ENY.WA-1A) yaban tipe aktarılmıştır. Fosfoglikoz izomeraz aktivitesinin azaltılması ve *B. subtilis*'ten *gspA* geninin aktarılması ile üç farklı mutant maya elde edilmiştir. Birinci mutantın fosfoglukoz izomeraz (PGI) aktivitesi azaltılmış, ikinci mutanta *B. subtilis*'in *gspA* geni aktarılmış ve üçüncü mutant ise bu iki genetik değişikliğe de maruz bırakılmıştır. Yaban tipin ve üç farklı mutantın, 2 litrelik biyoreaktördeki aerobik ve anaerobik fermantasyon işlemleri sonunda; hücre kuru ağırlıkları, gliserol, etanol, ortamda kalan glikoz, toplam protein, toplam RNA ve toplam karbonhidrat miktarları değerlendirilerek karşılaştırılmıştır. Sonuç olarak, fosfoglukoz izomeraz (PGI) aktivitesi azaltılmış ve *B. Subtilis*'in *gspA* geni aktarılmış mutantın (üçüncü mutant) gliserol üretim miktarının yaban tip ve diğer mutantlardan yüksek olduğu gözlemlenmiştir.

INVESTIGATION OF GROWTH PHYSIOLOGY AND METABOLISM OF MUTANT YEASTS OBTAINED BY METABOLIC ENGINEERING

SUMMARY

Glycerol which is a by-product of alcoholic fermentation is used in a wide variety of industrial areas. There are many studies aimed to obtain maximum yield of glycerol from the given substrate (glucose) concentration. At the present study, mutant *Saccharomyces cerevisiae* strains previously obtained by metabolic engineering strategies were used in order to obtain higher yield of glycerol against consumed glucose. In some of these mutants, phosphoglucose isomerase (PGI) activity was reduced. As a result, glucose catabolism was directed to pentose phosphate pathway (PPP) followed by increased nicotinamide adenine dinucleotide (NADPH) production. NADPH which would be obtained in high amount would be used in conversion of dihydroxyacetonephosphate (DHAP) to glycerol-3-phosphate (G3P) as a cofactor during the biosynthesis of glycerol. The gene whose product (glycerol-3-phosphate dehydrogenase, 'GPD') catalysis for this corresponding reaction was introduced to wild-type *S. cerevisiae* from *Bacillus subtilis* (*gspA*). Three mutants of wild-type *S. cerevisiae* were obtained by decreasing the phosphoglucose isomerase activity and introducing *gspA* gene of *Bacillus subtilis*. In the first mutant, phosphoglucose isomerase (PGI) activity has been reduced. The *gspA* gene of *Bacillus subtilis* was then introduced into the second mutant. At last, in the third mutant, both of these two genetic changes have been applied. Wild-type and these three mutant *S. cerevisiae* cells were grown under aerobic and anaerobic batch cultivation conditions in a 2 litre bioreactor. Furthermore, their glycerol, ethanol, total RNA, total protein, total carbohydrate, residual glucose concentrations and cell dry weight measurements were determined. The analysis results obtained from different mutants and wild-type cultivations under aerobic and anaerobic batch cultivation conditions were compared to each other. Finally, it was observed that glycerol yield of the third mutant was the highest when compared to other mutants and the wild-type.

1. INTRODUCTION

1.1 *Saccharomyces cerevisiae*: Brief information

The yeast *Saccharomyces cerevisiae*, known as ‘budding yeast’, has been used for making bread, beer, and wine since old ages. The word ‘*Saccharomyces*’ derives from Greek, and means ‘sugar mould’. ‘*Cerevisiae*’ comes from Latin and means ‘of beer’. Other names for the organism are Baker’s yeast, Ale’s yeast, Brewer’s yeast.

The taxonomy of *Saccharomyces cerevisiae* is shown:

Superkingdom	Eukaryota
Kingdom	Fungi
Phylum	Ascomycota
Subphylum	Saccharomycotina
Class	Saccharomycetes
Order	Saccharomycetales
Family	Saccharomycetaceae
Genus	<i>Saccharomyces</i>
Species	<i>Saccharomyces cerevisiae</i>

Saccharomyces cerevisiae is the most common yeast, which has been used as eukaryotic model organism for the last decades. It has been playing important role in genetics, molecular biology, and biochemistry and also for several biotechnological applications (Laget and Cantley, 2006). Moreover, *S. cerevisiae* is a very fascinating model organism in molecular and cell biology studies because of its non-pathogenic property. Genetic transformation of yeast was first achieved in 1978. Yeasts have then been used as one of the most suitable eukaryotic microorganisms for biological analyses (Akada, 2002). Another major reason of implementation of *S. cerevisiae* within the field of biotechnology is that it gives chance for recombinant DNA technology to make modifications on genes. Therefore, many researchers have

focused on the characterization of its cellular processes such as metabolic pathways since the accessibility of complete genome sequence (Ostergaard, et al., 2000).

S. cerevisiae can grow rapidly which provides the feasibility of acquisition time for researches. There are two forms in which yeast cells can survive and grow: haploid and diploid forms. Patterns of haploid forms and diploid forms are different to each other. In haploid form, there are two pheromones which are type a and type α , which are produced by MATa and MAT α genes, respectively. They can mate to form a MAT a/ α diploid form, which is more stable under extreme conditions (Dickinson, 2004). In addition, physiological properties of two different forms of *S. cerevisiae* show distinctive characters such as size and shape. The shape of the diploid form is ovoid and its size is larger than that of haploid form. However, the shape of the haploid form is round (Burke, et al., 2000).

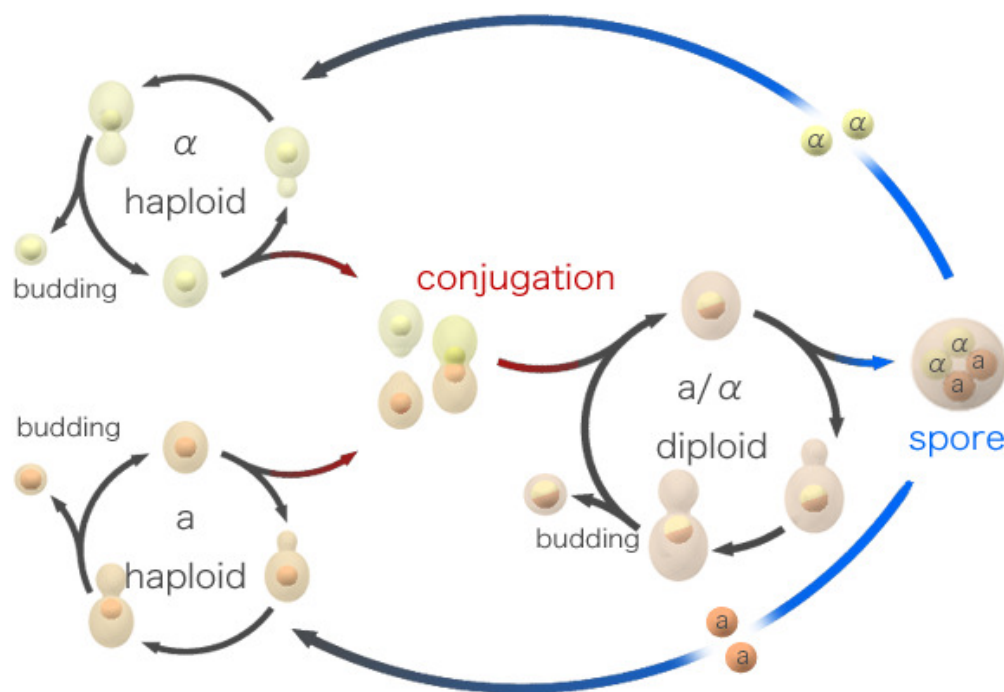


Figure 1.1: Life cycle of budding yeast (Sauer, 2006)

1.1.1 Industrial importance of *Saccharomyces cerevisiae*

S. cerevisiae has been used for several industrial applications such as the production of beer, wine, alcohol, baker's yeast, and heterologous proteins. Hundreds of years of use have resulted in development of several strains optimized for these specific applications. Different *S. cerevisiae* strains have different types of by-product and

secondary metabolite production. *S. cerevisiae* has been becoming an important tool in order to match the requirements of the industry by development of recombinant DNA technology and applying modern molecular techniques. Biotechnology, which is based on yeast *S. cerevisiae*, is classified in two patterns. One of them is traditional biotechnology which is related to produce bread, fermented by-products such as ethanol, glycerol, etc. The other one is modern biotechnology, whose domain is heterologous proteins of higher eukaryotes by genetic engineering. In the classical applications of *S. cerevisiae*, three main problems can be encountered:

- Glycerol formation under anaerobic conditions
- Crabtree effect (i.e. production of ethanol under aerobic conditions)
- Glucose repression of use of mixed carbon sources.

S. cerevisiae which is one of the most studied microorganisms has become a very significant organism for biotechnological applications (Murphy and Kavanagh, 1999). Moreover, *S. cerevisiae* has been used as a cloning tool in the biotechnology area. The most important advantage of this is the fact that *S. cerevisiae* can secrete the protein of interest into the growth medium, which makes the downstream processing easier and economical (Schreuder, et al., 1996).

S. cerevisiae has been used for many years for their dough leavening characteristics. It produces CO₂ that results in dough leavening and gives the flavour and crumb structure of bread. The yeast gassing rate is important in baking technology, and depends on the dough formulation, specific fermentation parameters and especially essential properties of *S. cerevisiae* that influence leavening in bread making (Randez-Gil, et al., 2000).

S. cerevisiae is also used in lactic acid fermentation upon modification of its metabolic pathways, deletion of pyruvate decarboxylase and overexpression of lactate dehydrogenase. Thus, fermentation is carried out at low pH successively (Compagno, et al., 1996).

Another major application area of *S. cerevisiae* is the brewing industry. The main properties of good fermenting yeast are listed below:

- A reproducible production of the correct levels of flavour and aroma compounds,
- A rapid fermentation rate without excessive yeast growth,
- An ability to resist the stress imposed by alcohol concentration and osmotic pressure encountered in breweries,
- An efficient use of maltose with good conversion to ethanol,
- Good handling characteristics (e.g. retention of viability during storage, genetic stability) (Priest and Campbell, 1996).

1.2 Aerobic and anaerobic metabolism of *Saccharomyces cerevisiae*

Yeasts can be classified in several groups according to their ways of energy production, utilizing cellular respiration or fermentation. It is important to note that these processes are mainly regulated by environmental factors. Hence, yeasts can adapt to varying growth environments, and even within a single species, the prevailing pathways will depend on the actual growth conditions. For example, glucose can be utilized in several different ways by *S. cerevisiae*, depending on the presence of oxygen and other carbon sources (Walker, 1998).

The main energy source in the yeast, *S. cerevisiae*, is glucose and glycolysis (Embden-Meyer-Parnas, EMP pathway), which is the general pathway for conversion of one molecule of glucose to two molecules pyruvate, followed by the Krebs cycle under aerobic conditions. Production of energy in form of ATP is coupled to the generation of intermediates and reducing power in form of NADH for biosynthetic pathways (Madigan, et al., 2000).

Two principal ways of use of pyruvate in later energy production can be distinguished as the cellular respiration under aerobic conditions and fermentation under anaerobic conditions. Under aerobic conditions, pyruvate enters the mitochondrial matrix where it is oxidatively decarboxylated to acetyl CoA by the pyruvate dehydrogenase (PDH) multi enzyme complex. This reaction links glycolysis to the citric acid cycle, in which the acetyl CoA is completely oxidized to give two molecules of CO₂ and reductive equivalents in form of NADH and FADH₂ (Figure 1.2). However, the citric acid cycle is an amphibolic pathway, which

combines both catabolic and anabolic functions. Thus, for example, amino acids and nucleotides can be produced from the production of intermediates (Walker, 1998).

Under anaerobic conditions, yeasts reoxidize NADH to NAD in a two step reaction from pyruvate, which is first decarboxylated by pyruvate decarboxylase (PDC) followed by the reduction of acetaldehyde, catalyzed by alcohol dehydrogenase (ADH). Ethanol and CO₂ are then produced. Therefore, ethanol production is tightly coupled with yeast cell growth. In addition to ethanol and CO₂, concomitantly, glycerol is generated from dihydroxyacetone phosphate to ensure production of this important compound (Figure 1.2).

An alternative way of glucose oxidation is the pentose phosphate pathway (PPP), which provides the cell with pentose sugars and cytosolic NADPH, necessary for biosynthetic reactions, such as the production of fatty acids, amino acids and sugar alcohols. The first step in this pathway is the dehydrogenation of glucose-6-phosphate to 6-phosphogluconolactone and generation of one mole of NADPH by glucose-6-phosphate dehydrogenase. Next, 6-phosphogluconate is decarboxylated by the action of phosphogluconate dehydrogenase to form ribulose-5-phosphate and a second mole of NADPH (Figure 1.2) (Ostergaard, et al., 2000). Thus, besides generating NADPH, the other major function of this pathway is the production of ribose sugars which serve in the biosynthesis of nucleic acid precursors and nucleotide coenzymes. Addition to these, percentage of glucose consumption by the PPP of *S.cerevisiae* varies from 2-20% up to 34% depending on if cells are growing actively or not (Walker, 1998).

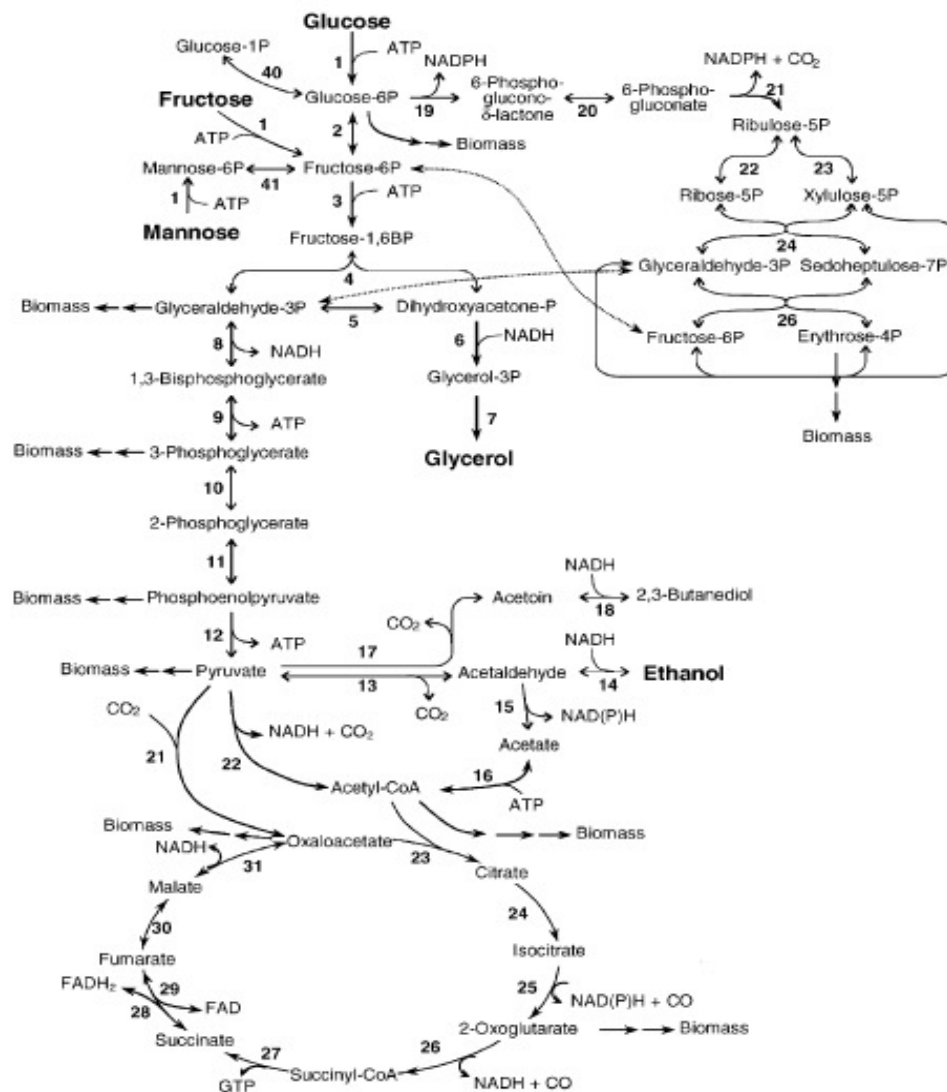


Figure 1.2: The sugar assimilation in glycolysis and TCA cycle in *S. cerevisiae*. (Taherzadeh, et al., 2002).

1.3 Glycerol production

Glycerol, a 1,2,3-propanetriol is a chemical compound with the formula $\text{HOCH}_2\text{CH}(\text{OH})\text{CH}_2\text{OH}$. It is a colourless, odourless, viscous liquid that is widely used in pharmaceutical formulations. Glycerol, also known as glycerin or glycerine, is a sweet-tasting sugar alcohol with low toxicity.

Glycerol is a precursor for synthesis of triacylglycerols and of phospholipids in the liver and adipose tissue in human. When the body uses stored fat as a source of

energy, glycerol and fatty acids are released into the bloodstream. The glycerol can be converted to glucose by the liver and provides energy for cellular metabolism.

Since the investigations of Pasteur in 1858, glycerol production by yeast fermentation has been known under aerobic or anaerobic conditions. Glycerol is a by-product of the fermentation of sugar to ethanol in a redox-neutral process in *S. cerevisiae*. The role of NADH-consuming glycerol formation is to maintain the cytosolic redox balance especially under anaerobic conditions, compensating for cellular reactions that produce NADH (van Dijken and Scheffers, 1986). Substantial overproduction of glycerol from monosaccharides can be obtained by yeast:

- Forming a complex of acetaldehyde with the bisulphite ion that limits ethanol production and that promotes reoxidation of glycolytically formed NADH by glycerol synthesis,
- Growing at pH values around 7 or above,
- By using osmotolerant yeasts (Agarwal, 1990).

1.3.1 Industrial importance of glycerol production

Glycerol is a simple alcohol with several uses in the cosmetic, paint, automotive, food, tobacco, pulp and paper, leather and textile industries or as a feedstock for the production of various chemicals (Wang, et al., 2001). In addition, glycerol is used in wrapping and packaging materials, lubricants, urethane polymers, gaskets, cork products, cement compounds, soldering compounds, compasses, cleaning materials, detergents, wetting agents, emulsifiers, skin protectives, asphalt, ceramics, photographic products, leather and wood treatment and adhesives (Morrison, 1994). The current world production of glycerol amounts to 600,000 t/year (Taherzadeh, et al., 2002). Glycerol has also been considered as a feedstock for new industrial fermentations in the future. For example, glycerol can be fermented to 1,3 propanediol, which is used for the chemical synthesis of poly(trimethylene terephthalate), a new polyester with novel fiber and for textile applications that combines excellent properties, like good resilience, inherent stain resistance, low static generation with an environmentally benign manufacturing process (Biebl, et al., 1999).

Glycerol has been produced either by microbial fermentation or by chemical synthesis from petrochemical feedstock or has been recovered as a by-product of soap manufacture from fats. Traditionally, glycerol has been produced as a by-product of the hydrolysis of fats in soap and other related materials. Nowadays, this process is less significant in industry in many developing countries, because of the replacement of soap with detergents (Agarwal, 1990). Now, approximately 25% of glycerol production occurs by the oxidation or chlorination of propylene to glycerol at worldwide. However, this way has been refused since the early 1970s because of environmental concerns (Hester, 2000). Furthermore, while the cost of propylene has increased and its availability has decreased, glycerol production by fermentation has become more attractive as an alternative way (Wilke, 1999).

Glycerol was produced by the fermentation for the first time on a large scale using the sulphite-steered yeast process during World War I, because glycerol was demanded in explosive manufacture (Prescott and Dunn, 1959). The process was short-lived and could not compete with post-war methods for synthetic glycerol production. The main problems of the fermentative processes have been the low yield of glycerol from carbohydrates, and difficulties in the recovery of glycerol from the fermentation broth (Taherzadeh, et al., 2002).

The important developments in biotechnology have led to a renewed interest in the production of chemicals from renewable sources of carbohydrates, in the past decades. Fermentative production of glycerol is one example of a process of interest in this respect. Technological advances in terms of downstream processing, cultivation technology and genetic engineering, in combination with increased consumer requirements, there has been a significant improvement in the microbial production of glycerol when compared with the processes that were used in the first half of the twentieth century.

Furthermore, improvements in the glycerol yields and productivities of yeast strains would help to improve the economics of the glycerol fermentation processes. The understanding of glycerol metabolism in yeast such as *S. cerevisiae* has made significant advance over the past ten years. The challenge for the biotechnologist will be to apply the fundamental knowledge on glycerol metabolism in order to manipulate yeast strains suitable for economic glycerol production by microbial fermentation (Wang, et al., 2001).

1.3.2 Glycerol production of microorganisms

Glycerol is a well-known metabolite formed by many microorganisms including bacteria, yeasts, moulds, and algae. As a result, there are a number of microorganisms, which are potential candidates for glycerol production (Taherzadeh, et al., 2002).

Table 1.1: Glycerol producing microorganisms (Taherzadeh, et al., 2002).

Genera	Strain
Yeast	Saccharomyces cerevisiae Saccharomyces ellipsoideus Zygosaccharomyces rouxii Saccharomyces mellis Saccharomyces formonensis Saccharomyces uvarum Candida stellata Candida kefyr Candida veratilis Pichia angusto Pichia anomala Pichia miso Kluyveromyces bulgaricus Kluyveromyces lactis Kluyveromyces marxianus Pachysolen tannophilus
Mould	Debaryomyces mogii Rhizopus nigricans Rhizopus javanicus Aspergillus niger
Algae	Penicillium italicum Dunaliella tertiolectra Dunaliella salina Dunaliella viridis
Protozoa	Trypanosoma cruzi Leishmania mexic
Bacteria	Bacillus subtilis Bacillus coli Bacillus welchii Bacterium orleanense Bacterium ascendens Lactobacillus lycopersici

Most of the studies about glycerol production have been carried out using *S. cerevisiae*. There are various reasons for the interest in glycerol production by Baker's yeast:

- Glycerol is a predominant by-product of the fermentative metabolism of *S. cerevisiae*,
- Glycerol is a desirable end product in wine, providing the quality of “mouth-feel”,
- Glycerol is typically an unwanted by-product in the production of distilled ethanol (Taherzadeh, et al., 2002).

1.3.3 Environmental effects on glycerol synthesis

Environmental factors such as temperature, aeration, phosphate and nitrogen content, sugar concentration and osmotic stress have been found to affect the rate and yield of glycerol produced by yeasts. The optimum temperature for glycerol production by yeasts is close to the optimum growth temperature of the organism. For example, the highest amount of glycerol was produced by *S. cerevisiae*, *C. magnoliae* and various strains of *Z. rouxii* between 30°C and 35°C (Spencer and Shu, 1957) and by *C. glycerinogenes* between 29°C and 33°C. Both the glycerol yield and cell growth were reduced at 40°C for *C. magnoliae* and at 35°C for *C. glycerinogenes* obviously. Nevertheless, wine yeast strains of *S. cerevisiae* have an optimum temperature between 20°C and 25°C for glycerol production (Gardner, et al., 1993).

The degree of aeration has an important effect on glycerol production especially in osmotolerant yeasts (Spencer and Shu, 1957). For example, at low levels of aeration, more glucose is converted to ethanol and ethyl acetate by *C. glycerinogenes*, on the other hand, glycerol production decreased appreciably. Under highly aerated conditions, glucose utilization increased with increased cell biomass and more rapid growth, however, the yield of ethanol and glycerol decreased (Jin, et al., 2000). Compared to processes using osmotolerant yeasts, steered glycerol production by *S. cerevisiae* requires oxygen-restricted conditions. For example, sparging with carbon dioxide or applying a vacuum to the bioreactor improved the glycerol concentration and fermentation productivity (Hecker, et al., 1990).

When sugar concentration increases, generally, synthesis of glycerol by yeasts increases. The increase in glycerol concentration results with the greater osmotic stress forced on the organism (Albertyn, et al., 1994).

On glycerol production, effect of nutrients has been examined at limited level. The nitrogen source has an important impact on the glycerol yield in the medium of anaerobically cultivated *S. cerevisiae* (Albers, et al., 1996). In ammonium-grown cultures, the glycerol yield is more than double that of cultures grown with an amino acid mixture as nitrogen source. As ammonium-grown cultures require *de novo* amino acid synthesis, the excess NADH formed is reoxidized via glycerol synthesis (Wang, et al., 2001).

1.3.4 Glycerol metabolism in *Saccharomyces cerevisiae*

Glycerol is synthesized in the cytosol of the yeast *S. cerevisiae* from the glycolytic intermediate dihydroxyacetone phosphate (DHAP) by a NAD⁺-dependent glycerol-3-phosphate dehydrogenase (GPD), followed by the dephosphorylation of glycerol-3-phosphate (glycerol-3-P) by a glycerol-3-phosphate phosphatase (GPP) (Albertyn, et al., 1994). *gpd1* and *gpd2* encode the two isozymes of GPD (Larsson, et al., 1993) and *gpp1* and *gpp2*, those of GPP (Pahlman, et al., 2001) (Figure 1.3). These genes are subject to different control, e.g., expression of *gpd1* and *gpp2* are increased in response to an osmotic stress, while *gpd2* and *gpp1* are induced under anaerobic condition (Albertyn, et al., 1994; Costenoble et al., 2000; Pahlman, et al., 2001). For example, only one iso-gene from each pair (*gpd2* and *gpp1*) is induced by shift to anaerobic conditions. Deletion of either of these two genes results in defective anaerobic growth, whereas deletion of either of the other iso-genes (*gpd1* or *gpp2*) is without anaerobic effects. Deletion of both *gpd1* and *gpd2* or *gpp1* and *gpp2* leads to blocked glycerol production and a completely inhibited anaerobic growth.

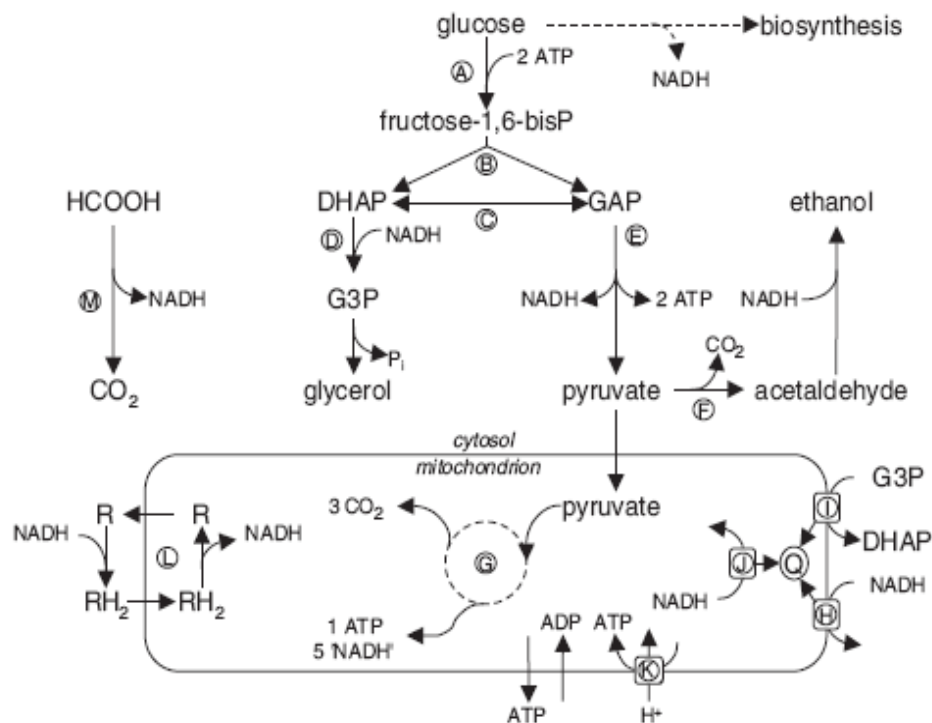


Figure 1.3: Schematic overview of fermentative and oxidative glucose metabolism of *S.cerevisiae*. (A) upper part of glycolysis, which includes two sugar phosphorylation reactions. (B) fructose-1,6-biphosphate aldolase, splitting the C₆-molecule into two triose phosphates (C) triosephosphate isomerase, interconverting DHAP and GAP. (D) glycerol pathway reducing DHAP to glycerol-3-phosphate (G3P) by G3P dehydrogenase, followed by dephosphorylation to glycerol by G3Pase. (E) The lower part of glycolysis converts GAP to pyruvate while generating 1 NADH and 2 ATP via a series of 5 enzymes. (F) Alcoholic fermentation; decarboxylation of pyruvate by pyruvate decarboxylase, followed by reduction of acetaldehyde to ethanol. (G) mitochondrial pyruvate-dehydrogenase converts pyruvate to acetyl-CoA, which enters the tricarboxylic acid cycle. (H) external mitochondrial NADH dehydrogenases. (I) mitochondrial G3P dehydrogenase. Electrons of these three dehydrogenases enter the respiratory chain that the level of the quinol pool (Q). (J) internal mitochondrial NADH dehydrogenase. (K) ATP synthase. (L) generalized scheme of NADH shuttle. (M) formate oxidation by formate dehydrogenase (Geertman, 2006).

1.3.4.1 Glycerol production under aerobic conditions

Yeast cells produce less amount of glycerol under aerobic conditions than under anaerobic conditions. On the other hand, if cells are placed to the hyperosmotic conditions and when they synthesize organic acids, yield of glycerol production is increased clearly owing to pooling NADH into the cell. Strain, medium and process conditions play a critical role to determine what fraction of the carbon source is converted to glycerol (Björkqvist, et al., 1997). Moreover, when cells are grown at

high glucose concentrations, cells produce glycerol in order to control respiration and eliminate the excess NADH which is produced by the respiratory chain (Nissen, et al., 2000).

1.3.4.2 Glycerol production under anaerobic conditions

Yeast cells produce glycerol to reoxidized NADH, one mole glycerol is then produced when one mole NADH molecule is reoxidized under anaerobic conditions. To increase biomass yield under anaerobic conditions, glycerol production should take place, because *gpd1/gpd2* mutants that cannot produce glycerol-3-phosphate dehydrogenase enzymes cannot grow under anaerobic conditions (Nissen, et al., 2000). Besides, several gene expressions are important with respect to glycerol requirement of glycerol to respond to and regulate hypoxic conditions. For example, *gpp* gene encodes glycerol-3-phosphatase which affects cell growth rate (Costenoble, et al., 2000).

1.3.5 Function of glycerol in microorganisms

A simplified scheme of the metabolism in *S. cerevisiae* is shown in Figure 1.3. Glycerol has at least two important functions in yeast: as an osmolyte balancing a high external osmotic pressure, and as a sink for the excess NADH which is produced by anabolic reactions during anaerobic conditions (Taherzadeh, et al., 2002).

In yeasts, glycerol plays a critical role as a compatible solute during osmoregulation. *S. cerevisiae* increases its rate of glycerol formation greatly in response to reduced extracellular water activity (Blomberg and Adler, 1992). Glycerol is produced by the reduction of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P), a reaction catalyzed by cytosolic NAD⁺-dependent glycerol-3-phosphate dehydrogenase (Andre, et al., 1991). The osmotic induction of NAD⁺-dependent G3P dehydrogenase occurs on a transcriptional level that is controlled by a specific signal transduction pathway called high osmolarity glycerol response pathway, HOG pathway. This pathway regulates *gpd1* gene and results in elevated formation of glycerol (Albertyn, et al., 1994).

The intracellular accumulation of glycerol is controlled by the Fps1p-channel in *S. cerevisiae* (Luyten, et al., 1995). Under hyperosmotic stress conditions, the channel

is closed in that way conserving the glycerol within the cell in order to maintain an osmotic equilibrium with the external environment. If there is no hyperosmotic stress, the channel remains open and glycerol goes out from the cell freely (Hohmann, et al., 2000).

Glycerol is produced by *S. cerevisiae* during fermentation of glucose to ethanol in order to maintain the redox balance. This metabolite can reach 0.1 g per g glucose consumed under anaerobic condition to equilibrate the redox balance by regenerating NADH associated with the biomass production during the fermentation (Overkamp, et al., 2002). When the genes *gpd2* and *gpp1* were deleted from the yeast, their importance was corroborated in cellular redox balancing. Besides, these strains were unable to grow under anaerobic conditions (Nissen, et al., 2000). Attempts to increase glycerol production in *S. cerevisiae* by overexpressing the gene *gpd1* disturb the redox balance in the cell. This results in an obvious increase in by-product synthesis such as pyruvate, acetate, acetoin, 2,3-butanediol and succinate in order to maintain the redox balance (Roustan, et al., 1999).

1.4 Metabolic engineering

Metabolic pathway manipulation is a very old concept for the purpose of providing microorganisms with desirable properties. This approach made use of chemical mutagens and creative selection techniques to identify strains with desired characteristics. Although it has been accepted widespread with remarkable successes, the genetic and metabolic profiles of mutant strains were characterized poorly and mutagenesis remained a random process (Stephanopoulos, 1999).

A new dimension to pathway modification is introduced by the development of molecular biological techniques for DNA recombination. Genetic engineering allowed particular modification of specific enzymatic reactions in metabolic pathways and leading to the construction of well-defined genetic background. After the feasibility of DNA recombination was established, the potential of directed pathway modification became apparent.

Metabolic engineering is defined as the directed improvement of product formation or cellular properties through the modification of specific biochemical reactions or introduction of new ones with the use of recombinant DNA technology. An

important element of this definition is the specificity of the biochemical reaction(s) targeted for modification or to be newly introduced. When such reaction target(s) are identified, established molecular biological techniques are applied in order to amplify, inhibit or delete, and transfer the corresponding gene(s) or enzyme(s) (Stephanopoulos, 1999). Metabolic engineering covers the whole biotechnology; it allows overproduction of industrially important metabolites. However there are some difficulties associated with metabolic engineering such as;

- Difficulties in determination of the flux-limiting step,
- Need on detailed knowledge of the pathway of interest,
- Information of the identity of the pathway and the identity of the enzymes involved in this pathway,
- Detailed information on the enzyme kinetics and the intermediate metabolites,
- The need for a high number of stimulus-response experiments (Bailey, 1991).

Metabolic engineering has emerged in the past decade as the interdisciplinary field aiming to improve cellular properties by using modern genetic tools to modify pathways. Metabolic engineering is a result of the synthesis of classical microbial genetics and selection, cell biology and genetic engineering. On the experimental side, applications have focused on pathway modifications in prokaryotic and eukaryotic systems (Bailey, 1991).

Metabolic engineering applications broaden to pathway optimization to include various combinatorial approaches such as gene shuffling and directed evolution that dramatically improve enzymatic and pathway properties (Stafford and Stephanopoulos, 2002).

1.5 Batch cultivations

Closed systems, which use a single volume of growth medium, are called batch fermentation. Batch fermentation using shake flask cultivations (semi-aerobic by using cotton-plugged flasks or anaerobic by using loop-traps) is most commonly used in laboratory experiments due to its simplicity. One clear disadvantage of batch fermentation system is that temperature and shaking are usually the only variables

that can be controlled exactly. Use of a bioreactor gives the extra possibility of exactly controlling the pH value, which has been found to be an important parameter in a number of genetic and physiological approaches of glycerol enhancement (Taherzadeh, et al., 2002).

1.6 High performance liquid chromatography (HPLC)

All chromatographic systems contain a stationary phase, which can be a solid gel, liquid or a solid/liquid mixture that is immobilised, and the mobile phase, which can be liquid or gaseous and which flows over or through the stationary phase. The choice of stationary and mobile phases is made so that the compounds to be separated have different distribution coefficients, which is defined as the total amount as distinct from the concentration of substance present in one phase divided by the total amount present in the other phase (Wilson and Walker, 2000).

The small particle size of the stationary phase causes better resolution for analytes. However, the small particle size causes greater resistance flow of the mobile phase. Thus, this creates a back-pressure in the column that is enough to damage the structure of the stationary phase. Eluent flow is then decreased and the resolution is impaired. There has been a development in column chromatography technology in the recent past. High Performance Liquid Chromatography (HPLC) technology provides smaller particle size stationary phase which can resist high pressure. HPLC guarantees that the analysis time is reduced and the results are displayed via online detectors. Additionally, several commercially available HPLC systems are microprocessor controlled to allow dedicated, continuous chromatographic separations (Wilson and Walker, 2000).

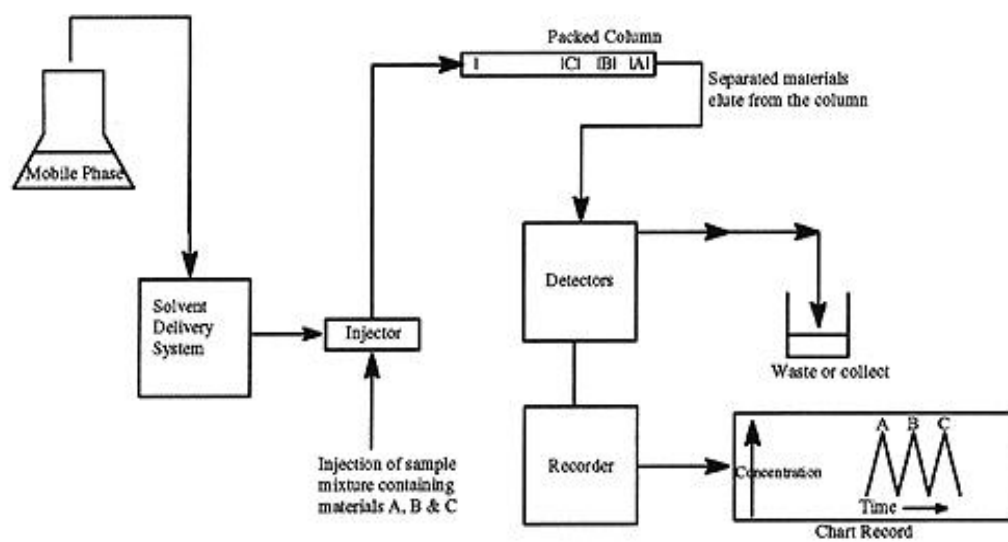


Figure 1.4: Components of an HPLC instrument (HPLC, 2004)

The principle of HPLC is based on partition theory and substances in a mixture can be separated from each other on a solid phase because of their different characteristics. Identifying low molecular weight molecules and separation of oligomer mixtures are some of application areas of HPLC (Brian, 1996).

The wide applicability, speed and sensitivity of HPLC have resulted in it becoming the most popular form of chromatography. In addition, all types of biological molecules have been assayed or purified using HPLC (Wilson and Walker, 2000).

1.7 Aim of the study

The aim of this study was to determine and compare the growth physiology, glycerol and other metabolite production characteristics of wild-type and recombinant *Saccharomyces cerevisiae* strains, which have previously been modified in their metabolic pathway for glycerol production using rational metabolic engineering approaches. Thus, the effects of different metabolic engineering approaches for glycerol pathway modification on glycerol production were compared to each other to identify the best approach for glycerol overproduction.

The wild-type yeast strain of *Saccharomyces cerevisiae* ENY.WA-1A was used as the control throughout the study. Three different yeast mutants were used in this study. They were obtained by reduction of phosphoglucose isomerase (PGI) activity

and insertion of *gpsA* gene, coding glycerol 3-phosphate dehydrogenase (GPD) enzyme, from *Bacillus subtilis* to yeast. Phosphoglucose isomerase activity of mutant 2 was reduced, *gpsA* gene of *B.subtilis* was transferred to mutant 3 and the last mutant 4 was exposed to both of these genetic changes. Aerobic and anaerobic cultivations of wild-type and three mutants were performed in a 2-liter bioreactor and growth physiology, glycerol and main metabolite production properties were compared.

2. MATERIALS and METHODS

2.1 Materials

2.1.1 Yeast strain

Four different *Saccharomyces cerevisiae* were used throughout this study, kindly provided by Dr. Elke Nevigout, Technical University of Berlin:

Table 2.1: Yeast strain used in this study.

Name	Explanation
Wild-type (mutant 1)	<i>Saccharomyces cerevisiae</i> WT (ENY.WA-1A) (MAT α <i>ura3-52 his3-Δ1 leu2-3,112 trp1-289 MAL2-8c MAL3 SUC3</i>) containing the control plasmid (p424GPD)
Mutant 2	<i>Saccharomyces cerevisiae</i> (ENY.WA-1A) (MAT α <i>ura3-52 his3-Δ1 leu2-3,112 trp1-289 MAL2-8c MAL3 SUC3</i>) with a plasmid containing <i>gpsA</i> from <i>Bacillus subtilis</i>
Mutant 3	<i>Saccharomyces cerevisiae</i> WT (ENY.WA-1A) (MAT α <i>ura3-52 his3-Δ1 leu2-3,112 trp1-289 MAL2-8c MAL3 SUC3</i>) whose phosphoglucose isomerase gene function is reduced (<i>pgi1Δ</i> (RBY 7-1))
Mutant 4	<i>Saccharomyces cerevisiae</i> (ENY.WA-1A) (MAT α <i>ura3-52 his3-Δ1 leu2-3,112 trp1-289 MAL2-8c MAL3 SUC3</i>) with reduced phosphoglucose isomerase gene and a plasmid containing <i>gpsA</i> from <i>Bacillus subtilis</i> .

2.1.2 Yeast culture media

Minimal medium I was used for pre-culturing. Its contents are shown below:

Fructose	20 g
Yeast nitrogen base without amino acids	6.7 g
Agar (for solid medium)	20 g
Distilled water	to 1 litre

Minimal medium II was used for batch cultivations. Its contents are given below:

Glucose	20 g
Yeast nitrogen base without amino acids	6.7 g
Agar (for solid medium)	20 g
Leucin	240 mg
Histidine	40 mg
Uracil	40 mg
Distilled water	to 1 litre

2.1.3 Chemicals

Table 2.2 shows the list of chemicals which were used in this study.

Table 2.2: Chemicals used in this study.

Chemical	Company	Country
Ethanol (absolute)	J.T. Baker	Holland
Yeast nitrogen base without amino acid	Fluka Cheme GmbH	Switzerland
Sulphuric acid 95-98 %	Merck	Germany
Potassium hydroxide	Merck	Germany
Ergosterol	Fluka	Switzerland
Polypropylene glycol P2000	Fluka	Switzerland
Uracil	AppliChem-Biochemica	Germany
L-Cysteinhydrochloride-monohydrate	Merck	Germany
Thiamin-hydrochloride	AppliChem-Biochemica	Germany
Yeast RNA	Roche Diagnostic	Switzerland
Sulfuric acid 95-98%	Merck	Germany
D-glucose	Riedel Haën	Germany
Tween 80	Merck	Germany
Glycerol	Merck	Germany
Bicinchoninic acid (BCA)	Merck	Germany
Phenol	Merck	Germany
MgSO ₄	Riedel Haën	Germany
Na-K tartrate	Riedel Haën	Germany
NaOH	Riedel Haën	Germany

2.1.4 Solutions and Buffers

Phenol	5 % (w/v)
Leucin	240 mg/l
Histidine	40 mg/l
Uracil	40 mg/l
NaCl	0.9 % (w/v)
MgSO ₄	10 mM
NaOH	4 M
CuSO ₄	0.01 M
Na-K-tartrate	0.06 M
KI	0.03 M
HClO ₄	3.0, 0.7, 0.5 and 0.3 M
KOH	0.3 M

2.1.5 Laboratory equipment

Laboratory equipment used in this study is listed in Table 2.3.

Table 2.3 Laboratory equipment used in this study.

Equipment	Brand/Model	Country
Autoclave	Tuttnauer Systec Autoclave Switzerland 2540 ml	
Deep freezers	Heto Ultra Freeze (-80°C) Arçelik (-20°C)	Denmark Turkey
Injector	Hayat (10 ml)	Turkey
Laminar flow	Faster BH-EN 2003	Italy
Light Microscope	Olympus CH30	Japan
Micropipettes	Eppendorf Precisa	Germany
Switzerland		
Orbital shaker incubator	Sartorius Certomat®SII	Germany
Refrigerator	Arçelik (+4°C)	Turkey
Allegra 25R™ Centrifuge	Beckman Coulter	USA
Rotor	Beckman Coulter, JA 30.50i rotor	USA
Syringe filter	Sartorius Minisart (pore size 0.2µm)	Germany
Microcentrifuge	Eppendorf Microcentrifuge	USA
UV-Visible Spectrophotometer	Shimadzu UV-1700	Japan
	Perkin Elmer 25 UV/VIS	USA
Vortex mixer	Heidolph REAX top	Germany
Biostat® B bioreactor systems		
Aeration	Airbag-Fiac	Italy
Dissolved oxygen (DO)	Mettler Toledo, Greifensee	
Switzerland		
MFCS/win Software	B. Braun Biotech International GmbH	Germany
pH probe	Mettler Toledo, Greifensee	
Switzerland		
Temperature and level control	B. Braun Biotech International GmbH	Germany
HPLC (High Performance Liquid Chromatography) System		Japan
Column Oven	Shimadzu CTO-10AC	
Degasser	Shimadzu DGU-14A	
Liquid Chromatography	Shimadzu LC-10AD	
Refractive Index Detector	Shimadzu RID10A	
System controller	Shimadzu SCL10A	

2.2 Methods

2.2.1 Medium preparation

Yeast minimal medium (YMM) was used throughout this study. However, YMM prepared for pre-cultures was supplemented with fructose (20 g/l) instead of glucose. Furthermore, leucine, histidine and uracil were prepared separately by filter sterilization (0.2 µm diameter pore size) and they were added to this medium in order to have the final concentration of 240 mg/l, 40 mg/L and 40 mg/l, respectively.

2.2.2 Cultivations

2.2.2.1 Shake flasks cultivation

In this study, stock cultures were inoculated to minimal medium I. However, YMM was used during the fermentation process.

The stock culture was cultivated in a total volume of 10 ml medium in a 50 ml sterile falcon tube. After an overnight incubation of the culture in an orbital shaker (Braun, Certomat S-2) at 30°C and 150 rpm, it was inoculated to a 500 ml Erlenmeyer flask with 100 ml culture volume by setting initial optical density (OD₆₀₀) to 0.3. This culture was incubated at 30°C and 150 rpm for 16 h, for aerobic and anaerobic batch cultivations. Moreover, 2 ml PPG 2000 (polypropylene glycol 2000) was added to the medium as antifoaming agent. Prior to inoculation of the seed cultures into bioreactor, the cultures were washed with YMM without glucose by centrifugation (Beckman).

2.2.2.2 Bioreactor cultivations

Cultivations were processed in a Biostat[®] B bioreactor system equipped with pH (Mettler Toledo, Greifensee), dissolved oxygen (DO) (Mettler Toledo Greifensee), and temperature control (B. Braun Biotech International GmbH). Cultivation data were collected by using the MFCS/win Software (B. Braun Biotech International GmbH).

Aerobic batch cultivation was performed with 1 litre working volume in a 2 litre bioreactor at 30°C and 250 rpm for 29 h. pH of the medium was kept constant at 4.5 by adding 3 M KOH. Additionally, airflow was set to 3 l/min during the cultivation. The aeration of the bioreactor system was maintained by an air compressor.

Anaerobic batch cultivations were performed for the control yeast and three different mutants using yeast minimal medium (YMM) with 2% (w/v) glucose concentration. The medium consisted of 6.7 g Yeast Nitrogen Base (YNB) without amino acids, with 40 mg/l Ura, 40 mg/l His, 240 mg/l Leu and 20 g glucose per 1 litre dH₂O. The culture volume was 1.5 litres.

Initial optical density (OD₆₀₀) of the culture upon inoculation was 1.0. Moreover, 3 ml PPG 2000 and 1.875 ml Tween 80/Ergosterol (8 g ergosterol and 336 g Tween 80 per litre ethanol) were added into the medium.

Anaerobic batch cultivations were performed with 1.5 litre working volume in a 2 litre bioreactor at 30°C and 150 rpm for 71 h. Medium pH was kept constant at 4.5 by adding 3 M KOH. Prior to anaerobic cultivation, the bioreactor was sparged with nitrogen gas to remove air, and the cultivation was then started.

2.2.3 Growth measurements of the aerobic and anaerobic cultures

2.2.3.1 Optical density measurements

Spectrophotometer was used for measuring cell growth during cultivation processes with regular time interval. For this purpose, optical densities of cultures were measured at 600 nm by using UV-Visible Spectrophotometer (Shimadzu UV-1700).

Optical densities of aerobic and anaerobic of wild type were set to 0.2 and 1 at the beginning of batch cultivations, respectively. Also, optical densities of anaerobic cultures of mutants were set to 1 at the beginning of batch cultivation.

2.2.3.2 Cell Dry Weight (CDW) measurements

For cell dry weight measurements, microfuge tubes (2 ml volume) were dried at 80°C for 48 h and cooled down in a desiccator for 15 minutes. Then, they were weighed before adding culture samples taken from aerobic of wild type cultures. Three replicate samples were taken for each set of cultures. The microfuge tubes were centrifuged at 14000 rpm (Beckman Coulter, JA 30.50i rotor, Allegra 25RTM Centrifuge, Beckman Coulter) for 5 minutes and supernatants were discarded. At the end, tubes containing the cell pellets were dried at 80°C for 48 h and cooled down in a desiccator for 15 minutes again. These microfuge tubes containing dried cell pellets were weighed and the cell dry weights were calculated by subtracting the weights of

empty tubes from the weight of tubes containing dried cell pellets. On the other hand, CDW determinations of anaerobic of wild-type and mutant cultures were calculated with the correlation between CDW and optical density (OD_{600}) of aerobic of wild-type culture. From the OD_{600} versus CDW graph of aerobic batch cultivation of wild-type, the slope and Equation 2.1 were obtained. This equation then applied to anaerobic of wild-type and all mutant cultures to calculate cell dry weights.

$$y=0.2261x \quad (2.1)$$

2.2.4 Metabolite analyses

2.2.4.1 Residual glucose, glycerol and ethanol production analysis

Residual glucose, glycerol, ethanol measurements were performed with High Performance Liquid Chromatography (HPLC) (Shimadzu systems). Samples were taken throughout the aerobic and anaerobic cultivations for wild type and anaerobic cultivations for mutants. These samples were centrifuged at 14000 rpm for 5 minutes. Then, supernatants were passed through 0.2 μ m syringe filter.

HPLC was calibrated for the measurement of residual glucose, ethanol and glycerol using the same method. HPLC grade water was used as the mobile phase. Meanwhile, the flow rate was 0.6 ml/min and the oven temperature was at 80°C. Mobile phase and samples were passed through Aminex HPX-87H column using refractive index detector. The injection volume was 20 μ l. The retention times for glucose, glycerol and ethanol were 8.29 min, 12.85 min and 20.3 min, respectively.

2.2.4.2 Total soluble protein determination from whole cells

Total soluble protein concentration determination of batch cultivation samples was done as follows. The pellets of 1 ml of the samples were resuspended in 0.9 % NaCl and 10 mM $MgSO_4$ solution. 500 μ l of this solution was transferred into a glass tube and 400 μ l NaOH was added. This solution was incubated in boiling water for 10 minutes. After cooling down to room temperature, another solution containing 0.01 M $CuSO_4$, 0.06 M Na-K tartrate, 4 M NaOH, and 0.03 M KI was added to this solution. After incubating the solution at 37°C for 30 min, it was centrifuged at 4000 rpm for 20 min. The spectrophotometric measurement was then taken at 546 nm with the standard BSA solution.

2.2.4.3 Total carbohydrate analysis

Phenol determination method (Herbert et al., 1971) was applied to measure the total carbohydrate concentration of the samples taken throughout the aerobic and anaerobic cultivations for wild-type and anaerobic cultivations for mutants. In order to obtain dextrose standard curve, 5 tubes with dextrose concentration of 0 µg/ml, 25 µg/ml, 50 µg/ml, 75 µg/ml, 100 µg/ml were prepared as standard solutions, and their optical densities were measured at 489 nm (UV-Visible Spectrophotometer; Shimadzu UV-1700) together with other those of the obtained from cultivations.

The total carbohydrate analysis was applied to samples that were collected as pellets throughout aerobic and anaerobic cultivations for wild-type and anaerobic for mutants. These pellets were washed twice with 1 ml ddH₂O, by centrifugation at 14000 rpm for 5 minutes in a benchtop centrifuge (Eppendorf, 30.50.i rotor), and the supernatant was discarded. The washed pellets of the samples were resuspended in 1 ml and 10 ml ddH₂O for anaerobic and aerobic fermentation samples, respectively. One ml phenol solution (5% w/v) and 5 ml H₂SO₄ solution (99%) were added to glass tubes that contained 1 ml diluted sample solution and each standard solutions as well (final total volume was 7 ml) and vortexed carefully. First, all samples were incubated at room temperature for 10 min and then at 30°C for 10 min. Finally, OD₄₈₉ values of standard solutions and samples were measured in the spectrophotometer.

2.2.4.4 Total RNA determination from whole cells

In order to obtain a standard curve of total RNA from whole cells, 10 mg RNA of yeast was dissolved in 100 ml 0.3 M KOH. Then, 1:1, 1:2, 1:5, 1:10, 1:50 and 1:100 dilutions were prepared in 0.3 M KOH solution, to be used as the standard solution. Their optical density values were measured at 260 nm (UV-Visible Spectrophotometer; Shimadzu UV-1700,) together with those obtained from cultivations.

Analysis of the total RNA from whole cells was applied to samples that were collected as pellets throughout aerobic and anaerobic cultivations for wild-type and anaerobic cultivation for mutants. These pellets were washed twice with 1 ml 0.7 M HClO₄, by centrifugation at 14000 rpm for 2 min in a benchtop centrifuge (Eppendorf, 30.50.i rotor) followed by discarding supernatant. After pellets and RNA

standards had been digested with 1 ml 0.3 M KOH at 37°C for 60 min, they were neutralized with 100 μ l 3 M HClO₄ and centrifuged at 14000 rpm for 2 minutes. While supernatants were being stored, pellets were washed twice with 0.45 ml 0.5 M HClO₄. Stored and washed supernatants were collected in the same microfuge tube. Finally, OD₂₆₀ values of standard solutions and samples were measured using the spectrophotometer.

3. RESULTS

3.1 Aerobic cultivation results for wild-type

Aerobic batch cultivation was performed for *Saccharomyces cerevisiae* WT (ENY.WA-1A) (MAT α *ura3-52 his3- Δ 1 leu2-3,112 trp1-289 MAL2-8c MAL3 SUC3*) containing control plasmid (p424GPD) in Biostat[®] B bioreactor systems. The batch cultivation was stopped at 29th h.

Optical density (OD₆₀₀) measurements were taken regularly to follow growth. Dissolved O₂ (pO₂%) and pH were also monitored online. The pre-culture OD₆₀₀ was 4.36, whereas the initial OD₆₀₀ of the culture was set to 0.275. Table 3.1 shows the numerical results of OD₆₀₀, lnOD₆₀₀, pO₂% and pH for the aerobic batch cultivation of the *S. cerevisiae* WT (ENY.WA-1A).

Table 3.1: OD₆₀₀, ln OD₆₀₀, pO₂% and pH results for the aerobic batch cultivation of wild type *S. cerevisiae* (ENY.WA-1A)

Time (h)	OD ₆₀₀	lnOD ₆₀₀	pO ₂ %	pH
0	0.27	-1.29	100.00	5.55
6	1.18	0.17	96.00	4.50
8	1.96	0.67	89.90	4.61
10	3.42	1.23	74.90	4.42
12	6.89	1.93	22.00	4.41
13	8.25	2.11	6.50	4.47
14	10.54	2.36	1.60	4.48
16	22.80	3.13	0.00	4.46
18	26.40	3.27	0.00	4.45
20	32.22	3.47	0.00	4.48
22	37.50	3.62	0.00	4.42
23	41.04	3.71	0.00	4.44
24	46.57	3.84	30.00	4.49
25	49.97	3.91	39.90	5.39
26	46.84	3.85	48.20	6.15
27	52.30	3.96	61.20	6.29
28	57.00	4.04	73.70	6.13
29	51.20	3.94	85.30	6.00

After incubation for 8 h, the culture was in the log phase. In addition, pH decreased sharply before 14th h and in log phase its variation was very small between 13th and

23rd h of incubation. After 23rd h, when O₂ consumption started to decrease, pH began to increase.

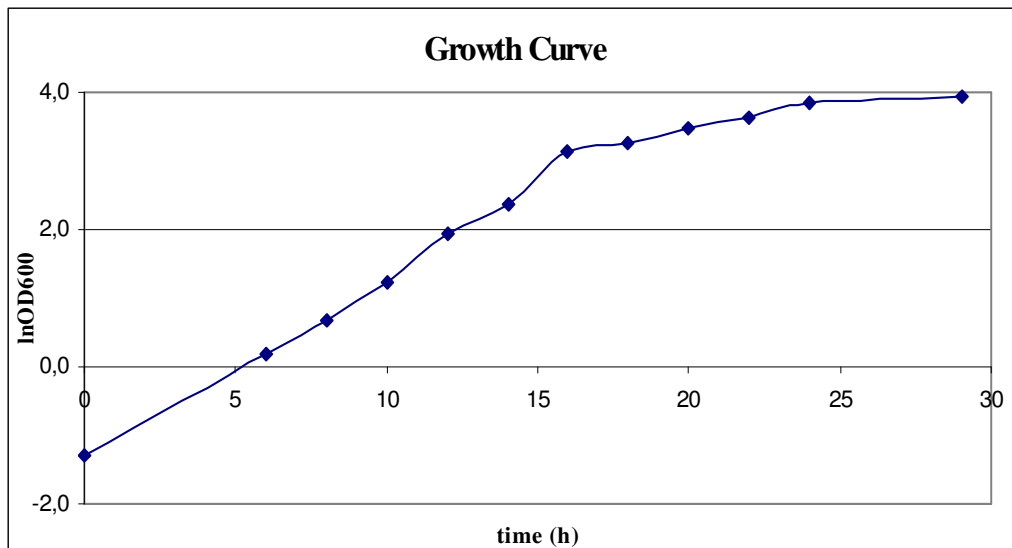


Figure 3.1: Growth behaviour of *Saccharomyces cerevisiae* WT with control plasmid for aerobic batch cultivation.

Three cell dry weight (CDW) samples were taken for each sampling time for aerobic batch cultivation of wild-type. The average CDW was calculated from these 3 corresponding measurements. These results were then divided to the sample volume to calculate CDW (mg)/ml for aerobic batch cultivation.

Table 3.2: CDW, total carbohydrate, total RNA and total soluble protein results for aerobic cultivation of wild-type.

Time (h)	CDW(mg)/ml	ToCh (mg/ml)	RNA (mg/l)	Protein (mg/l)
0	0.05±0.00	0.07±0.00	0.00±0.00	760.50±22.82
6	0.45±0.02	0.14±0.01	37.34±1.87	655.80±19.67
8	0.65±0.03	0.20±0.01	52.47±2.62	672.30±20.17
10	1.13±0.06	0.30±0.01	115.40±5.77	869.50±26.09
12	1.92±0.10	0.45±0.02	142.99±7.15	1239.70±37.19
14	2.88±0.14	0.63±0.03	208.77±10.44	1407.00±42.21
16	4.48±0.22	0.98±0.05	270.13±13.51	1912.50±57.38
18	6.22±0.31	1.44±0.07	333.90±16.69	2224.00±66.72
20	7.37±0.37	2.31±0.12	399.67±19.98	2599.10±77.97
22	8.80±0.44	2.54±0.13	374.03±18.70	2872.20±86.17
24	10.48±0.52	3.05±0.15	449.19±22.46	3113.00±93.39
29	11.32±0.57	3.23±0.16	584.42±29.22	2903.10±87.09

Cell dry weights of the aerobic batch cultures increased with cultivation time. Although during the first 8 h there was no significant increase in cell dry weights, after 14th h, a significant increase was observed (Table 3.2).

Total carbohydrate (ToCh) concentrations of the samples were determined by using “Phenol Method” and the results are shown in Table 3.2. Total carbohydrate concentration of the wild-type increased continuously, especially after the 10th h. However, after the 24th h, there was no further increase in total carbohydrate concentration.

Total RNA concentrations of aerobic batch cultivation are shown in Table 3.2 and the change of total RNA concentration with cultivation time in aerobic batch cultivations is shown in Figure 3.2. Total RNA concentration graph is similar to total carbohydrate concentration graph, where the concentrations started to increase from the 10th h until the 24th h, and then did not change significantly.

Protein amounts of the samples were determined by using “BCA Method”. The total protein concentrations of aerobic batch cultivation are shown in Table 3.2. The changes in total protein concentration with cultivation time are shown in Figure 3.2. After the 8th hour of incubation, protein concentration of the wild-type started to increase until 24th h regularly like CDW graph.

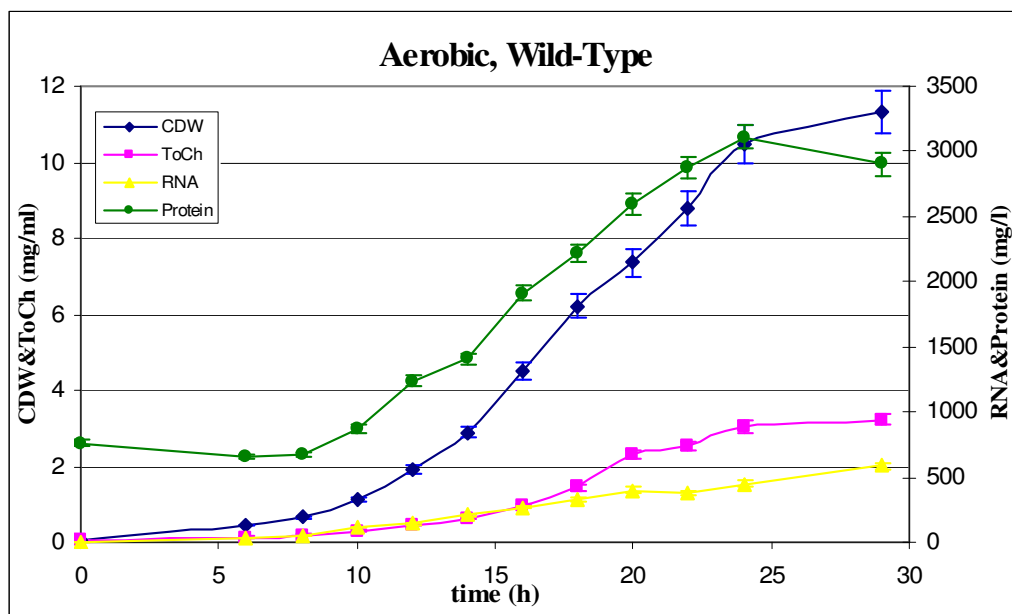


Figure 3.2: CDW, total carbohydrate, total RNA and total soluble protein results for aerobic cultivation of wild-type.

Using HPLC analysis, residual glucose and glycerol concentrations were determined for aerobic batch cultivation of the wild-type (Table 3.3).

Table 3.3: Residual glucose and glycerol concentrations of aerobic cultivation of wild-type samples are determined by HPLC analysis.

Time (h)	Residual glucose (g/l)	Glycerol (g/l)
0	21.03	0.000
6	20.48	0.040
8	20.37	0.039
10	20.05	0.067
12	17.58	0.097
14	14.92	0.088
16	13.63	0.203
18	10.67	0.272
20	7.28	0.315
22	3.16	0.356
24	0.95	0.393
29	0.00	0.420

Glycerol production started from the beginning of cultivation gradually. But, from the 14th h of incubation, glycerol production increased sharply to the final sampling time, the 29th h, (Figure 3.3). On the other hand, residual glucose concentration started to decrease faster from the 10th h on. At the end of the cultivation, no glucose was left in the medium.

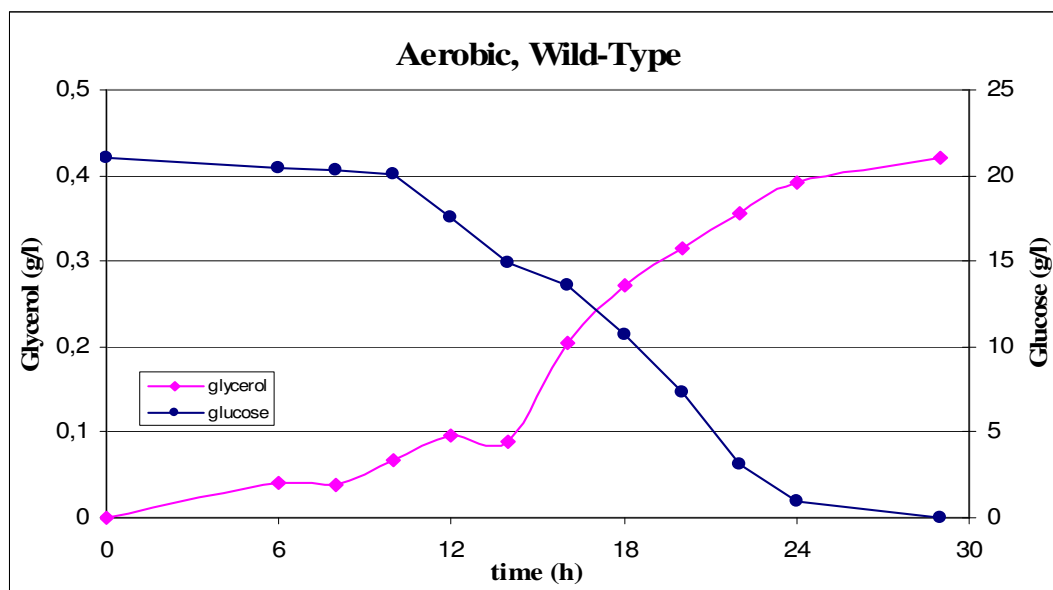


Figure 3.3: Residual glucose and glycerol results for aerobic cultivation of wild-type.

3.2 Results of anaerobic cultivations

Anaerobic batch cultivations were performed for *Saccharomyces cerevisiae* WT (ENY.WA-1A) (MAT α *ura3-52 his3- Δ 1 leu2-3,112 trp1-289 MAL2-8c MAL3 SUC3*) containing control plasmid (p424GPD) (mutant 1), *Saccharomyces cerevisiae* (ENY.WA-1A) (MAT α *ura3-52 his3- Δ 1 leu2-3,112 trp1-289 MAL2-8c MAL3 SUC3*) with a plasmid containing *gpsA* from *Bacillus subtilis* (mutant 2), *Saccharomyces cerevisiae* WT (ENY.WA-1A) (MAT α *ura3-52 his3- Δ 1 leu2-3,112 trp1-289 MAL2-8c MAL3 SUC3*) the phosphoglucose isomerase gene function of which was reduced (*pgi1 Δ* (RBY 7-1) (mutant 3), and *Saccharomyces cerevisiae* (ENY.WA-1A) (MAT α *ura3-52 his3- Δ 1 leu2-3,112 trp1-289 MAL2-8c MAL3 SUC3*) with reduced phosphoglucose isomerase gene function and a plasmid containing *gpsA* from *Bacillus subtilis* (mutant 4), in a Biostat[®] B bioreactor system.

All anaerobic batch cultivations were stopped at the 71st h of cultivation. However, samples were removed every 3 h for 54 h during cultivations. For each mutant, optical density (OD₆₀₀), pH, PO₂% measurements were monitored online. Residual glucose, ethanol and glycerol concentrations in the culture medium were measured by using on HPLC system. Furthermore, total soluble protein, total carbohydrate (ToCh) and total RNA measurements were done for all anaerobic cultivations, as well. Cell dry weights of anaerobic of wild-type and mutant cultures were calculated by Equation 2.1.

3.2.1 Anaerobic cultivation results for wild-type (mutant 1)

Anaerobic batch cultivation was performed for *Saccharomyces cerevisiae* WT (ENY.WA-1A) (MAT α *ura3-52 his3- Δ 1 leu2-3,112 trp1-289 MAL2-8c MAL3 SUC3*) containing control plasmid (p424GPD), in a Biostat[®] B bioreactor system. Table 3.4 shows the numerical results of OD₆₀₀, lnOD₆₀₀, pO₂% and pH for the anaerobic batch cultivation of the *S. cerevisiae* WT (ENY.WA-1A).

Table 3.4: OD₆₀₀, ln OD₆₀₀, pO₂% and pH results for the anaerobic batch cultivation of wild type *S. cerevisiae* (ENY.WA-1A).

Time (h)	OD₆₀₀	lnOD₆₀₀	pO₂ %	pH
0	0.85	-0.16	0	5.48
3	1.01	0.01	0	5.28
6	1.21	0.19	0	5.07
9	1.20	0.19	0	4.89
12	1.23	0.21	0	4.75
15	1.20	0.18	0	4.61
18	1.25	0.22	0	4.51
21	1.18	0.16	0	4.45
24	1.27	0.24	0	4.49
27	1.23	0.21	0	4.47
30	1.20	0.18	0	4.45
33	1.38	0.32	0	4.51
36	1.41	0.34	0	4.54
39	1.32	0.28	0	4.51
42	1.40	0.34	0	4.47
45	1.33	0.28	0	4.48
48	1.29	0.25	0	4.50
51	1.42	0.35	0	4.46
54	1.30	0.26	0	4.47
71	1.38	0.32	0	4.47

In anaerobic batch cultivation of wild-type, pH value decreased gradually from the beginning of the incubation until 18th h. It then it fluctuated between 4.51 and 4.46 until the end of the incubation (Table 3.4). Meanwhile, wild-type cells could not reach a typical log phase in anaerobic batch cultivation, unlike aerobic batch cultivation. Cell concentration fluctuated in a very narrow range from the 6th h to the end of the incubation according to the OD₆₀₀ results. The fluctuation in cell growth is shown in Figure 3.4.

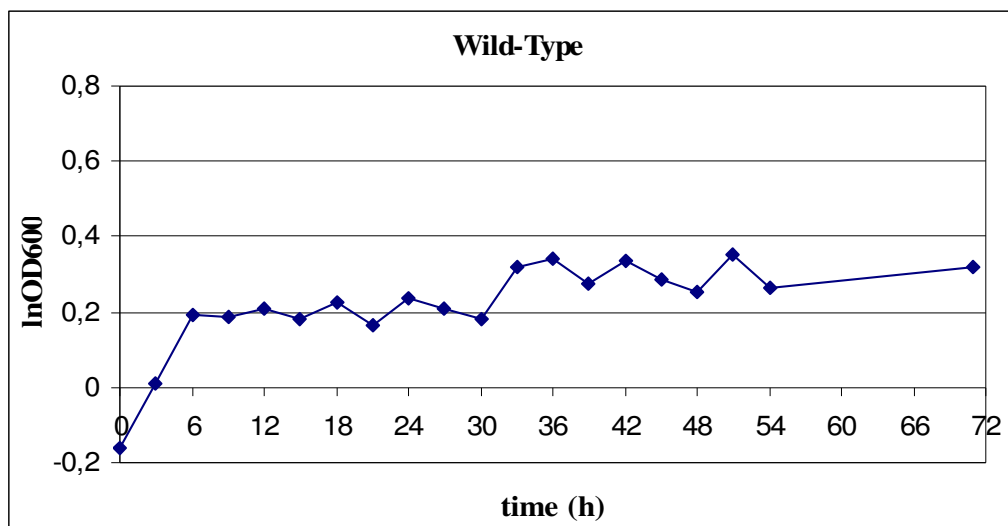


Figure 3.4: Growth behaviour of *Saccharomyces cerevisiae* WT with control plasmid for anaerobic batch cultivation.

Cell dry weight, total carbohydrate, total RNA and total soluble protein concentrations of anaerobic batch cultivation of wild-type are listed in Table 3.5. Like growth curve, cell dry weight increased until the 6th h of incubation, and then it fluctuated until 71st h (Figure 3.5). Maximum CDW concentrations were obtained at 36th, 42nd, and 51st h similar to OD₆₀₀ values.

Total carbohydrate concentration decreased from the beginning of the cultivation to the 9th h, unlike CDW concentration. Afterwards, small fluctuations in CDW level were observed until the end of cultivation, similar to other measurements. Total RNA concentrations showed large variations during the cultivation time (Figure 3.5). Moreover, total soluble protein concentration did not change significantly throughout the cultivation. Maximum protein concentration was observed at the 71st h.

Table 3.5: CDW, total carbohydrate, total RNA and total soluble protein results for anaerobic cultivation of wild-type.

Time (h)	CDW(mg)/ml	ToCh (mg/ml)	RNA (mg/l)	Protein (mg/l)
0	0.19±0.01	0.19±0.01	17.62±0.88	0.72±0.02
3	0.23±0.01	0.16±0.01	18.34±0.92	0.65±0.02
6	0.27±0.01	0.15±0.01	14.44±0.72	0.62±0.02
9	0.27±0.01	0.07±0.00	16.69±0.83	0.68±0.02
12	0.28±0.01	0.09±0.00	19.73±0.99	0.68±0.02
15	0.27±0.01	0.09±0.00	20.00±1.00	0.62±0.02
18	0.28±0.01	0.10±0.01	18.21±0.91	0.64±0.02
21	0.27±0.01	0.14±0.01	20.86±1.04	0.75±0.02
24	0.29±0.01	0.14±0.01	20.46±1.02	0.74±0.02
27	0.28±0.01	0.12±0.01	20.33±1.02	0.67±0.02
30	0.27±0.01	0.10±0.00	20.07±1.00	0.68±0.02
33	0.31±0.02	0.15±0.01	13.25±0.66	0.75±0.02
36	0.32±0.02	0.12±0.01	19.34±0.97	0.77±0.02
39	0.30±0.01	0.11±0.01	15.89±0.79	0.76±0.02
42	0.32±0.02	0.12±0.01	16.42±0.82	0.76±0.02
45	0.30±0.02	0.20±0.01	14.83±0.74	0.81±0.02
48	0.29±0.01	0.16±0.01	14.90±0.75	0.82±0.02
51	0.32±0.02	0.17±0.01	18.54±0.93	0.77±0.02
54	0.29±0.01	0.19±0.01	20.20±1.01	0.79±0.02
71	0.31±0.02	0.14±0.01	14.90±0.75	0.86±0.03

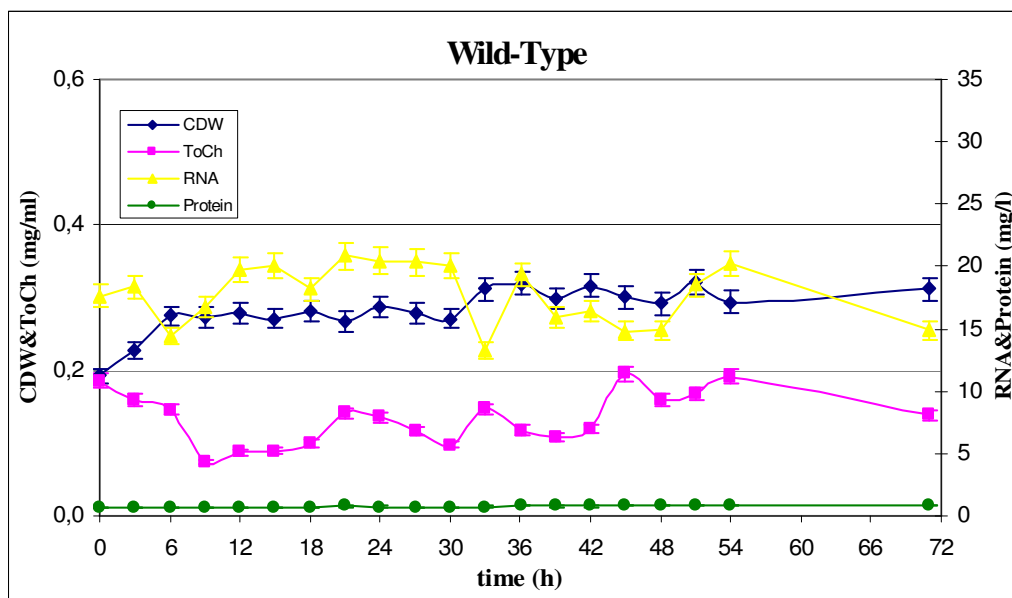


Figure 3.5: CDW, total carbohydrate, total RNA and total soluble protein results for anaerobic cultivation of wild-type.

Using HPLC analysis, residual glucose, glycerol and ethanol concentrations were determined as shown in Table 3.6 for anaerobic batch cultivation for wild-type. Although glucose consumption started at the beginning of cultivation, it was not consumed much during cultivation of the wild-type. Glucose consumption occurred mostly between 54th h and 71st h of incubation. However, glucose was not completely depleted at end of cultivation (Figure 3.6).

Even though glycerol production started at first hours of cultivation, it increased slowly until the end of cultivation. Furthermore, glycerol production reached its maximum during the last hours of the cultivation (Table 3.6). Ethanol concentrations increased from the beginning to 42nd h of the cultivation, and continued similarly until the end of cultivation (Figure 3.6).

Table 3.6: Residual glucose, glycerol and ethanol concentrations of anaerobic cultivation of wild-type are determined by HPLC analysis.

Time (h)	Residual Glucose (g/l)	Glycerol (g/l)	Ethanol (g/l)
0	20.89	0.000	0.451
3	19.86	0.022	0.553
6	19.81	0.033	0.574
9	19.79	0.043	0.594
12	19.36	0.054	0.576
15	19.37	0.059	0.586
18	18.66	0.064	0.597
21	18.61	0.066	0.635
24	18.61	0.064	0.644
27	18.67	0.070	0.606
30	18.74	0.072	0.640
33	18.63	0.074	0.594
36	19.62	0.075	0.605
39	19.76	0.075	0.584
42	18.80	0.081	0.589
45	18.71	0.075	0.585
48	19.08	0.073	0.623
51	18.76	0.081	0.621
54	18.34	0.080	0.595
71	15.90	0.085	0.352

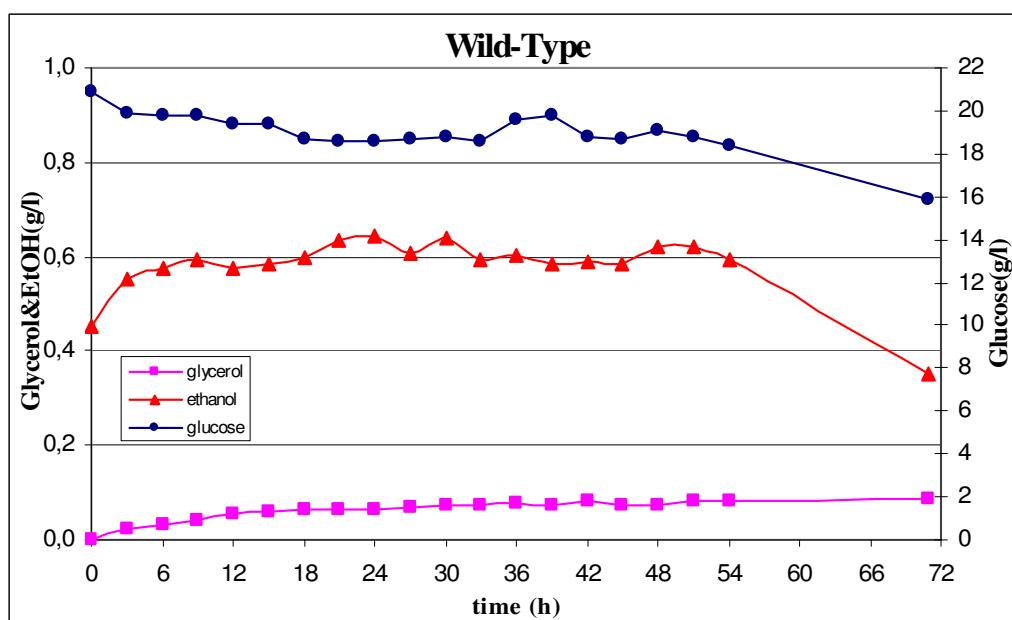


Figure 3.6: Residual glucose, glycerol and ethanol results for anaerobic cultivation of wild-type.

3.2.2 Anaerobic cultivation results for mutant 2

Anaerobic batch cultivation was performed for *Saccharomyces cerevisiae* WT (ENY.WA-1A) (MAT α *ura3-52 his3- Δ 1 leu2-3,112 trp1-289 MAL2-8c MAL3 SUC3*) with a plasmid containing *gpsA* from *Bacillus subtilis* in Biostat[®] B bioreactor systems. Table 3.7 shows the numerical results of OD₆₀₀, lnOD₆₀₀, pO₂% and pH for the anaerobic batch cultivation of mutant 2.

In anaerobic batch cultivation of mutant 2, pH decreased gradually from the beginning of the cultivation until the 21st h. But, it increased from 4.40 to 5.52 at the 24th h suddenly. It then started to decrease again gradually until the end of the cultivation (Table 3.7). Meanwhile, mutant 2 could not reach a typical log phase in anaerobic batch cultivation, like anaerobic batch cultivation of wild-type. Although mutant 2 cell concentration increased from the 12th h to the 27th h regularly, It then fluctuated to the end of incubation according to the OD₆₀₀ results. Cell growth is shown in Figure 3.7.

Table 3.7: OD₆₀₀, ln OD₆₀₀, pO₂% and pH results for anaerobic batch cultivation of mutant 2.

Time (h)	OD ₆₀₀	lnOD ₆₀₀	pO ₂ %	pH
0	0.89	-0.12	0	5.42
3	1.03	0.03	0	5.30
6	0.92	-0.08	0	5.13
9	1.02	0.02	0	4.91
12	0.97	-0.03	0	4.63
15	1.02	0.02	0	4.45
18	1.11	0.10	0	4.50
21	1.28	0.25	0	4.40
24	1.30	0.26	0	5.52
27	1.38	0.32	0	5.43
30	1.32	0.28	0	5.37
33	1.25	0.22	0	5.33
36	1.28	0.25	0	5.28
39	1.15	0.14	0	5.26
42	1.36	0.31	0	5.25
45	1.15	0.14	0	5.23
48	1.45	0.37	0	5.20
51	1.32	0.28	0	5.18
54	1.40	0.34	0	5.15
71	0.98	-0.02	0	5.00

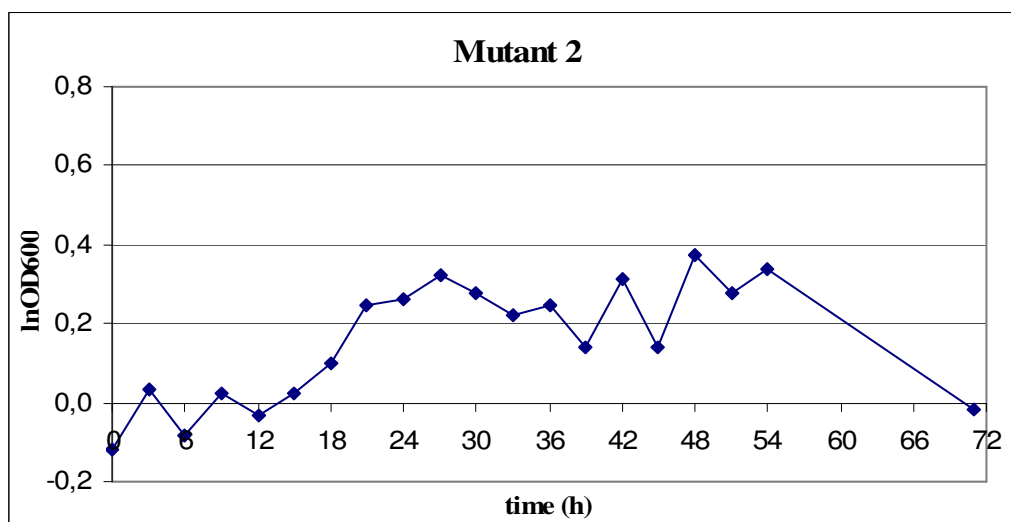


Figure 3.7: Growth behaviour of mutant 2 for anaerobic batch cultivation.

Cell dry weight, total carbohydrate, total RNA and total soluble protein concentrations of anaerobic batch cultivation of mutant 2 are listed in Table 3.8. Cell dry weight concentrations of mutant 2 started to increase from the 12th hour to 27th hour of incubation regularly, and then they fluctuated to the end of the cultivation,

(Figure 3.8). Total carbohydrate concentration did not change significantly throughout the cultivation (Figure 3.8). However, total RNA concentration showed large fluctuations during the cultivation time (Figure 3.8). Total soluble protein concentration showed small changes throughout the cultivation, similar to the total soluble protein result of anaerobic cultivation of wild-type.

Table 3.8: CDW, total carbohydrate, total RNA and total soluble protein results for anaerobic cultivation of mutant 2.

Time (h)	CDW(mg)/ml	ToCh (mg/ml)	RNA (mg/l)	Protein (mg/l)
0	0.20±0.01	0.15±0.01	28.01±1.40	1.84±0.06
3	0.23±0.01	0.19±0.01	23.36±1.17	2.02±0.06
6	0.21±0.01	0.16±0.01	24.96±1.25	1.91±0.06
9	0.23±0.01	0.20±0.01	21.76±1.09	1.84±0.06
12	0.22±0.01	0.18±0.01	23.43±1.17	1.83±0.05
15	0.23±0.01	0.20±0.01	23.74±1.19	2.00±0.06
18	0.25±0.01	0.27±0.01	23.74±1.19	1.99±0.06
21	0.29±0.01	0.21±0.01	25.57±1.28	1.99±0.06
24	0.29±0.01	0.22±0.01	27.10±1.35	1.96±0.06
27	0.31±0.02	0.24±0.01	26.18±1.31	2.17±0.07
30	0.30±0.01	0.25±0.01	26.03±1.30	1.97±0.06
33	0.28±0.01	0.26±0.01	27.63±1.38	1.85±0.06
36	0.29±0.01	0.21±0.01	26.11±1.31	1.98±0.06
39	0.26±0.01	0.28±0.01	25.34±1.27	2.13±0.06
42	0.31±0.02	0.20±0.01	20.92±1.05	2.02±0.06
45	0.26±0.01	0.25±0.01	18.60±0.93	1.96±0.06
48	0.33±0.02	0.19±0.01	27.31±1.37	2.06±0.06
51	0.30±0.01	0.24±0.01	19.24±0.96	2.10±0.06
54	0.32±0.02	0.24±0.01	18.78±0.94	2.03±0.06
71	0.22±0.01	0.22±0.01	24.81±1.24	2.03±0.06

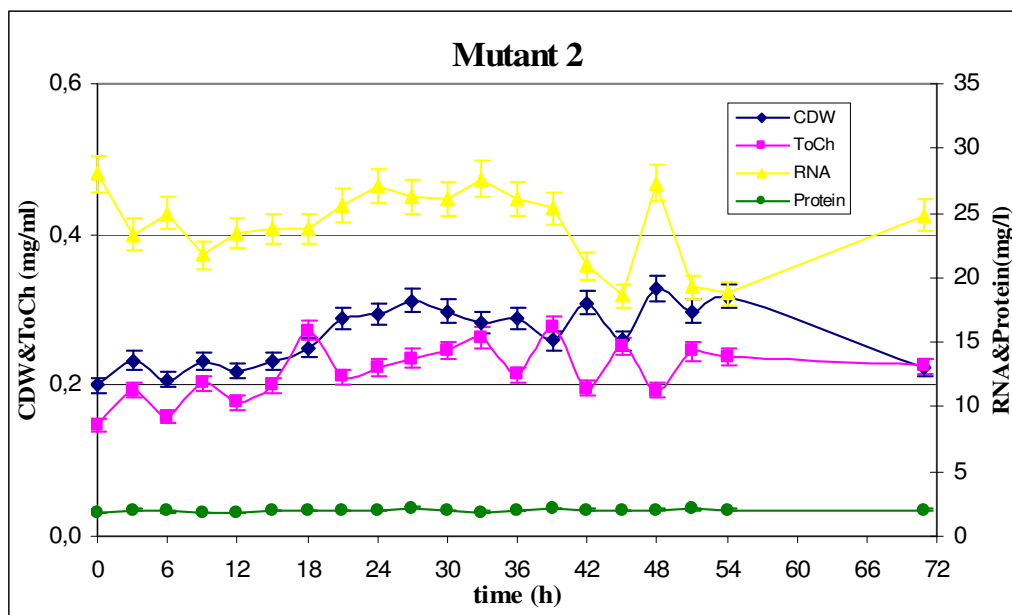


Figure 3.8: CDW, total carbohydrate, total RNA and total soluble protein results for anaerobic cultivation of mutant 2.

Using HPLC analysis, residual glucose, glycerol and ethanol concentrations are determined and these values are listed in Table 3.9 for anaerobic batch cultivation for mutant 2.

Although glucose consumption started from the beginning of cultivation, compared to the wild-type, less glucose was consumed. There was still same glucose left at the end of the cultivation (Figure 3.9).

Even though glycerol production started during the first hours of cultivation, it increased gradually until the 21st h of incubation. After that, glycerol production did not change much to the end of the cultivation, (Table 3.9).

Ethanol concentration increased rapidly during the first 12 hours; however, it decreased gradually to the end of the cultivation like glucose consumption. Besides, ethanol seems to be consumed starting from the 54th h of incubation.

Table 3.9: Residual glucose, glycerol and ethanol concentrations of anaerobic cultivation of mutant 2 as determined by HPLC analysis.

Time (h)	Residual Glucose (g/l)	Glycerol (g/l)	Ethanol (g/l)
0	21.71	0.000	0.673
3	20.27	0.014	0.630
6	19.85	0.023	0.654
9	19.32	0.040	0.708
12	20.34	0.058	0.869
15	19.27	0.066	0.726
18	18.69	0.072	0.786
21	18.75	0.078	0.654
24	17.66	0.077	0.652
27	17.64	0.083	0.655
30	17.44	0.081	0.601
33	18.01	0.072	0.629
36	18.32	0.079	0.645
39	18.28	0.075	0.593
42	17.82	0.082	0.589
45	18.12	0.083	0.600
48	17.88	0.074	0.767
51	17.99	0.081	0.585
54	17.64	0.081	0.565
71	16.77	0.085	0.354

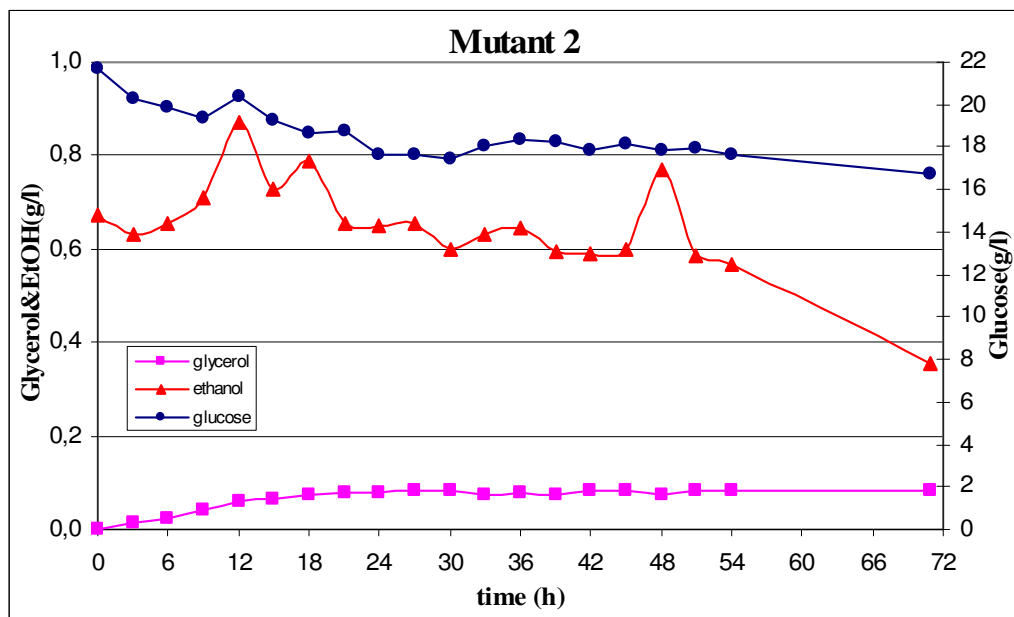


Figure 3.9: Residual glucose, glycerol and ethanol results for anaerobic cultivation of mutant 2.

3.2.3 Anaerobic cultivation results for mutant 3

Anaerobic batch cultivation was performed for *Saccharomyces cerevisiae* WT (ENY.WA-1A) (MAT α *ura3-52 his3- Δ 1 leu2-3,112 trp1-289 MAL2-8c MAL3 SUC3*) whose phosphoglucose isomerase gene function of which was reduced (*pgi1 Δ* (RBY 7-1), in a Biostat[®] B bioreactor system. The numerical results of OD₆₀₀, lnOD₆₀₀, pO₂% and pH for the anaerobic batch cultivation of mutant 3 are listed in Table 3.10.

In anaerobic batch cultivation of mutant 3, pH decreased gradually from the beginning of incubation until the 24th h. But, it increased gradually until the end of the incubation (Table 3.10). Mutant 3 could not reach a typical log phase in anaerobic batch cultivation. Cell concentration gradually increased from the beginning of cultivation to the 27th hour, and decreased at the 36th h. There were small fluctuations until the end of the cultivation (Figure 3.10).

Table 3.10: OD₆₀₀, ln OD₆₀₀, pO₂% and pH results for anaerobic batch cultivation of mutant 3.

Time (h)	OD ₆₀₀	lnOD ₆₀₀	pO ₂ %	pH
0	0.91	-0.09	0	5.51
3	0.95	-0.05	0	5.40
6	1.05	0.05	0	5.29
9	1.11	0.10	0	5.16
12	1.17	0.15	0	4.97
15	1.17	0.16	0	4.77
18	1.17	0.16	0	4.56
21	1.24	0.22	0	4.53
24	1.26	0.23	0	4.54
27	1.42	0.35	0	4.67
30	1.43	0.36	0	4.89
33	1.43	0.36	0	5.09
36	1.33	0.28	0	5.20
39	1.31	0.27	0	5.14
42	1.30	0.26	0	5.09
45	1.31	0.27	0	5.25
48	1.21	0.19	0	5.32
51	1.30	0.26	0	5.35
54	1.32	0.27	0	5.35
71	1.14	0.13	0	5.20

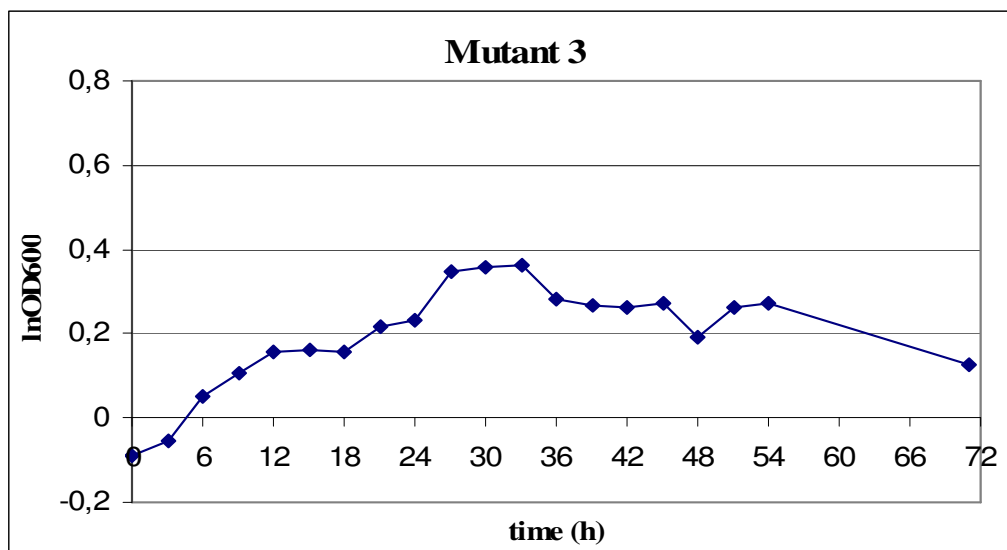


Figure 3.10: Growth behaviour of mutant 3 for anaerobic batch cultivation.

Cell dry weight, total carbohydrate, total RNA and total soluble protein concentrations of anaerobic batch cultivation of mutant 3 are listed in Table 3.11. Cell dry weight concentration of mutant 3 increased gradually, reached the maximum amounts between 27th and 33rd h of incubation, (Figure 3.11). Cell dry weight graph is parallel to the growth curve (lnOD₆₀₀ vs. time graph). Even though total carbohydrate concentration increased regularly until the 18th h, it then fluctuated until the end of the cultivation, (Figure 3.11). Total RNA concentration increased until the 15th h; however, it showed fluctuations to the end of cultivation time, similar to total carbohydrate concentration (Figure 3.11). Like in the case of the other mutants, total soluble protein concentration remained almost constant (Figure 3.11).

Table 3.11: CDW, total carbohydrate, total RNA and total soluble protein results for anaerobic cultivation of mutant 3.

Time (h)	CDW(mg/ml)	ToCh (mg/ml)	RNA (mg/l)	Protein (mg/l)
0	0.21±0.01	0.10±0.00	14.92±0.75	2.19±0.07
3	0.21±0.01	0.10±0.00	19.52±0.98	2.42±0.07
6	0.24±0.01	0.11±0.01	18.15±0.91	2.29±0.07
9	0.25±0.01	0.14±0.01	20.16±1.01	2.22±0.07
12	0.26±0.01	0.16±0.01	22.10±1.10	2.27±0.07
15	0.26±0.01	0.15±0.01	25.16±1.26	2.53±0.08
18	0.26±0.01	0.17±0.01	23.47±1.17	2.27±0.07
21	0.28±0.01	0.14±0.01	22.26±1.11	2.29±0.07
24	0.29±0.01	0.15±0.01	24.60±1.23	2.36±0.07
27	0.32±0.02	0.25±0.01	26.85±1.34	2.50±0.07
30	0.32±0.02	0.17±0.01	25.97±1.30	2.37±0.07
33	0.32±0.02	0.18±0.01	23.39±1.17	2.25±0.07
36	0.30±0.01	0.23±0.01	23.63±1.18	2.36±0.07
39	0.30±0.01	0.19±0.01	28.31±1.42	2.57±0.08
42	0.29±0.01	0.17±0.01	29.03±1.45	2.40±0.07
45	0.30±0.01	0.21±0.01	22.66±1.13	2.34±0.07
48	0.27±0.01	0.21±0.01	20.97±1.05	2.40±0.07
51	0.29±0.01	0.15±0.01	27.82±1.39	2.60±0.08
54	0.30±0.01	0.18±0.01	23.02±1.15	2.47±0.07
71	0.26±0.01	0.17±0.01	27.42±1.37	2.18±0.07

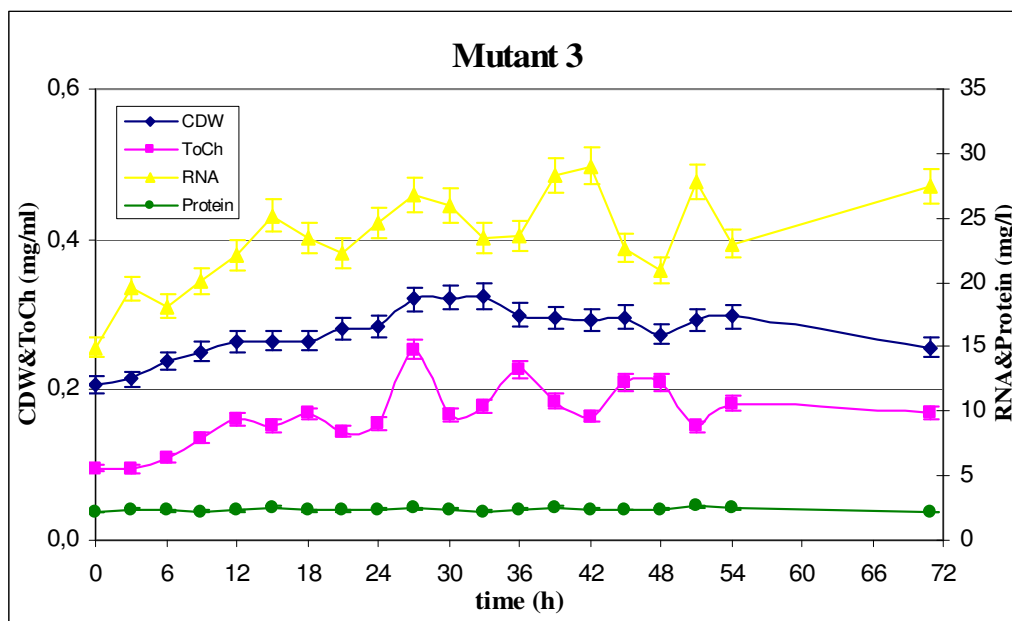


Figure 3.11: CDW, total carbohydrate, total RNA and total soluble protein results for anaerobic cultivation of mutant 3.

Using HPLC analysis, residual glucose, glycerol and ethanol concentrations were determined for anaerobic batch cultivation for mutant 3 (Table 3.12). Although glucose consumption started from the beginning of cultivation, there was little consumption after the 12th h until the 54th h by mutant 3. On the other hand, most glucose was consumed after the 54th h to the 71st h of incubation (Table 3.12). Besides, glucose was not completely depleted by the end of cultivation (Figure 3.12).

Glycerol was produced from the beginning of the cultivation by mutant 3; and glycerol levels increased gradually until the end of cultivation (Figure 3.12). In addition, glycerol amount increased quickly at the 12th h and the 27th h of the cultivation (Table 3.12).

After ethanol concentration had increased during the first 3 hours, it decreased gradually until the 9th h. Later, it increased until the 18th h, and continued smoothly until the 36th h. Finally, ethanol concentration decreased by the end of the cultivation time (Figure 3.12).

Table 3.12: Residual glucose, glycerol and ethanol concentrations of anaerobic cultivation of mutant 3 are determined by HPLC analysis.

Time (h)	Residual Glucose (g/l)	Glycerol (g/l)	Ethanol (g/l)
0	18.29	0.000	0.620
3	17.18	0.012	0.641
6	16.33	0.017	0.579
9	16.60	0.017	0.555
12	16.83	0.047	0.631
15	16.67	0.048	0.672
18	16.79	0.056	0.654
21	16.62	0.060	0.667
24	16.65	0.064	0.662
27	16.69	0.081	0.648
30	16.55	0.087	0.643
33	16.61	0.084	0.652
36	16.48	0.085	0.651
39	16.43	0.088	0.588
42	16.51	0.097	0.551
45	16.58	0.096	0.483
48	16.54	0.093	0.492
51	16.57	0.090	0.551
54	16.54	0.096	0.511
71	14.54	0.096	0.257

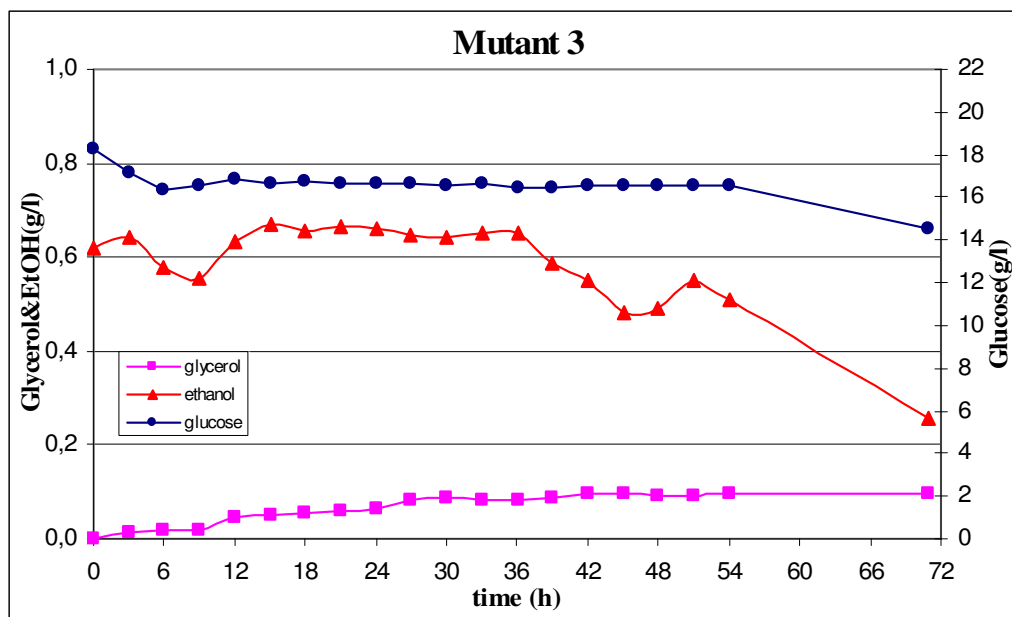


Figure 3.12: Residual glucose, glycerol and ethanol results for anaerobic cultivation of mutant 3.

3.2.4 Anaerobic cultivation results for mutant 4

Anaerobic batch cultivation was performed for *Saccharomyces cerevisiae* WT (ENY.WA-1A) (MAT α *ura3-52 his3- Δ 1 leu2-3,112 trp1-289 MAL2-8c MAL3 SUC3*) with reduced phosphoglucose isomerase gene and a plasmid containing *gpsA* from *Bacillus subtilis* in a Biostat[®] B bioreactor system. The numerical results of OD₆₀₀, lnOD₆₀₀, pO₂% and pH for the anaerobic batch cultivation of mutant 4 are listed in Table 3.13.

In anaerobic batch cultivation of mutant 4, after pH value had decreased gradually from the beginning of incubation until the 15th h, it fluctuated until the end of the incubation (Table 3.13). Mutant 4 could not reach a typical log phase in anaerobic batch cultivation as other anaerobic cultures. After the 3rd h, cell concentration increased until the 36th h of incubation. It then fluctuated to the end of cultivation as seen Figure 3.13.

Table 3.13: OD₆₀₀, ln OD₆₀₀, pO₂% and pH results for anaerobic batch cultivation of mutant 4.

Time (h)	OD ₆₀₀	lnOD ₆₀₀	pO ₂ %	pH
0	1.04	0.03	0	5.53
3	1.00	0.00	0	5.33
6	1.11	0.10	0	5.11
9	1.20	0.18	0	4.85
12	1.33	0.29	0	4.56
15	1.43	0.36	0	4.51
18	1.50	0.40	0	4.60
21	1.76	0.56	0	4.53
24	1.86	0.62	0	4.51
27	1.70	0.53	0	4.50
30	2.03	0.71	0	4.51
33	2.08	0.73	0	4.57
36	2.09	0.74	0	4.50
39	1.78	0.58	0	4.54
42	1.62	0.48	0	4.50
45	1.93	0.66	0	4.48
48	1.87	0.63	0	4.46
51	1.77	0.57	0	4.78
54	2.01	0.70	0	4.74
71	1.45	0.37	0	4.50

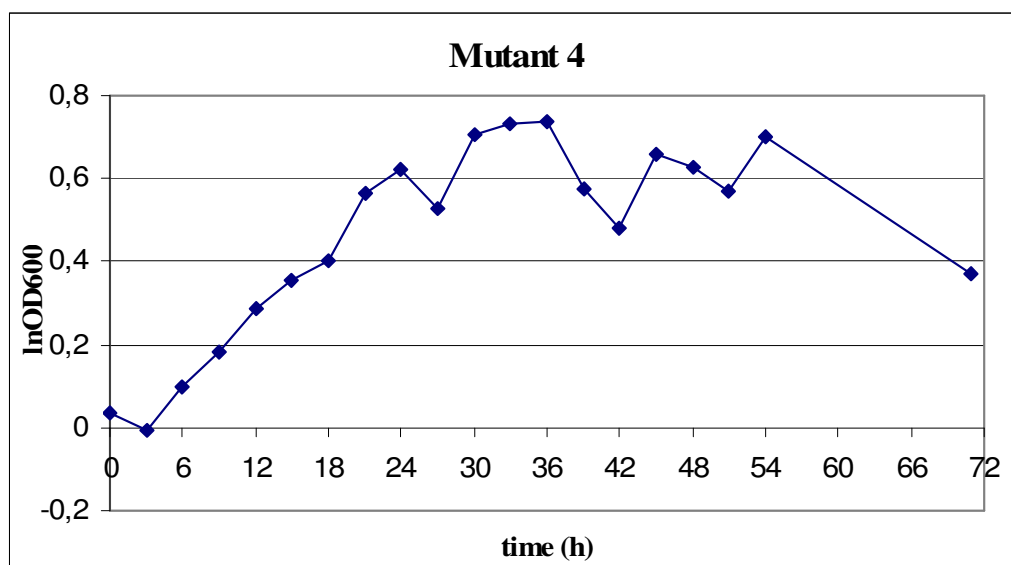


Figure 3.13: Growth behaviour of mutant 4 for anaerobic batch cultivation.

Cell dry weight, total carbohydrate, total RNA and total soluble protein concentrations of anaerobic batch cultivation of mutant 4 are listed in Table 3.14. Cell dry weight of mutant 4 increased gradually, reached the maximum amounts

between at 33rd and 36th hours, which is similar to growth curve (Figure 3.14). After the 36th h, it fluctuated to the end of cultivation. Total carbohydrate concentration increased until the end of cultivation time (Figure 3.14). Total RNA concentration increased with fluctuations until the end of the cultivation time, like total carbohydrate concentration (Figure 3.14). Like all mutants tested, the total soluble protein concentration of mutant 4 was rather constant with slight fluctuations (Figure 3.14). The maximum protein amount was obtained at the 39th h, (Table 3.14).

Table 3.14: CDW, total carbohydrate, total RNA and total soluble protein results for anaerobic cultivation of mutant 4.

Time (h)	CDW(mg)/ml	ToCh (mg/ml)	RNA (mg/l)	Protein (mg/l)
0	0.23±0.01	0.13±0.01	18.19±0.91	2.12±0.06
3	0.22±0.01	0.16±0.01	22.58±1.13	2.72±0.08
6	0.25±0.01	0.23±0.01	21.29±1.06	2.26±0.07
9	0.27±0.01	0.17±0.01	24.77±1.24	2.24±0.07
12	0.30±0.02	0.30±0.02	21.48±1.07	2.37±0.07
15	0.32±0.02	0.20±0.01	25.93±1.30	2.27±0.07
18	0.34±0.02	0.30±0.01	23.48±1.17	2.64±0.08
21	0.40±0.02	0.27±0.01	34.58±1.73	2.53±0.08
24	0.42±0.02	0.37±0.02	27.10±1.35	2.64±0.08
27	0.38±0.02	0.21±0.01	24.00±1.20	2.71±0.08
30	0.46±0.02	0.32±0.02	28.52±1.43	2.70±0.08
33	0.47±0.02	0.34±0.02	24.45±1.22	2.78±0.08
36	0.47±0.02	0.36±0.02	28.77±1.44	2.80±0.08
39	0.40±0.02	0.31±0.02	27.61±1.38	3.03±0.09
42	0.37±0.02	0.26±0.01	29.55±1.48	2.66±0.08
45	0.44±0.02	0.29±0.01	25.29±1.26	2.75±0.08
48	0.42±0.02	0.29±0.01	33.35±1.67	2.53±0.08
51	0.40±0.02	0.36±0.02	33.74±1.69	2.67±0.08
54	0.45±0.02	0.23±0.01	28.64±1.43	2.57±0.08
71	0.33±0.02	0.25±0.01	28.39±1.42	2.61±0.08

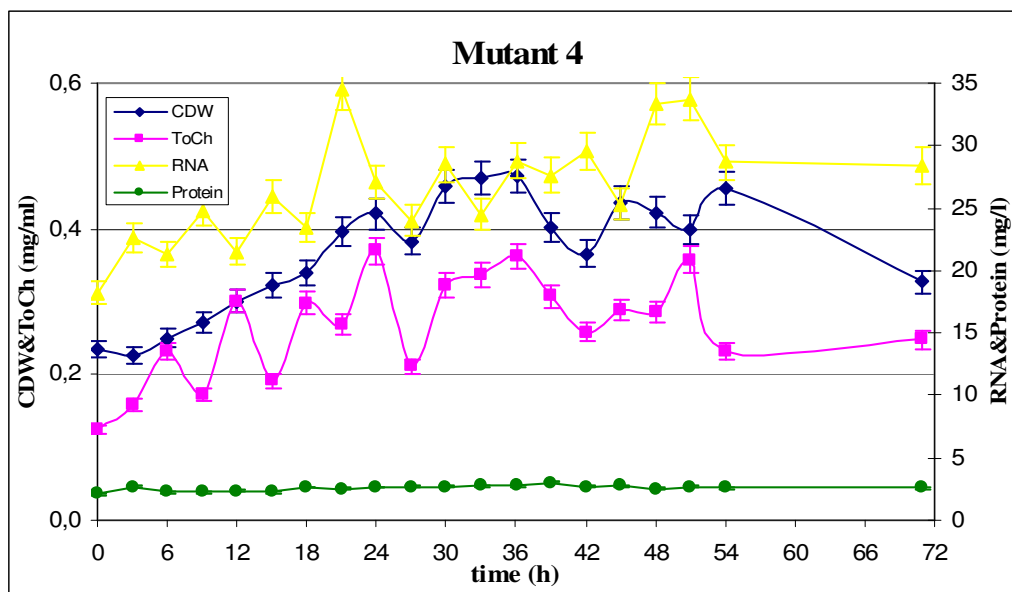


Figure 3.14: CDW, total carbohydrate, total RNA and total soluble protein results for anaerobic cultivation of mutant 4.

Residual glucose, glycerol and ethanol concentrations were determined by using HPLC analysis for anaerobic batch cultivation for mutant 4 (Table 3.15).

Again, although glucose consumption started from the beginning of cultivation, there was not much glucose consumption until the 54th h of cultivation of the mutant 4. However, most glucose was consumed after the 54th h to the 71st h, (Table 3.15). Also, glucose was not completely consumed by the end of cultivation by mutant 4, (Figure 3.15).

Glycerol was produced from the beginning of the cultivation by mutant 4; and it increased gradually to the end of cultivation (Figure 3.15). In addition, glycerol concentration increased quickly at the 12th h and the 24th h, and the highest glycerol production was observed at the 71st h (Table 3.15).

During the first 6 hours of incubation, ethanol concentration was rather constant; however it then increased gradually until the 15th h, after which it decreased until the end of the cultivation (Figure 3.15).

Table 3.15: Residual glucose, glycerol and ethanol concentrations of anaerobic cultivation of mutant 4 as determined by HPLC analysis.

Time (h)	Residual Glucose (g/l)	Glycerol (g/l)	Ethanol (g/l)
0	17.41	0.000	0.614
3	17.24	0.001	0.600
6	16.83	0.003	0.610
9	16.86	0.007	0.626
12	16.92	0.016	0.733
15	16.82	0.021	0.760
18	16.86	0.030	0.754
21	16.62	0.038	0.700
24	16.23	0.054	0.651
27	16.45	0.055	0.640
30	16.63	0.068	0.667
33	16.77	0.065	0.654
36	16.21	0.078	0.591
39	16.66	0.081	0.632
42	16.49	0.090	0.614
45	16.68	0.098	0.622
48	16.81	0.100	0.600
51	16.56	0.102	0.563
54	16.35	0.103	0.564
71	9.31	0.109	0.295

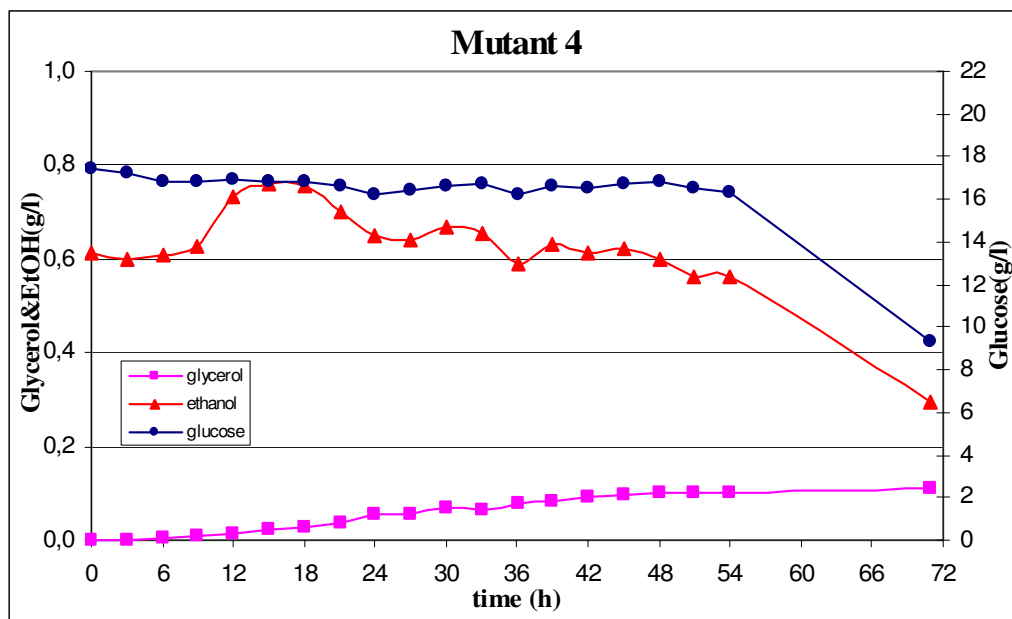


Figure 3.15: Residual glucose, glycerol and ethanol results for anaerobic cultivation of mutant 4.

3.2.5 Comparison of glycerol productions of all mutants under anaerobic condition

Table 3.16 compares glycerol production for wild-type and mutants under anaerobic conditions. The highest glycerol production was obtained with mutant 4, 0.109 g/l, with reduced phosphoglucose isomerase gene and a plasmid containing *gpsA* from *Bacillus subtilis*. Additionally, wild-type and mutant 2 produced the same amount of glycerol, 0.085 g/l, at the end of the cultivations (Figure 3.16).

Table 3.16: Glycerol productions of wild-type and mutants

Time (h)	Wild-Type	Mutant 2	Mutant 3	Mutant 4
0	0.000	0.000	0.000	0.000
3	0.022	0.014	0.012	0.001
6	0.033	0.023	0.017	0.003
9	0.043	0.040	0.017	0.007
12	0.054	0.058	0.047	0.016
15	0.059	0.066	0.048	0.021
18	0.064	0.072	0.056	0.030
21	0.066	0.078	0.060	0.038
24	0.064	0.077	0.064	0.054
27	0.070	0.083	0.081	0.055
30	0.072	0.081	0.087	0.068
33	0.074	0.072	0.084	0.065
36	0.075	0.079	0.085	0.078
39	0.075	0.075	0.088	0.081
42	0.081	0.082	0.097	0.090
45	0.075	0.083	0.096	0.098
48	0.073	0.074	0.093	0.100
51	0.081	0.081	0.090	0.102
54	0.080	0.081	0.096	0.103
71	0.085	0.085	0.096	0.109

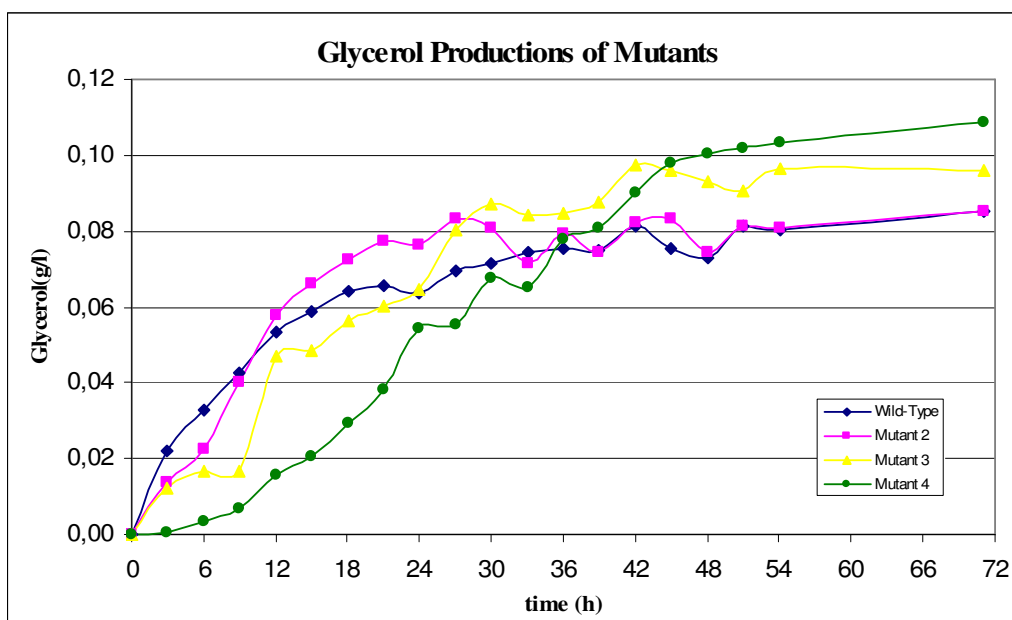


Figure 3.16: Glycerol productions of all mutants.

Glycerol yield was obtained by taking the ratio of produced glycerol (mol) to consumed glucose as substrate (mol) and this value was calculated for the samples taken at the 24th and 54th hours, as listed in Table 3.17. According to these calculations, the highest glycerol yield was obtained with mutant 4, and the lowest glycerol yield with mutant 2. Glycerol yield of mutant 4 was about 3 fold of that of the wild-type at the 54th h (Figure 3.17).

Table 3.17: Glycerol yields of wild-type and mutants at 24th and 54th hours (mol glycerol/mol glucose)

	Glycerol yield (mol glycerol / mol glucose)	
	24 th hour	54 th hour
Wild-Type	0,054	0,061
Mutant 2	0,037	0,039
Mutant 3	0,077	0,107
Mutant 4	0,090	0,190

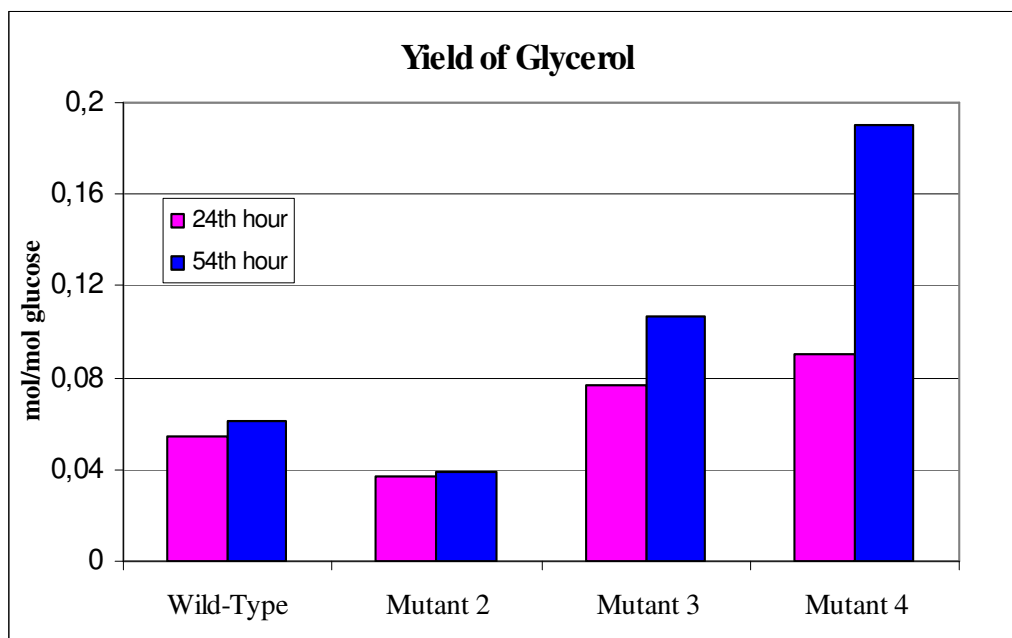


Figure 3.17: Glycerol yields of wild-type and mutants under anaerobic condition (mol glycerol/mol glucose)

4. DISCUSSION AND CONCLUSIONS

In this study, growth physiology and glycerol production of mutant *Saccharomyces cerevisiae* strains obtained by rational metabolic engineering strategies were compared to each other, in order to determine the best strain (construct) and the best metabolic engineering strategy for optimum glycerol yield. Wild-type yeast of *Saccharomyces cerevisiae* (ENY.WA-1A) was used as control. Three different recombinant yeasts were previously obtained by reduction of phosphoglucose isomerase (PGI) activity and insertion of *gpsA* gene, coding glycerol 3-phosphate dehydrogenase (GPD) enzyme, from *Bacillus subtilis* to yeast. Phosphoglucose isomerase activity of the first mutant (mutant 2) was reduced, *gpsA* gene of *B. subtilis* was transferred to another mutant (mutant 3) and the last mutant (mutant 4) was exposed to both of these genetic changes. Wild-type and these three mutant *S. cerevisiae* strains were grown under aerobic and anaerobic batch cultivation conditions in a 2-litre bioreactor. Moreover, their glycerol, ethanol, total RNA, total protein, total carbohydrate, residual glucose concentrations and cell dry weights were determined. The analysis results obtained from different mutants and wild-type cultivations under aerobic and anaerobic batch cultivation conditions were compared to each other.

During aerobic batch cultivation, wild-type *S. cerevisiae* cells showed a classical growth behaviour. At the 8th hour of incubation, cells entered the logarithmic phase. Between 16th and 25th hours of incubation, deceleration phase took place (Figure 3.1). Meanwhile, the dissolved oxygen levels decreased rapidly during the logarithmic growth phase, and were finished at the beginning of deceleration phase. After the 25th hour, during the stationary phase, dissolved oxygen levels increased gradually until the end of the cultivation (Table 3.1). This demonstrates that while cells were growing, they consumed oxygen fast. During the stationary phase, growth stopped, and amount of dissolved oxygen increased in the bioreactor. Moreover, cell dry weight analyses results and concentrations of total carbohydrate, total soluble protein, and total RNA were parallel to the growth curve (Figure 3.2). At the lag

phase, they did not increase significantly. However, during the logarithmic phase, they increased rapidly, and remained constant during the stationary phase.

Glucose was consumed mostly during the logarithmic phase (from the 10th to 24th hour of incubation), as expected. It is known that during the exponential phase nutrients are in excess and the organism is growing at its maximum specific growth rate for prevailing conditions (Stanbury, et al., 2000). Also, glycerol was mostly produced again in the logarithmic phase. At the end of aerobic batch cultivation of wild-type, all glucose was consumed and 0.420 g/l glycerol was produced (Figure 3.3). On the other hand, there was no ethanol production observed at aerobic batch cultivation.

At anaerobic batch cultivation, wild-type *S.cerevisiae* (mutant 1) cells did not show classical growth curve, unlike aerobic batch cultivation. After 6 hours of incubation, cell concentration fluctuated during the cultivation (Figure 3.4). Besides, concentrations of cell dry weight, total carbohydrate, total soluble protein, and total RNA were parallel to the growth curve results. Cell concentration and concentrations of other metabolites at anaerobic cultivation was not increased as much as at aerobic cultivation. On the other hand, glucose was not finished during cultivation time; actually, it was not consumed much. At the 54th hour (at the end of the sampling time), 2.55 g/l glucose was consumed and at the 71st hour (at the end of the incubation time), only 4.99 g/l glucose was consumed. Then, at the end of sampling time of anaerobic batch cultivation of wild-type, 0.080 g/l glycerol was produced. Meanwhile, ethanol was produced during anaerobic cultivation, as expected (Figure 3.6).

Cell dry weight results of anaerobic batch cultivations of all mutants were between 0.192 and 0.473 mg/ml. Even though the results of cell dry weight analyses of the mutants were similar, mutant 4 had the highest and mutant 1 had the lowest values.

Total carbohydrate concentrations of anaerobic batch cultivations of all mutants are between 0.095 and 0.370 mg/ml. In spite of the small fluctuations of total carbohydrate concentrations, the highest values were obtained from mutant 4, and the lowest values were obtained from mutant 1, again. Total RNA concentrations of anaerobic batch cultivations of all mutants were between 13.252 and 34.581 mg/l and total soluble protein concentrations of anaerobic cultivations of all mutants were between 0.616 and 2.798 mg/l. Although both metabolite concentrations had small

fluctuations for all mutants, the highest amounts were obtained from mutant 4, and the lowest amounts were obtained from mutant 1, like other metabolite amounts.

Using HPLC analysis, residual glucose, glycerol and ethanol concentrations were determined for anaerobic batch cultivation for all mutants. Unlike aerobic batch cultivation of wild-type, ethanol production was observed at anaerobic batch cultivations of all mutants. Under anaerobic conditions, yeasts reoxidize NADH to NAD in a two step reaction from pyruvate, which is first decarboxylated by pyruvate decarboxylase followed by the reduction of acetaldehyde, catalyzed by alcohol dehydrogenase. Ethanol and CO₂ were then produced (Walker, 1998). Ethanol concentrations of anaerobic batch cultivations of all mutants were between 0.451 and 0.760 g/l during sampling time. Generally, ethanol concentrations of the mutants did not change. However, there was a decrease in ethanol concentrations from the 54th hour to the end of the cultivation time, possibly indicating the consumption of ethanol as a carbon source.

Glucose consumption levels were low during anaerobic batch cultivations of all mutants, when compared with the aerobic batch cultivation of wild-type. At the end of the cultivation, mutant 1, mutant 2, mutant 3 and mutant 4 consumed 2.55 g/l, 4.07 g/l, 1.75 g/l and 1.06 g/l of glucose, respectively. As expected, because of the lack of oxygen, glucose cannot be fully oxidized and consumed to obtain energy for growth (biomass production). Fermentative pathways (ethanol production) were active during anaerobic conditions.

According to Figure 3.16, the highest glycerol production was obtained at mutant 4 at the end of cultivations. Until the 36th hour of incubation, glycerol production of mutant 4 was at the lowest level among all mutants. After the 45th hour of incubation, amount of glycerol produced by mutant 4 was higher than the other mutants (Table 3.16). It was shown that glycerol was produced under anaerobic conditions as a by-product that compensates for cellular reactions that produce NADH (van Dijken and Scheffers, 1986). Thus, this demonstrates that the biomass yield of mutant 4 was higher than that of the other mutants after the 36th hour. Furthermore, at the end of sampling time, mutant 1, mutant 2, mutant 3 and mutant 4 produced 0.080 g/l, 0.081 g/l, 0.096 g/l and 0.103 g/l of glycerol, respectively.

The key difference between the mutants tested was based on their glycerol yields. As mentioned in the results section, glycerol yield was obtained by taking the ratio of

produced glycerol (mol) to consumed glucose as substrate (mol). Thus, the highest glycerol yield was obtained with mutant 4 with reduced phosphoglucose isomerase gene function and a plasmid containing *gpsA* from *Bacillus subtilis*. The yield calculations were done for the 24th hour and the 54th hour of incubation (Table 3.17). According to 24th hour results, glycerol yield in mutant 4 was about 67% higher when compared to wild-type. Moreover, according to the 54th hour results, glycerol yield in mutant 4 was about 211.5% higher when compared to the wild-type. Additionally, at the 54th hour, glycerol yield of mutant 4 increased 111.2% in comparison to the 24th hour of incubation. This result is expected when glucose consumption and glycerol production results were taken into consideration. Meanwhile, until the end of cultivation (according to the sampling time), NAD(P)H could increase and glycerol production increased to reoxidize NADH.

Glycerol is produced in two steps in *S.cerevisiae*. The first step is the reduction of glycolytically formed dihydroxyacetone phosphate (DHAP) to glycerol 3-phosphate (G3P) concomitant with the oxidation of NADH. The reaction is catalyzed by glycerol 3-phosphate dehydrogenase (GPD). The next step is the dephosphorylation of G3P to glycerol by glycerol 3-phosphatase (GPP) (Overkamp, et al., 2002), as shown in Figure 1.3. There were two mutations for glycerol overproduction at mutant 4. Aims of these mutations were replacement of the cofactor requirement for glycerol formation from NADH to NADPH and transporting the NADPH by redirecting the sugar catabolism through the pentose phosphate pathway (PPP). For this reason, first, the main flux of the glucose catabolism was redirected through the PPP by strongly reducing the activity of the phosphoglucose isomerase and thereby generating an excess of NADPH. Secondly, the *gpsA* gene from *B. subtilis* encoding a NADPH-dependent dihydroxyacetone phosphate (DHAP) reductase was introduced (Nguyen, et al., 2006). DHAP reductase of *B. subtilis* is heterologous NADPH-dependent GPD. Furthermore, the *gpsA* gene of *B.subtilis* seems to be controlled at the transcriptional level strongly, so the expression of the gene can be improved by a strong promoter. On the other hand, all GPDs of yeasts have been strictly NADH-dependent (Schomburg, et al., 1995).

At previous study, the first mutation, reducing the *pgil* gene activity, was done for mutant 3, so most of the carbon flux from glucose should readdress through the PPP. Even though mutant 3 produced NADPH from PPP, glycerol production was less

than in mutant 4, because it has not NADPH-dependent DHAP reductase activity at glycerol biosynthesis pathway. The second mutation, introducing the *gpsA* gene from *B. subtilis*, was done for mutant 2 whose glycerol production was also less than mutant 3 and mutant 4. At last, both mutations were done for mutant 4. Therefore, most carbon flux from glucose should be redirected through the PPP and more NADPH should be produced. More DHAP should then be converted to G3P by NADPH-dependent DHAP reductase. Consequently, glycerol production and yield was the highest in the case of the mutant 4.

To summarize, redirection of the glycerol biosynthesis pathway by metabolic engineering was shown to increase glycerol yield markedly in this study. Further studies to determine the metabolic flux distributions could give more insight into the glycerol and carbon metabolism of the metabolically engineered strains.

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