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ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

PHYSIOLOGY AND BIOCHEMICAL STORAGE CHARACTERISTICS OF *E. coli* K12

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E. coli K12'NİN FİZYOLOJİSİ VE BİYOKİMYASAL DEPOLAMA MEKANİZMALARININ İNCELENMESİ

YÜKSEK LİSANS TEZİ

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ABBREVIATIONS

ATP	: Adenosine Triphosphate
CDW	: Cell Dry Weight
CoA	: Coenzyme A
DNA	: Deoxyribonucleic Acid
DO	: Dissolved Oxygen
EBPR	: Enhanced Biological Phosphorus Removal
ED	: Entner-Doudoroff Pathway
EMP	: Embden-Meyerhof-Parnas Pathway
G6P	: Glucose-6-phosphate
GC	: Gas Chromatography
HB	: β-hydroxybutyric Acid
HCl	: Hydrochloric Acid
HPLC	: High Performance Liquid Chromatography
NAD^+	: Nicotinamide adenine dinucleotide (oxidized form)
NADH	: Nicotinamide adenine dinucleotide (reduced form)
NADPH	: Nicotinamide adenine dinucleotide phosphate (reduced form)
OD_{600}	: Optical Density (Abs. 600 nm)
OUR	: Oxygen Uptake Rate
PHA	: Polyhydroxyalkanoate
PHB	: Poly-β-hydroxybutyrate
PHV	: Polyhydroxyvalerate
RNA	: Ribonucleic Acid
SBR	: Sequencing Batch Reactor
SUR	: Substrate Uptake Rate
VFA	: Volatile Fatty Acid

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ÖZET

Çeşitli mikroorganizmalarda dinamik yaşam koşullarında (bolluk/kıtlık rejimi) içsel depolama ürünleri oluşmaktadır. Büyümenin azot, oksijen gibi anahtar faktörler tarafından kısıtlandığı durumlarda bir rezerv maddesinin oluşumu kabul görmüştür. Aktif çamur sistemleri dinamik yaşam koşullarında çalışan bir biyolojik atıksu arıtma prosesidir. Aktif çamur mikroorganizmaları bolluk/kıtlık döngüsü ile muamele edildiklerinde glikojen ve poli-β-hydroksialkonat gibi depolama polimerleri üretmektedir. Polihidroksialkonatlar bütünüyle biyodegrade edilebilen termoplastiklerdir. Polihidroksibütiratlar en iyi karakterize edilmiş başlıca komponentidir. Birincil substratın glikoz veya diğer şekerler (örneğin karbohidratlar, gliserin veya proteinler) olduğu durumlarda oluşan glikojen düz zincirli bir polimerdir.

E. coli gram-negatif, çomak şeklinde, hareketli, fakültatif anaeroplar grubuna üye bakteridir. Birincil habitatı insan olduğu için aktif çamur sistemlerinde ve atıksu arıtma tesislerinde *E. coli*'ye sık rastlanmaktadır. Bu nedenle aktif çamur sistemlerinin depolama mekanizmalarının incelenmesi amacıyla model organizma olarak *E. coli* kullanılmıştır.

Bu çalışmada, kesikli erlen deneyleri ve kesikli, yarı kesikli ve respirometrik analizler içeren ardışık kesikli biyoreaktör deneyleri gibi farklı kültivasyon koşulları ile çeşitli deneyler gerçekleştirilmiştir. Glikoz ve gliserin kimyasal olarak tanımlanmış (M9) besiyerinde tek karbon kaynağı olarak kullanılmıştır. Optik yoğunluk, hücre kuru ağırlığı, toplam protein ve uçucu yağ asidi konsantrasyonları gibi anahtar parametreler büyüme fizyolojisinin tanımlanması amacıyla kullanılmıştır. Glikojen ve PHA gibi depolama polimerleri YPSK (Yüksek Performans Sıvı Kromatografi) ve GK (Gaz Kromatografi) teknikleri kullanılarak direk olarak ölçülmüştür. Farklı kültivasyon koşullarında *E. coli*'nin fizyolojisi ve biyokimyasal depolama mekanizmaları karakterize edilmiştir. Glikozun tek karbon kaynağı olarak kullanıldığı yarı kesikli ve ardışık kesikli kültivasyon deneyleri sırasında ve NH_4Cl'nin tek azot kaynağı olarak kullanıldığı azot limitasyon/açlık deneylerinde glikojen depolama ve tüketimi gözlenmiştir. Diğer taraftan gliserinin tek karbon kaynağı olarak kullanıldığı kesikli erlen deneyleri sonucunda glikojen depolanmasına rastlanmamıştır. Glikozun ve gliserinin tek karbon kaynağı olarak kullanıldığı deneysel koşulların hiçbirinde PHB depolanması tespit edilmemiştir

Anahtar Kelimeler: Depolama, Polihidroksialkonat, Polihidroksibütirat, Glikojen, *Escherichia coli*.

SUMMARY

Internal storage products occur under dynamic conditions (feast/famine regimes) in various microorgansims. It is generally accepted that under growth-limited conditions by some other key factors such as nitrogen, oxygen, etc., accumulation of a reserve material is most likely observed. Activated sludge system is a biological wastewater treatment process, which generally occurs under dynamic conditions. When activated sludge microorganisms are subjected to feast/famine regimes, microorganisms produce storage polymers, like glycogen and poly-β-hydroxyalkanoates. Polyhydroxyalkanoate (PHA) is a thermoplastic with complete biodegradability. Polyhydroxybutyrate (PHB) is the most characterized form and also one of the major components. Glycogen is a straight chain polymer formed when the primary substrate is glucose or other sugars, like different carbohydrates, glycerol or proteins.

E. coli is gram-negative, rod-shaped, mobile bacterium that belongs to the group of facultative anaerobes. Since humans are the major habitat of *E. coli*, it is highly found in the activated sludge systems and also in wastewater treatment plants. Therefore, in this study, *E. coli* was used as the model organism to investigate the storage mechanisms of activated sludge systems.

Various experiments were performed in this study under different cultivation conditions, e.g. shake flask batch experiments and batch, fed-batch and sequencing batch cultivations including respirometric analysis in a bioreactor. A chemically defined medium was used with either glucose or glycerol as the sole carbon source. The key parameters like optical density, cellular dry weight, total protein and volatile fatty acid concentrations were used to monitor the growth physiology. The storage polymers, glycogen and PHA were measured by High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) techniques, respectively. The biochemical storage and physiological properties of *E. coli* were characterized under different experimental conditions. During fed-batch and SBR cultivations of *E. coli* with glucose as the sole carbon source, glycogen storage and consumption was observed. Also during batch shake flask experiments where varying NH_4Cl concentrations were used for nitrogen limitation/starvation, glycogen production was observed. On the other hand, no glycogen formation was observed at varying concentrations of glycerol in batch shake flask and bioreactor cultivations. No storage of PHB was observed under any of the cultivation conditions with neither glycerol nor glucose as the sole carbon source.

Keywords: Storage, Polyhydroxyalkanoate, Polyhydroxybutyrate, Glycogen, *Escherichia coli*.

1. INTRODUCTION

1.1. The Relevance of the Subject and The Aim of the Study

Industrial and domestic wastewaters contain a number of living microorganisms, which can be harmful to the environment and pathogenic to human health. Additionally, the sewage originated from these industrial and domestic wastes consists of carbohydrates, fatty acids, amino acids, volatile fatty acids, non-volatile fatty acids, detergents, uric acid, creatine, amino sugars, as well as nutrients like nitrogen, phosphorus, sulfur, etc. This material has to be efficiently treated without giving harm to the natural environment.

Among the systems constructed for the wastewater treatment, activated sludge systems have attributed much of the success. Biological wastewater treatment process generally occurs under dynamic conditions. The activated sludge microorganisms are subjected to feast/famine regimes. It has been observed that under these conditions microorganisms produce storage polymers, like glycogen and poly-β-hydroxyalkanoates (Van Loosdrecht *et al.*, 1997). In these cases, the storage polymer acts like a buffer for the substrate that is taken up but not directly used for growth. When the microorganisms are able to store during the feast period and subsequently use it for growth, they then have a competitive advantage for their survival (Beccari *et al.,* 1998). The performance of the activated sludge process depends on the role played by these storage compounds (Chiesa *et al.*, 1985; Majone *et al.,* 1996; Van Loosdrecht *et al.,* 1997).

The metabolism of various storage polymers in mixed cultures under different culturing conditions were investigated in detail. However, how different substrates and culturing conditions affect microorganisms individually is still a black box. It is not yet understood how microorganisms shift to storage response from growth situation. The aim of the present research was therefore to study the biochemical storage metabolism of pure cultures by using *Escherichia coli (E. coli)* as the model organism of the activated sludge microorganisms. Also the effects of previous

culturing conditions to metabolic activities after the addition of substrate were investigated. Although *E. coli* has been known to accumulate no polyhydroxyalkanoates (PHA), it is recently being discussed that under suitable conditions all microorganisms can accumulate storage polymers (van Loosdrecht, personal communication). Thus, the storage metabolism, i.e. accumulation of glycogen and PHA of *E. coli* was investigated as well as its biochemical and physiological properties. Additionally, the effects of varying concentrations of different carbon sources on polyhydroxybutyrate (PHB) and glycogen storage were studied. Especially, as the sole carbon source in the growth medium, glucose, a simple sugar highly abundant in the environment and glycerol -a by-product from the production of biofuels from oilseeds and palm oil (a plant oil)- were investigated at different concentrations. The growth physiology was determined by measuring key parameters like; optical density, cellular dry weight, total protein and volatile fatty acid concentrations. Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC) techniques were used for the determination of PHB and glycogen, respectively. The biochemical storage and physiological properties of *E. coli* have been characterized under different experimental conditions.

1.2. The Substrate Storage Phenomena

1.2.1. The Importance of Storage

The various components in the domestic and industrial wastewater serve as the substrate for the microbial community of that wastewater. Treatment of wastes with the activated sludge process represents a component of the largest biotechnology industry in the world. The activated sludge systems are mixed cultures in which an enormous diversity of organic compounds enter the system, involve in different biochemical reactions and result as different chemical compositions and molecular particles. It is thought that when cell growth is inhibited by the limitation of a key factor such as oxygen, nitrogen, sulfate or phosphate (Dawes, 1992; Lafferty *et al.*, 1988); storage occurs. It is shown that these storage products are subsequently utilized as an energy and/or carbon source and/or electron donor.

Microorganisms are exposed to feast (presence of substrate) and famine (absence of substrate) conditions many times. The experimental studies and the literature indicate

that the response of microorganisms, exposed to such conditions is to accumulate storage polymers in the feast period. In the absence of substrate, these storage polymers are used for growth and survival. Bacteria that can take up and accumulate organic substrates in the feast period and grow in the famine conditions have a strong competitive advantage over the organisms without the storage capacity (Satoh *et al.,* 1999).

Recent studies were conducted under growth-limited conditions. It is thought that storage occurs independently. It is obvious that formation of a storage molecule in the bacteria gives the advantage of a balanced metabolism when sudden changes occur in the substrate availability. When the substrate uptake is limited, the organisms would need relatively large amounts of substrate uptake enzymes. Therefore, decrease in the amount of enzymes would be uncontrolled. If the substrate concentration suddenly rises, this will result in the rapid uptake of the substrate. But the growth processes of the organism cannot directly consume this amount of substrate and this may result in a metabolic imbalance. Consequently, the storage cannot be defined as an independent mechanism from the growth (van Loosdrecht *et al.*, 1997). In addition to this, the substrate is transported into the cell and maintained in an almost unchanged form or transformed into low molecular weight metabolic intermediates. In other words, the storage can be also explained as a mechanism for preventing the accumulation of internal metabolites at toxic levels during the growth lags (Majone *et al.,* 1998).

1.2.2. The Importance of Pure Culture Studies

The growth of bacteria on storage products has recently gained interest. It is well recognized that in activated sludge and mixed cultures under dynamic conditions a storage response is usually triggered i.e. the substrate is removed and transformed into specialized internal polymers (usually polysaccharides or polyhydroxyalkanoates), which can later be used as an internal carbon source for growth. The stoichiometry and kinetics of storage have been described in detail, at least for PHB formation from acetate. However, how the microorganisms individually respond under aerobic conditions can affect the storage response in dynamic systems is quite less known. Therefore, a pure culture study under conditions that is typical of environmental cases, i.e. on cells cultured under unsteady

conditions (feast/famine) must be constructed. These could be better understood with the aid of pure culture studies.

1.3. Microbial Response

Microbial response to dynamic conditions can include several different phenomena; in particular growth response and storage response (Daigger and Grady, 1982). These responses are discussed in the next section.

1.3.1. Growth Response

Microorganisms as cells are considered to be the fundamental units of life. A cell is a dynamic unit; it constantly undergoes changes and replaces its parts. At the beginning of the cultivation, therefore, a physiological adaptation of the microorganisms to the particular environment occurs.

All cells are made up of four chemical components; proteins, nucleic acids, lipids and polysaccharides that are collectively called macromolecules. The changes in the cellular compositions like deoxyribonucleic acids (DNA), ribonucleic acid (RNA) and proteins indicate the adaptation process. As the environment becomes relatively steady, the protein synthesis reaches to an optimal level for cell growth in that environment (Roels 1983, Grady *et al.,* 1996). If the substrate in the environment is sufficient for the microorganisms, the growth occurs at a constant rate. On the other hand, the growth response varies if the microorganisms are exposed to substrate-limited conditions. If the culture is adapted to grow under a substrate-limited condition, the protein synthesis rate will not be high enough to quickly increase the growth rate when the substrate limitation is removed.

Like any other cellular organisms, microorganisms need energy for growth and storage. The energy required is generally obtained from the previously stored and conserved forms of chemical adenosine triphosphate (ATP). ATP is generally used as an energy carrier between energy-generating reactions (catabolism) and energy–consuming reactions (anabolism) in the cell (Gottschalk, 1985). The metabolic events that take place inside the cell are summarized in Fig. 1.1.

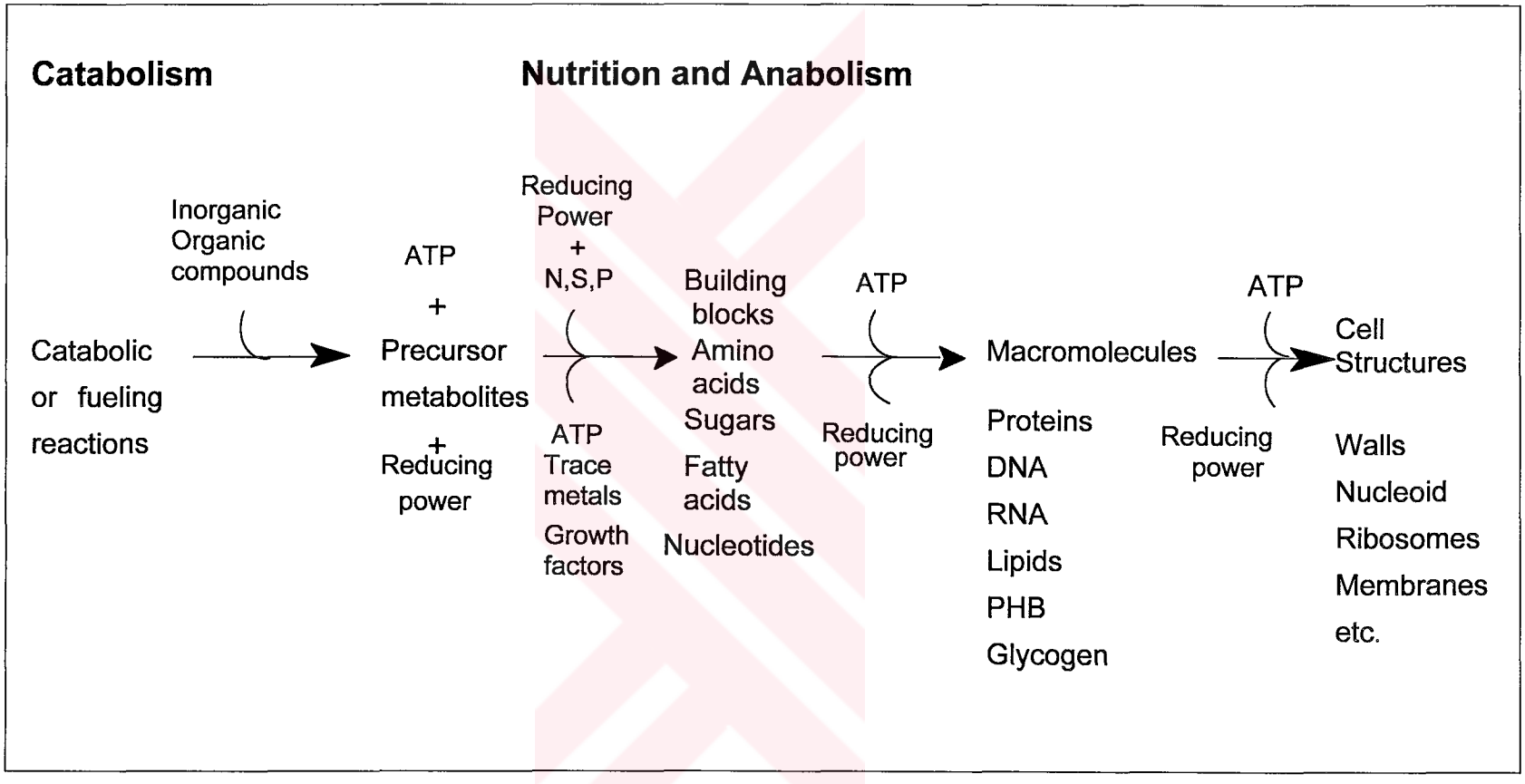


Figure 1.1 The metabolic events taking place inside the cell (Gottschalk, 1985)

1.3.2. Determination of Cell Growth

Basically cell growth is achieved in batch or continuous culturing conditions. Determination of cell growth, product yield and the stoichiometry in a culture media is based on the quantification of cell concentration. The methods in the cell concentration quantification are classified in two categories: direct and indirect. In many cases, the direct methods are not feasible due to the presence of suspended solids or other interfering compounds in the medium (Shuler and Kargi, 1992).

Determination of *cellular dry weight* is the most commonly used direct method for determining cell mass concentration. In a solids-free medium cells are separated from the medium and the resulting weight obtained give the number of cells. Another rapid method is based on the absorption of light by suspended cells in sample culture media. The intensity of the transmitted light is measured using a spectrometer. Cell mass concentration is often measured by the cell number determination by *optical density*. The extent of light transmission in a sample chamber is a function of cell density. The procedure entails using a wavelength-minimized absorption by medium components (600- to 700- nm), "blanking" against medium, and the use of a calibration curve. The calibration curve relates optical density (OD) to dry-weight measurements.

In many fermentation processes indirect methods are used, which are mainly based on the measurement of substrate consumption and/or product formation during the course of growth. Intracellular components of cells such as RNA, DNA, and protein can be measured as indirect measures of cell growth. During a batch growth cycle, the concentrations of these intracellular components change with time. Concentration of RNA (RNA/cell weight) varies significantly during a batch growth cycle, whereas DNA and protein concentrations remain fairly constant. Therefore, protein and DNA concentrations measurements can be used as a measure of microbial growth.

After the inoculation of a liquid medium with a seed culture, the microorganisms selectively take up dissolved nutrients from the medium and convert them into biomass. In a typical batch growth, the following phases are observed; (1) lag phase, (2) logarithmic or exponential growth phase, (3) deceleration phase, (4) stationary phase, and (5) death phase. Fig. 1.2 describes the batch cycle growth correlated with optical density measurements.

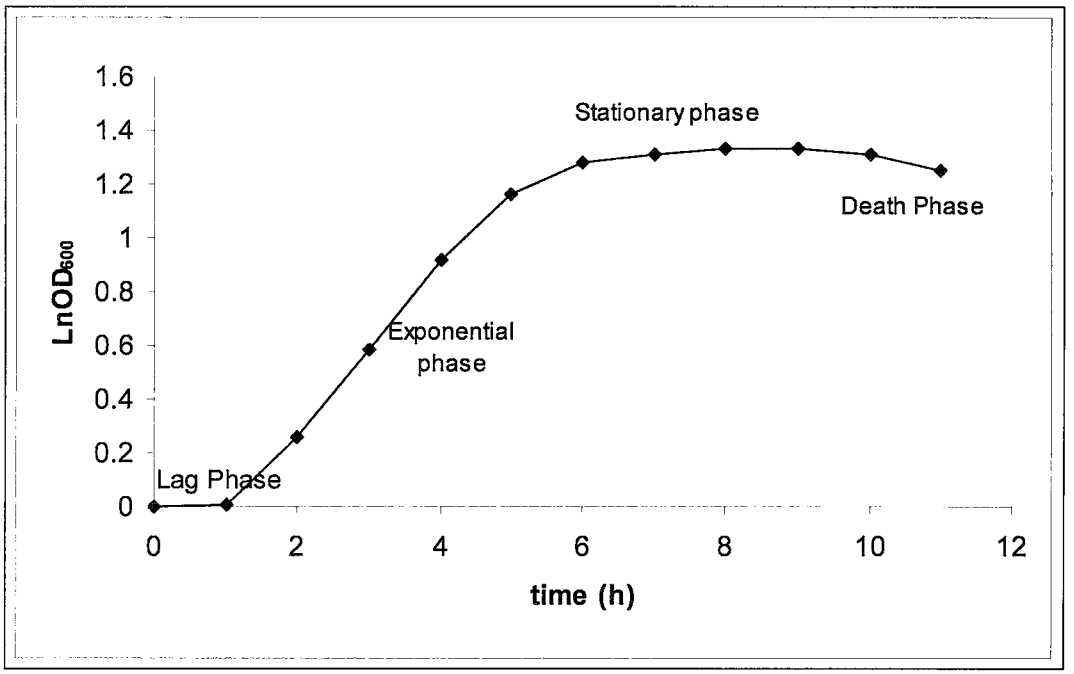


Figure 1.2 A typical growth curve of bacterial pure batch cultivation

The lag phase occurs immediately after inoculation and is a period of adaptation of cells to a new environment. During this phase cell mass may increase a little, without an increase in cell number density. In the exponential growth phase, the cells have adjusted to their new environment. After this adaptation period, cells can multiply rapidly, and cell mass and cell number density increase exponentially with time. This is a period of balanced growth in which all components of a cell grow at the same rate. The deceleration growth phase follows the exponential phase. In this phase, growth decelerates due to either depletion of one or more essential nutrients or the accumulation of toxic by-products of growth. In the exponential phase, the cellular metabolic control system is set to achieve maximum rates of reproduction. In the deceleration phase, the stresses induced by nutrient depletion or waste accumulation cause a restricting of the cell to increase the prospects of cellular survival in a hostile environment. The stationary phase starts at the end of deceleration phase, when the net growth rate is zero or when the growth rate is equal to the death rate.

For aerobic growth, it is necessary to provide extensive aeration. This is because O_2 is only poorly soluble in water and the O_2 used up by the organisms during growth is not replaced fast enough by diffusion from the air. Forced aeration of cultures is therefore frequently desirable and can be achieved either by vigorously shaking the flask or tube on a shaker or by bubbling sterilized air into the medium through a fine

glass tube or porous glass disc. Usually aerobes grow much better with forced aeration than when O_2 is provided by simple diffusion (Madigan *et al.*, 2003).

Dissolved oxygen (DO) is an important substrate in aerobic fermentations and may be a limiting substrate, since oxygen is a spargingly soluble gas in water. At high cell concentrations, the rate of oxygen consumption may exceed the rate of oxygen supply, leading to oxygen limitations.

1.3.3. Storage Response

The dynamic conditions can also lead the cells to a storage response, even in the absence of any external limitation for the growth. The kinetics and the yield in the storage process are different than the growth. The substrate uptake rate (SUR) rapidly increases whereas new solids are formed in the cell. The solid composition differs according to the substrate and the mechanism of the microorganism, as well as the environmental conditions. Main storage polymers are considered to be polysaccharides and lipids in general. Because synthesis of storage polymers is simpler than that of whole cell, less physiological adaptation is required and storage response can be considered as more rapid than the growth response (Daigger and Grady, 1982a).

In literature, organic and inorganic storage metabolites have been reported. Polyphosphates are considered to be an example for inorganic storage molecules whereas poly-β-hydroxybutyric acid (PHB), glycogen and lipids are reported to be the important organic storage polymers (Zevenhuizen and Ebbink, 1974).

Storage product composition depends on the carbon source used. Among the inorganic storage compounds, PHB is probably the most dominant polymer as it is directly formed out of the central metabolite acetyl-CoA. On the other hand, glycogen is determined to form only in the presence of sugars in the influent. The literature notifies a few reports on the presence of lipids as the storage molecules. They are present in small, constant amounts in all bacteria. They are generally considered as structural components of membranes (phospholipids) and cell walls (polysaccharides).

1.3.4. Accumulation Response

Microorganisms can also remove the substrate quickly by accumulation. The substrate that is transported into the cell is maintained in an almost unchanged form or it can be transformed into a low molecular weight metabolic intermediate. Mostly, these intermediates can be accumulated at toxic levels. In the storage mechanism the accumulation of the substrate in toxic levels is prevented.

1.4. Storage Metabolism

1.4.1. PHB Metabolism

The fermentative bacteria metabolize the carbon sources that are available to produce end products, in particular short-chain fatty acids such as acetate. These acids are taken up by the biomass and converted into storage molecules (Seviour and Blackall, 1999). One group of these storage molecules is named as "polyhydroxyalkanoates" and is supposed to be the most important carbon storage materials. Polyhydroxyalkanoate (PHA) is a thermoplastic with complete biodegradability. The homopolymer of 3-hydroxybutyrate and 3-hydroxyvalerate are isolated as the major components, and 3-hydroxyhexanoate and possible 3-hydroxyheptanoate as the minor components (Satoh *et al.*, 1999).

Poly-β-hydroxybutyrate (PHB) is generally considered to be the more common prokaryotic storage polymer under conditions of carbon source excess (van den Eynde *et al.*, 1984; Smolders *et al.*, 1995). Fig. 1.3 demonstrates the chemical structure of PHB. It is widespread in bacilli, in chemolithotrophic and phototrophic bacteria, and in pseudomonades. PHB is a polymer of D(-)β-hydroxybutyrate and has a molecular weight between 60,000 and 250,000 (Gottschalk, 1985).

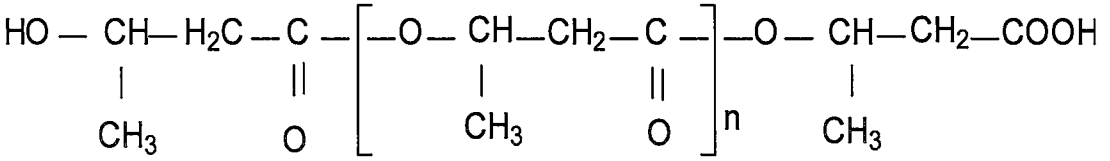


Figure 1.3 The chemical structure of poly-β-hydroxybutyric acid (Gottschalk, 1985).

It is accumulated in the cells as granules surrounded by membranes. Under appropriate conditions, bacterial cells may accumulate so much PHB that it accounts for approximately 60% of their dry weight (Gottschalk, 1985). In Fig. 1.4 the central carbon metabolism and the entry point for PHB synthesis in a microbial cell is demonstrated.

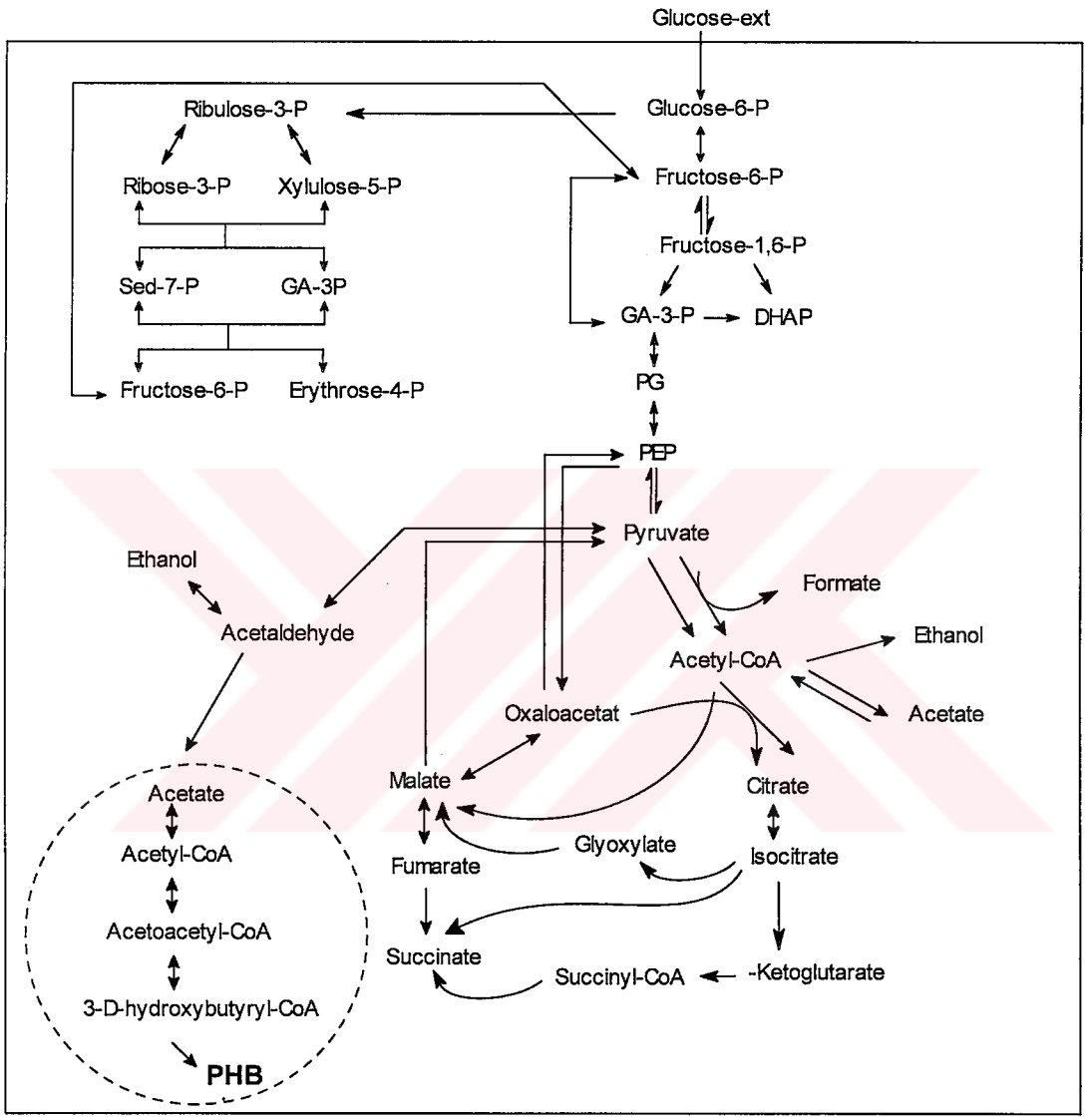


Figure 1.4 The central carbon metabolism and the entry point for PHB synthesis in a bacterium (Klamt, S. and Stelling, J, 2004).

Under feast/famine conditions, the substrate uptake rate will be higher than required for growth. The substrate uptake results in $NADH_2$ formation and it is later consumed by oxidative phosphorylation, which leads to ATP formation. If the ATP consumption for growth processes is limited, then ATP will accumulate and result in an accumulation of $NADH_2$. The production of a more reduced storage polymer like

PHB (which requires $NADH_2$) is then more likely than the production of glycogen (which leads to formation of $NADH_2$). In such a case PHB does act as a $NADH_2$ sink.

1.4.2. Biochemical Pathway and Regulation of PHB Production and Consumption under Aerobic Conditions

The biochemical pathway of PHB synthesis has been studied in mixed activated sludge culture and also in pure culture (Fig.1.5). Anderson *et al.* conducted a study on *A. eutrophus* and it was found that inside the cell physical restrictions exist and the cell is unable to accommodate more polymers within the fixed existing amount of cell wall material. It is found that the number of PHB granules per cell is fixed at the early stage of polymer accumulation (Anderson *et al.,* 1990). The diameter of these granules increases in order to accommodate the PHB produced.

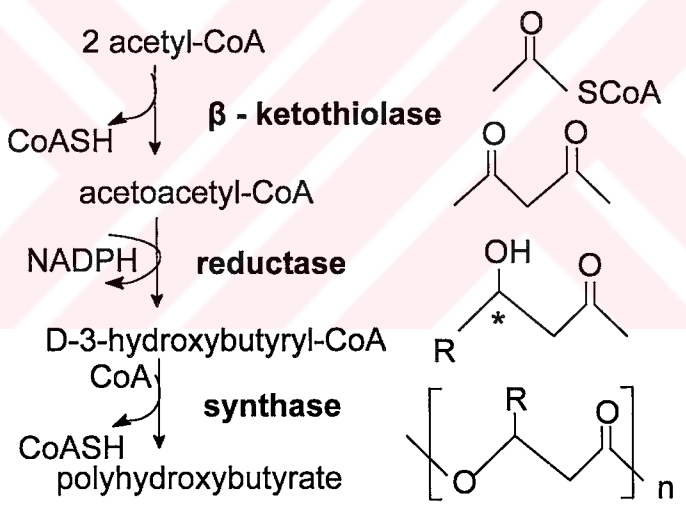


Figure 1.5 The pathway of PHB synthesis from acetyl-CoA found in numerous bacteria (Klamt, S. and Stelling, J, 2004).

Although a cyclic metabolic pathway has been presented in many organisms, a little is known about the location and regulation of the enzymes. The cyclic metabolic pathway of PHB is shown in Fig. 1.6. Acetyl-CoA is a key intermediate compound in the PHB synthesis and degradation pathway. It can be used for two purposes; it can be used in the TCA cycle to produce ATP and $NADH_2$, and to form biomass or can be used to produce PHB.

In the PHB synthesis two molecules of acetyl-CoA condense to form acetoacetyl-CoA and release a free coenzyme. The enzyme 3-ketothiolase catalyzes this condensation reaction. This reaction does not lead to PHB synthesis direction. The concentration of intermediate compounds in bacterial cells is maintained in micromolar range. If there is more external acetate available (in the range of mM) for the biomass than needed for growth process, the concentration of acetyl-CoA will increase. This will direct the reaction of 3-ketothiolase to PHB synthesis.

Subsequently, the accumulation of PHB occurs in order to keep the concentration of intermediates in the cells at a balanced level. In the case of the high ratio of acetyl-CoA/free coenzyme A, the cells will produce more anabolic enzymes. They may increase their growth rate. If this excess external substrate period is long enough, the specific growth rate of the biomass increases whereas the PHB synthesis rate decreases (VanAalst-vanLeeuwen *et al.*, 1997). The enzyme involved in the last step of PHB synthesis, P(3HB) synthase, is thought to be located in the membrane surrounding the granule.

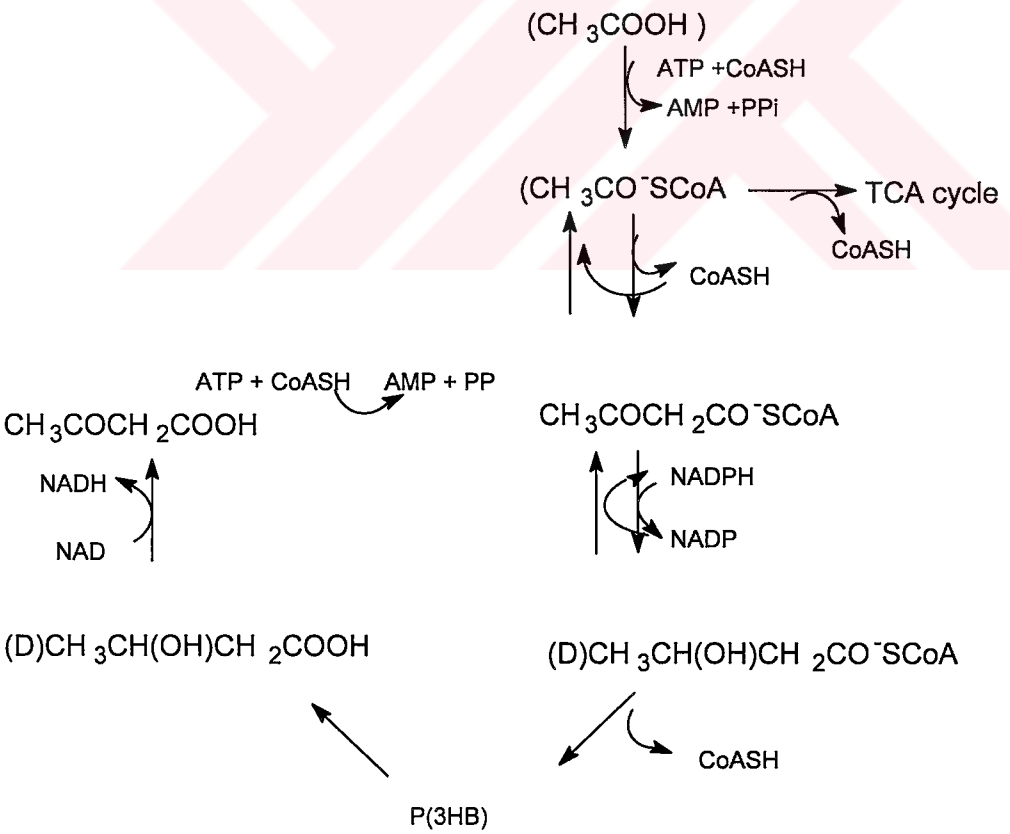


Figure 1.6 The cyclic metabolic pathway of PHB (Klamt, S. and Stelling, J, 2004).

It is also observed that in the famine period the growth rate changes depending on the PHB degradation rate. This shows that one of the steps in the biochemical pathway of PHB degradation is rate limiting. Although the knowledge of the PHB degradation pathway is very poor, the rate-limiting step is thought to be the conversion of acetate to acetyl-CoA step shown in Fig. 1.6.

van Aalst-van Leeuwen *et al.* (1997) studied the PHB production by a steady-state culture of *P. pantotrophus* after a pulse addition of substrate. They also investigated the growth on the internally accumulated storage polymer after depletion of the added substrate, and formed a metabolic model for PHB formation and consumption. They have described the aerobic metabolism of the bacterium capable of storing PHB growing on ammonia as the sole nitrogen source and either acetate or PHB as carbon and energy source. Basically seven internal reactions take place in this metabolic pathway (Fig.1.7).

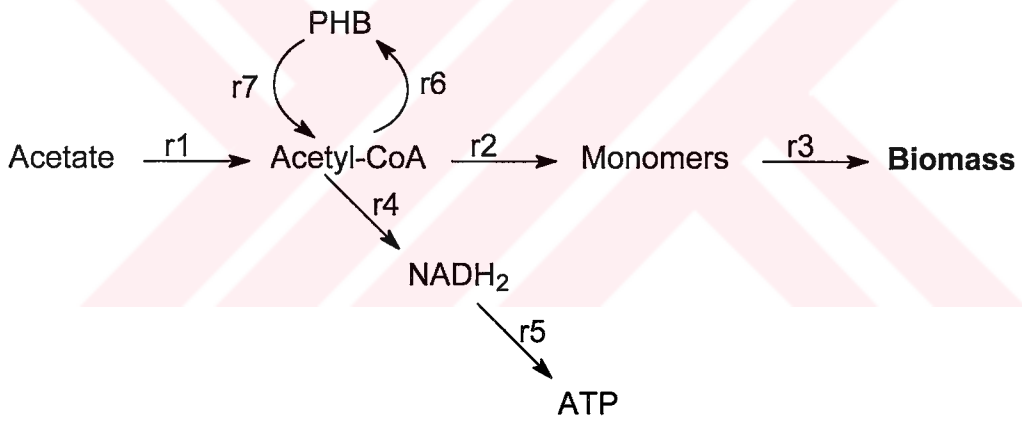


Figure 1.7 Schematic representations of PHB production and consumption introduced by van Aalst-van Leeuwen *et al.* (1997)

In the first reaction (r1) acetate is taken up by the cell by means of active transport and activated to form acetyl-P*i*. This is then converted into acetyl-CoA. This acetyl-CoA is used for anabolism, synthesis of active biomass. This reaction takes place in two steps. First the biomass monomers are synthesized (r2) and then the biomass precursors are polymerized into active biomass (r3). The $NADH_2$ formed by the carbon source catabolism (r4) is used in the oxidative phosphorylation to produce ATP (r5). The substrate is not only used for biomass synthesis and catabolism but

also used for PHB synthesis. No decarboxylation occurs during the production of PHB (Doi, 1990). From 2 moles of acetyl-CoA, 1 mole of β-hydroxybutyrate can be made, which is polymerized into PHB. No additional ATP is required in the formation of PHB from acetyl-CoA. (Doi, 1990) The accumulated PHB is utilized as the carbon and energy source in the absence of exogenous energy source (r7).

PHB is hydrolyzed and converted into acetyl-CoA. A depolymerase releases D(-)-β-hydroxybutyrate from the granules, which is subsequently oxidized to acetoacetate by a NAD^+-specific D(-)-β-hydroxybutyrate dehydrogenase. Acetoacetate is then channeled into the intermediary metabolism by a CoA transferase reaction. (Fig.1.8) Thus, CoA esters are the substrates for PHB synthesis and acids are the products of PHB degradation. This separation facilitates the regulation of both processes. One mole of ATP is needed for every mole of β-hydroxybutyric acid (HB) converted into acetyl-CoA (Dawes and Senior, 1973).

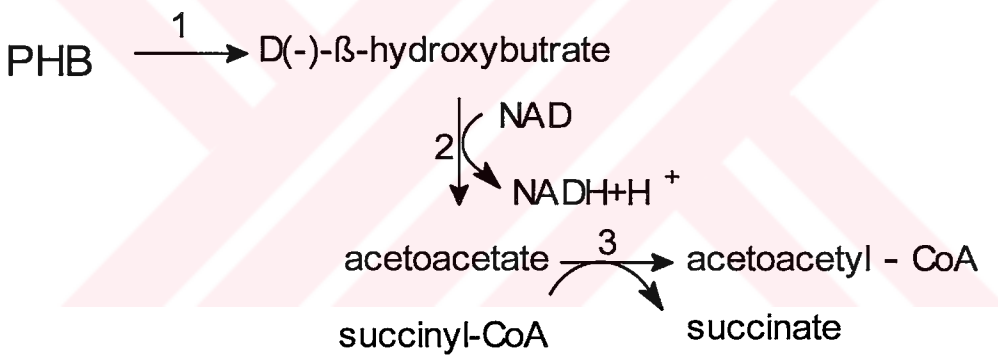


Figure 1.8 PHB utilization (Gottschalk, 1985).

1.4.3. Glycogen Metabolism

Unlike PHB, glycogen is a straight-chain polymer of linear α-1,4-linked D-glucose subunits with some α-1,6 branches (Fig. 1.9). Branching -because it increases the number of terminal points at which the degradation enzymes can attack- will enhance the rate of glycogen synthesis and degradation (Stryer, 1995). This is contradictory to experimental results obtained by Zevenhuizen and Ebbink, (1974), which show that faster glycogen consumption corresponds to a lower degree of branching. Dircks *et al.*, (2000) demonstrated a probable reason for this; the branching also increases the

density of glycosyl units in the tiers as the glycogen molecule grows in size. The free space available for the enzymes to attack the bonds is dramatically reduced after the tiers of the bonds increase.

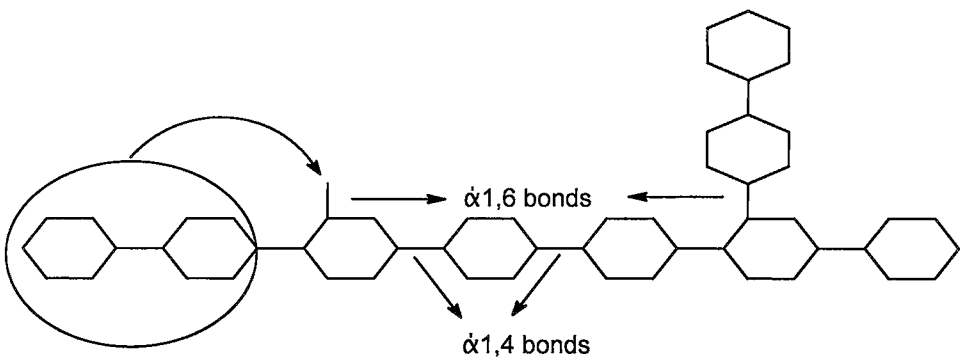


Figure 1.9 Schematic representation of glycogen branching (Gottschalk, 1985).

1.4.4. Biochemical Pathway and Regulation of Glycogen Production and Consumption under Aerobic Conditions

Organic storage polymers are, in general, divided into two major groups of compounds; polysaccharides and lipids (including polyhydroxyalkonates) (Dawes and Senior, 1973). The different storage polymers originate from different metabolites. When the primary substrate is glucose or other sugars or a compound that can be converted into pyruvate with an increase in the reducing power; for example, different carbohydrates, glycerol or proteins; glycogen is formed as the storage metabolite. This is because the rate of glycolysis will be reduced when the $NADH_2$ level inside the cell is increased.

Dircks *et al.* (2000) performed a study in order to describe the aerobic metabolism of glycogen in the activated sludge and they compared their data with the metabolism of PHA, which is identified by Beun *et al.*, (2000). Dircks *et al.,* (2000) concluded their data by a metabolic model (Fig. 1.10).

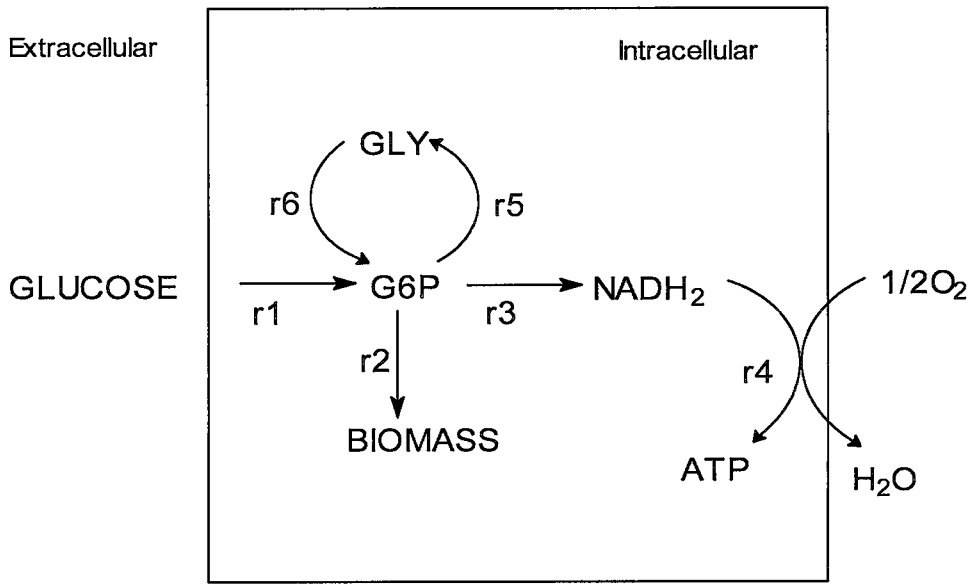


Figure 1.10 The metabolic model of glycogen metabolism with its six internal reactions (Dircks *et al.*, 2000).

Glucose metabolism (glycolysis) will be discussed in detail in the following sections. Briefly, the cell takes up the glucose and phosphorylates into glucose-6-phosphate (G6P) (r1) by using ATP. The required amount of this G6P is used for the synthesis of biomass (r2). $NADH_2$ produced in the primary catabolism of the carbon source (r3) is used in the electron transport phosphorylation (r4) in order to produce energy (ATP) for the cell. The synthesis of the storage polymer glycogen from G6P (r5) requires $^1/_6$ ATP per G6P (Stryer, 1995).

When the exogenous glucose source is depleted, the bacterium utilizes the stored glycogen as the energy and carbon source. The phosphorolysis of glycogen into G6P (r6) is considered to proceed without consumption of energy. Glycogen degradation is considered not to occur when the primary substrate glucose is present in the environment.

1.4.5. PHB and Glycogen Storage Under Anaerobic-Aerobic Conditions

PHA biosynthesis requires a source of reducing power without direct involvement of ATP, and occurs in bacteria during unbalanced aerobic growth. Among all the models suggested, a more likely source of reducing power is offered by Arun *et al.* (1988), who proposed that metabolism of carbohydrate storage reserves produces the NADH. In recent metabolic models a pathway for PHA synthesis from stored glycogen reserves has been proposed (Satoh *et al.*, 1992), where glycogen is

metabolized to pyruvate. The glycolysis of this glycogen results in the formation of varying ratio of PHV and PHB. This glycolysis may occur via Embden-Meyerhof-Parnas (EMP) pathway or via the Entner-Duodoroff (ED) pathway (Maurer *et al.*, 1997). Some of the pyruvate is converted to acetyl-CoA, which is reduced to PHA, while the rest is converted to oxaloacatete and then to propionyl-CoA through a propionate fermentation pathway. The propionyl-CoA is then incorporated into the PHA.

Hesselmann *et al.*, (1999) examined the stoichiometry and biochemical pathways of the anaerobic EBPR (Enhanced Biological Phosphorus Removal) metabolism in mixed cultures of activated sludge with acetate as the organic substrate. They have concluded that acetate is taken up by diffusion and activated by acetyl-CoA synthetase. Also they have demonstrated that glycogen is degraded via the Entner-Duodoroff (ED) pathway. Glucose was largely converted into gluconic acid and excreted into medium before being deposited in the form of PHB as the primary product of assimilation. Subsequently, PHB was metabolized being partly transformed into glycogen and ether-soluble lipid (Fig. 1.11).

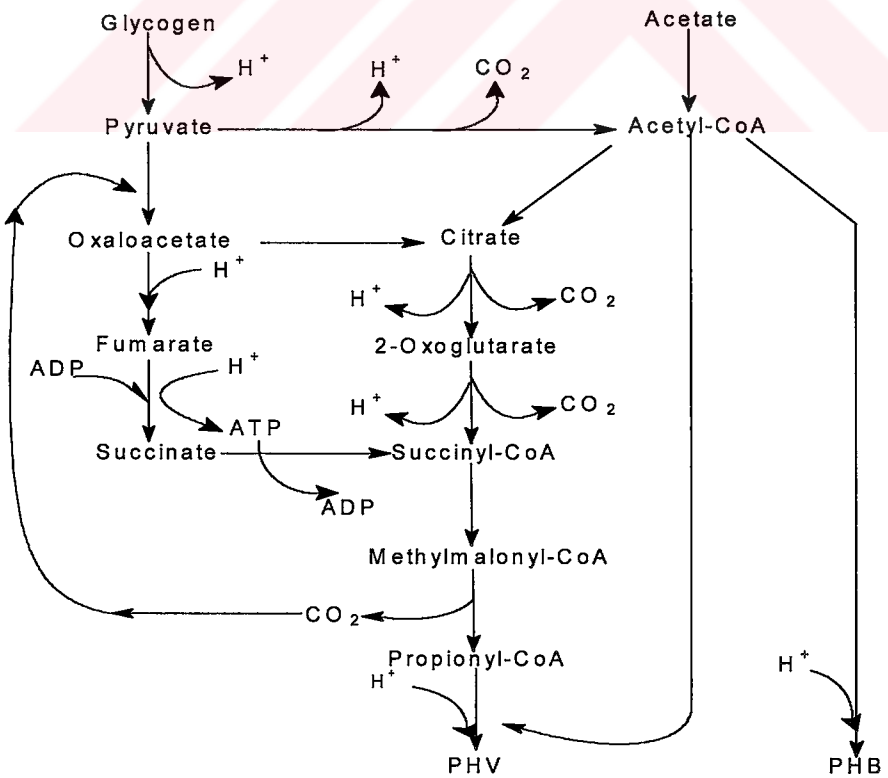


Figure 1.11 Behaviors of microorganisms under anaerobic-aerobic conditions in the model proposed by Hesselmann *et al.* (1999).

1.5. *Escherichia coli* (*E. coli*) as the Model Organism

Recently, it is accepted that the storage mechanisms of the microorganisms for various substrates have an important role on the treatment of the wastewaters. Moreover, studies performed on the mixed culture of the activated sludge microorganisms have also supported these findings. However, it is not yet identified which organisms are mostly responsible and which pathways are used for these storage mechanisms. From this point of view, pure culture studies with model organisms at various conditions are very important in order to elucidate the mechanisms. Therefore, in this present study the storage mechanisms of *E. coli*, as a model organism, were investigated under different cultivation conditions and on different substrates. Although *E. coli* has been known to accumulate no storage metabolites, recent debates on the PHA and glycogen accumulation at suitable conditions has increased our interest in this microorganism.

Moreover, *E. coli* is one member of a group of bacteria that comprise the fecal coliform bacteria group and is used as an indicator organism to identify the potential for the presence of pathogenic organisms in a water sample (Hayes, J., 2004). *E. coli* and other associated bacteria come from the feces of any warm-blooded animal, and their presence within the digestive tract is normal. *E. coli* and other intestinal pathogens are nearly or completely absent from unpolluted waterbodies.

The presence of specific indicator organisms signals that a given water source is contaminated with pathogens. The most widely used indicator is the coliform group of organisms. Coliforms are defined in water bacteriology as aerobic and facultative anaerobic, gram-negative, nonspore-forming, rod-shaped bacteria that ferment lactose with gas formation within 48 h at 35°C (Madigan *et al.*, 2003). This is an operational definition rather than a taxonomic definition, and the coliform group includes a variety of organisms, but most are members of the enteric bacterial group (Madigan *et al.*, 2003).

E. coli -generally named as the lab rat of the bacterial world- is gram-negative, rod-shaped, mobile bacterium that belongs to the group of facultative anaerobes. It prefers to grow in the presence of oxygen when it uses respiratory energy metabolism, but also grows in the absence of oxygen while using fermentative

energy metabolism. The optimum living temperature and the pH are 30-40°C and 6.5-7.5, respectively.

1.6. The Faith of Glucose in *E. coli* during Aerobic Growth

E. coli is able to grow with a number of substrates in both the presence and the absence of oxygen. Under aerobic conditions, part of the substrate is oxidized to CO_2 with oxygen as the terminal electron acceptor. The cytoplasmic membrane of *E. coli* cells is not simply permeable to glucose; there is no free diffusion of glucose in and out of bacterial cells, which would allow the concentration of the sugar inside and outside become equal. Instead *E. coli* possesses a transport system that recognizes glucose specifically. It picks up glucose at the medium side of the membrane and releases it on the cytoplasmic side. This transport process is coupled to a chemical conversion of the substrate, i.e., the phosphorylation of glucose to glucose-6-phosphate (Glucose-6-P).

The basic reactions that take place after the transport of glucose into *E. coli* cells are shown in Fig. 1.12. Embden-Meyerhof-Parnas (EMP) is the most commonly used sequence of reactions for the conversion of glucose into pyruvate (Equation 1.6.1) in animals, plants, and many bacteria. *E. coli* contains high activities of the necessary enzymes. This pathway can be thought of as comprising three stages. Stage 1, which is the conversion of glucose into fructose 1,6-bisphosphate, consists of three steps: a phosphorylation, an isomerization, and a second phosphorylation reaction. The strategy of these initial steps in glycolysis is to trap the glucose in the cell and form a compound that can be readily cleaved into phosphorylated three-carbon units. Stage 2 is the cleavage of the fructose 1,6-bisphosphate into two three-carbon fragments. These resulting three-carbon units are readily interconvertible. In stage 3, ATP is harvested when the three-carbon fragments are oxidized to pyruvate (Berg *et al.*, 2002).

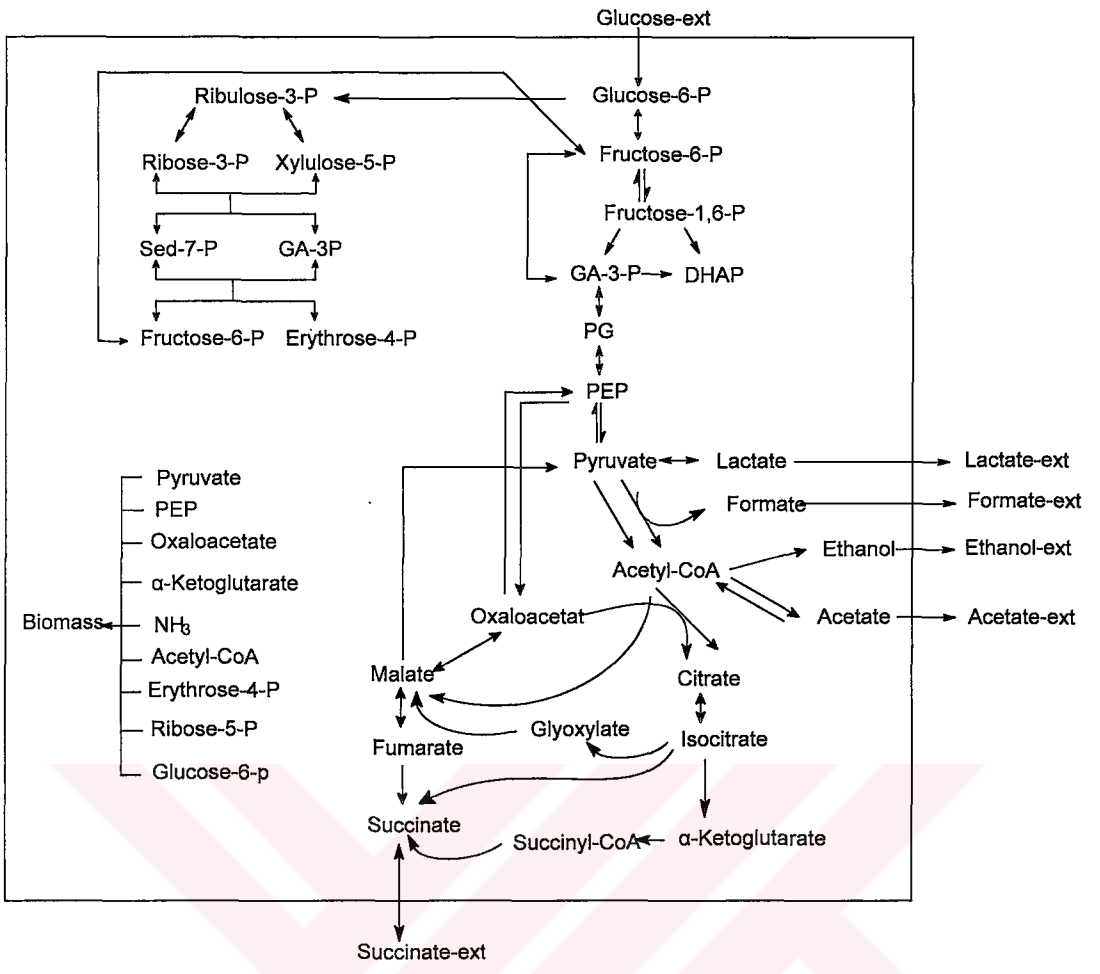


Figure 1.12 Central carbon metabolism of *E. coli* (Klamt, S. and Stelling, J, 2004).

After the phosphorylation of glucose to Glucose-6-P, it is converted into D-fructose-1,6-bisphosphate (Fructose-1,6-P), which is the characteristic intermediate of this pathway. It is split into two C_3 fragments -dihydroxyacetonephosphate (DHAP) and D-glyceraldehyde-3-phosphate (GAP)- by the interested enzyme. Both compounds are in equilibrium with each other due to the presence of the enzyme that converts them to each other. Consequently, if only the cells need little DHAP, most of the C_3 fragments originating from Fructose-1,6-P can be further metabolized via GAP. This is what normally happens; one molecule of glucose is transformed into two molecules of GAP (Gottschalk, 1985).

In the following reactions in this sequence the GAP is converted into 1,3-bisphosphoglycerate (1,3-BPG), which is an acyl phosphate. Such compounds have a high phosphoryl-transfer potential; one of its phosphoryl group is transferred to ADP in the next step in glycolysis. This reaction is really the sum of two

processes; the oxidation of the aldehyde to a carboxylic acid by NAD^+ and the joining of the carboxylic acid and the orthophosphate to form the acyl-phosphate product (Berg *et al.,* 2002).

The final stage in the glycolysis is the generation of ATP from the phosphorylated three-carbon metabolites of glucose. When the phosphoryl group of the acyl phosphate of 1,3-BPG is transferred to ADP, the products are ATP and 3-phosphoglycerate (3-PG) (Berg *et al.,* 2002).

In the remaining steps of glycolysis, 3-PG is converted into pyruvate with the concomitant conversion of ADP into ATP. The first reaction is a rearrangement the position of the phosphoryl group shifts in the conversion of 3-phopsphoglycerate into 2-phosphoglycerate. In the next reaction, an enol is formed by the dehydration of 2-phosphoglycerate. Thus, phosphoenolpyruvate (PEP) is formed. When the phosphoryl group of PEP has been donated to ATP, the enol undergoes a conversion into the more stable ketone- namely, pyruvate (Berg *et al.,* 2002).

The net reaction in the transformation of glucose into pyruvate is (Gottschalk, 1985):

$$\text{Glucose} + 2P_i + 2\ \text{ADP} + 2\ NAD^+ \rightarrow 2\ \text{pyruvate} + 2\ \text{ATP} + 2\ \text{NADH} + 2\ H^+ + 2\ H_2O \quad (1.6.1)$$

Thus, two molecules of ATP are generated in the conversion of glucose into two molecules of pyruvate (Berg *et al.,* 2002).

As in most aerobic growth pyruvate is converted into acetyl-coenzyme A (acetyl-CoA) (Equation 1.6.2). Acetyl-CoA is then oxidized in the tricarboxylic acid cycle (TCA) (Fig.1.12). The combined action of the enzymes of the EMP pathway and the pyruvate conversion to acetyl-CoA leads to the degradation of glucose to CO_2 and acetyl-CoA according to the equation (Gottschalk, 1985):

$$\text{Glucose} + 2\ \text{ADP} + 4\ NAD^+\ 2\ P_i + 2\ \text{CoA} \rightarrow 2\ \text{acetyl CoA} + 2\ CO_2 + 2\ \text{ATP} + 4\ \text{NADH} + 4\ H^+ \quad (1.6.2)$$

When growing aerobically on glucose, *E. coli* oxidizes about 50% of the glucose to CO_2 for the production of ATP. The remaining 50% is converted into cellular material (Gottschalk, 1985). The synthesis of these cellular materials requires most of the energy produced inside the cell. The faith of the extracellular glucose transported into the cell depends on the oxygen concentration of the environment. Fig. 1.13 represents a scheme for the formation of cellular constituents from glucose.

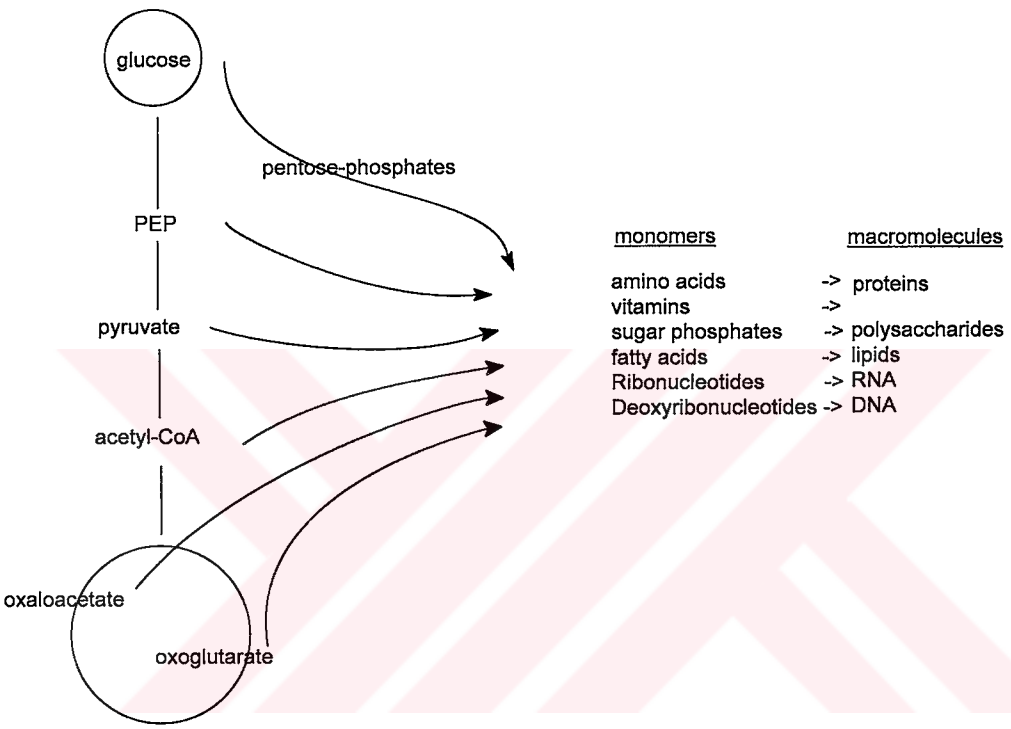


Figure 1.13 Cellular components formation from glucose (Gottschalk, 1985)

More than 95% of the cellular material of *E. coli* consists of macromolecules. Proteins account for approximately 52% and nucleic acids for 19% of the dry weight. About 3% of the dry weight of the cells includes low-molecular-weight organic compounds and salts (Table 1.1).

Table 1.1 General content of macromolecules of microbial cells (Stouthamer, 1973).

Macromolecule	Amount (g/100g dried cells)
Protein	52.4
Polysaccharide	16.6
Lipid	9.4
RNA	15.7
DNA	3.2
TOTAL	**97.3**

1.7. *E. coli* Cells Growing on Acetate, a by-product of Glucose Catabolism

Diauxic growth was first discovered by Monod in 1942 in a culture of *Bacillus subtilis* supplemented with glucose and arabinose as the carbon and energy source. The bacteria first utilized glucose and then arabinose (Gottschalk, 1985). This resulted in a biphasic growth curve. The same growth behavior is observed for a great variety of substrate combinations utilized by *B. subtilis, E. coli* and many other organisms. In all cases, one readily utilizable substrate represses the utilization of other substrates.

It has been observed in the budding yeast, *Saccharomyces cerevisiae*, that when plenty of sugar is available, fermentation is the preferred pathway, even in the presence of oxygen (Gonçalves et al., 1998). This is called the Crabtree effect, and this behavior is observed for some bacteria. In mixed-acid fermentation, three acids are formed in significant amounts- acetic, lactic, and succinic; whereas ethanol, CO_2, and H_2O are also formed (Madigan *et al.*, 2003). Thus, even in the presence of oxygen, *E. coli* can produce a significant amount of acetate and can also utilize it with a diauxic growth, after it has consumed the readily biodegradable substrate, which is glucose (Fig. 1.14).

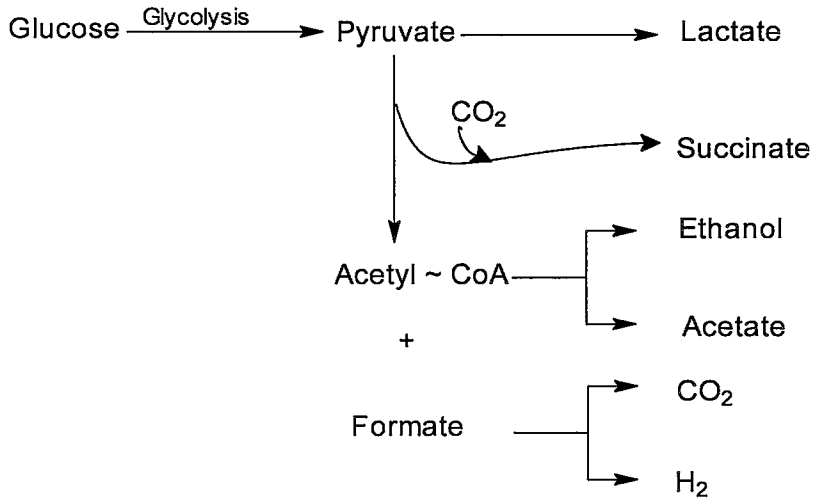


Figure 1.14 Mixed acid fermentation in *E. coli* (Madigan *et al.*, 2003)

Acetate, which is previously produced and excreted from the cell is actively transported into the cell and subsequently converted into acetyl-CoA. Acetyl-CoA then is fed into the TCA. The problem during growth on acetate, however, is how intermediates of the cycle that serve as starting materials biosynthesis are regenerated. Clearly, there is no direct way to synthesize PEP from acetate. Two other enzymes are involved, where the first one catalyzes the cleavage of isocitrate to succinate and glyoxylate and the second one catalyzes the condensation of acetyl-CoA with glyoxylate to yield L-malate. Together with enzymes of TCA these reactions form the so-called Glyoxylate Cycle (Fig. 1.15).

Thus, extra oxaloacetate becomes available (actually formed from two acetyl-CoA), which can be used to form L-glutamate (via citrate and α-oxoglutarate) and other compounds derived from cycle intermediates. Moreover, oxaloacetate serves as starting material for all the carbohydrates required for polymer synthesis. The ultimate precursor of gluconeogenesis is PEP, and it is formed from oxaloacetate and ATP.

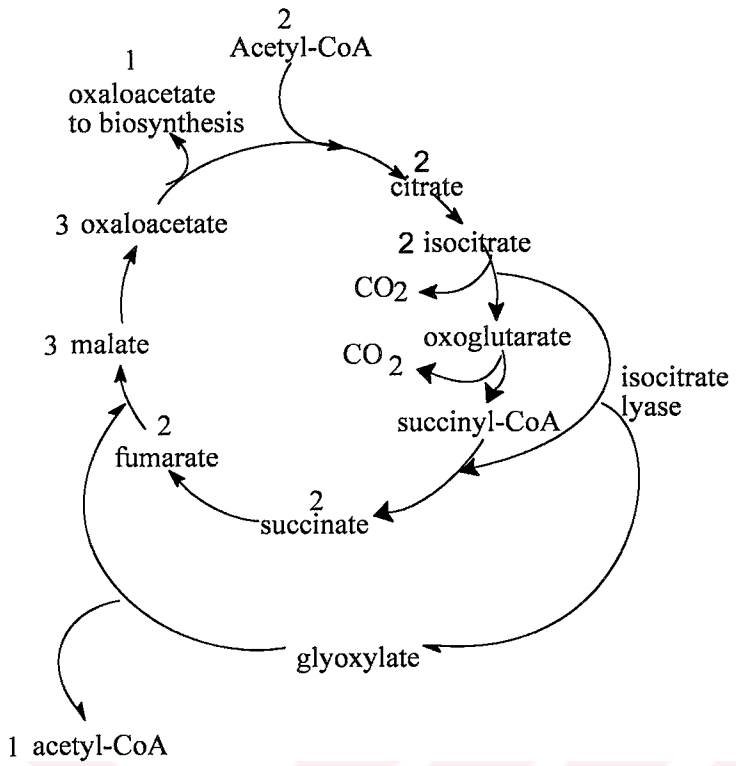


Figure 1.15 The function of the TCA and the Glyoxylate cycle reactions during growth of *E. coli* on acetate (Gottschalk, 1985)

1.8. Types of Cultivation

Growth is a result of both replication and changes in cell size. Microorganisms can grow under a variety of physical, chemical, and nutritional conditions. In a suitable nutrient medium, organisms extract nutrients from the medium and convert them into biological compounds. Part of these nutrients is used for energy production and part is used for biosynthesis and product formation. The rate of growth is directly related to cell concentration and is characterized by the specific growth rate (μ).

Growth operations may be carried out as batch, fed-batch and sequencing batch processes. The type of product being produced, the conditions being investigated, etc dictates the mode of operation.

1.8.1. Batch Growth

Batch growth refers to culturing cells in a vessel with an initial charge of medium that is not altered by further nutrient addition or removal (Gottschalk, 1985). It is a closed culture system, which contains an initial, limited amount of nutrient. The inoculated culture will pass through a number of phases, as illustrated in the previous sections in Fig. 1.2. After inoculation there is a period during which it appears that no growth takes place; this period is referred to as the lag phase and may be considered as a time of adaptation. Following a period during which the growth rate of the cells gradually increases, the cells grow at a constant, maximum, rate and this period is known as the log, or exponential phase. The stationary phase in batch culture is that point where the growth rate has declined to zero. During this phase, the population is still metabolically active (Stanbury *et al.*, 2000).

1.8.2. Fed-batch Growth

Yoshida *et al.* (1973) introduced the term fed-batch culture to describe batch cultures, which are fed continuously, or sequentially, with medium, without the removal of culture fluid. A fed-batch culture is established initially in batch mode and is then fed with a very concentrated solution of the media containing the substrate at a rate and resulting in an insignificant increase in volume. A system employing this strategy is described as fixed volume. This operation system is considered as a batch culture in which the growth of the process organism has depleted the substrate to a limiting level. If the substrate is then added in a concentrated feed such that the broth volume remains almost constant then the substrate will be consumed as soon as it enters the fermentor.

1.8.3. Sequencing Batch Growth

Sequencing batch reactors (SBR) have been identified as time-oriented periodic unsteady state cyclical processes (Irvine and Ketchum, 1989; Irvine et al., 1997; Ketchum, 1997). SBR is an activated sludge system operating on a fill-and-draw (batch) basis (Orhon and Artan, 1994). SBR systems consist of a series of one, or sometimes more than one, reactors, each of which operates under batch mode. Each cycle consists of a number of separate operational phases of fill, react, settle, draw and idle (Fig. 1.16), so that after the react stage, the biomass is allowed to settle and

the clear treated supernatant can be removed. The length and imposed operational parameters for these phases can be manipulated.

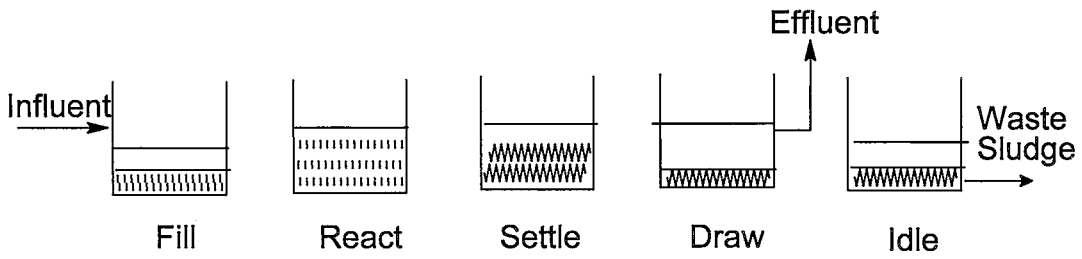


Figure 1.16 The cyclic operation of the SBR (Orhon and Artan, 1994).

In activated sludge reactors, the tank is filled with waste and mixed with the biomass (fill). Waste is consumed by the biomass and biological conversions took place (react), then microorganisms are separated from the treated waste (settle) and discharged from the tank. The reactor is left idle before refilling it with waste (idle) (Orhon and Artan, 1994). In pure culture studies, microorganism cannot settle down in a short period of time. Therefore, this cyclic operation is modified and the settling time is passed. Drawing is performed from the mixed liquor.

1.9. Respirometric Analysis

Directly measurement of the oxygen consumption rate in biological systems provides information about the metabolism of microorganisms. The relation between the oxygen and substrate consumption can be used to calculate the consumed substrate amount. The first important step of the oxygen uptake rate (OUR) measurements for a batch culture, to which an amount of substrate was added, is to know the respiration due to biomass itself, called the endogenous respiration. This value is assumed to be caused by maintenance of the biomass. The general basis is then to subtract this respiration from the measured respiration in order to determine the true yield coefficient (Dircks *et al.,* 1999). If the endogenous respiration is known, then the exogenous respiration, which is due to added substrate, can be calculated. If a storage polymer like PHA and/or glycogen was produced from the added substrate, then it is assumed that a metabolic shift was occurred to that more "slowly" readily biodegradable substrate –the storage polymer. This shift can also be observed in a typical respirogram.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Bacterial strain

Escherichia coli K12 wild type strain was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany. *E. coli* K12 pure culture was used as the model organism of activated sludge cultures.

2.1.2. Medium

For all cultivations a chemically defined medium (M9 minimal medium) was prepared as follows: 8.421 g $Na_2HPO_4.2H_2O$; 3 g KH_2PO_4; 0.5 g NaCl; 1g NH_4Cl; 1 ml of 1M $MgSO_4$ stock solution and 1 ml of 0.1M $CaCl_2$ stock solution per 1 liter of distilled water was separately autoclaved at 121°C, 1.1 bar for 15 min. The pH of the medium was adjusted to 7.4 using 3 M KOH. Glucose (D(+)-Glucose, anhydrous, puriss, Riedel-de Haën, Sigma-Aldrich Laborchemikalien GmbH) and glycerol (30 Bé, Carlo Erba Reagenti, France) were separately autoclaved at 121°C, 1.1 bar for 10 min. and added to the medium.

2.1.3. Equipment

Studies were performed with Biostat® B bioreactor systems equipped with pH (Mettler Toledo, Greifensee, Switzerland), dissolved oxygen (DO) (Mettler Toledo Greifensee, Switzerland), temperature and level control (B. Braun Biotech International GmbH). Cultivation data were collected by using the MFCS/win Software (B. Braun Biotech International GmbH). The aeration of the bioreactor system was maintained by an air compressor (Airbag-Fiac, Italy).

UV-Visible Spectrophotometer Shimadzu UV-1601 (Japan) was used for the spectrophotometric measurements of growth (OD_{600} values) and also for protein

analysis (Abs. 570 nm). Chromatographic analysis was performed using Shimadzu HPLC Systems (Japan) and HP- Agilent Technologies 6890N GC Systems (USA).

2.2. Methods

2.2.1. Shake flask experiments

Three parallel shake flask sets were constructed by using 1-liter baffled Erlenmeyer flasks (Sigma) with 250 ml working volume where glycerol and glucose were used separately as the substrates. The inoculum size of the batch cultivations was 6%. The cultivations were performed at 37°C and 250 rpm in an orbital shaker (Certomat S-2, Germany).

For experiments where glycerol was used as the sole carbon source, six parallel shake flask sets were constructed by using 250 ml Erlenmeyer flasks with 50 ml working volume at 0/30/50/200/500/1000 mg/L glycerol concentrations. The inoculum size of the batch cultivations was 6%. The cultivations were performed at 37°C and 250 rpm in an orbital shaker (Certomat S-2, Germany). Final OD_{600} measurements, glycogen and PHB staining were performed.

Limitation of nitrogen as a key nutrient was observed by preparing varying concentrations of NH_4Cl -the only N source- in the standard minimal media. The concentrations were as follows: 25/50/100/500/1000 mg/L. The inoculum size of the batch cultivations was 6%. The cultivations were performed at 37°C and 250 rpm in an orbital shaker (Certomat S-2, Germany). OD_{600}, glycogen and PHB samples for microscopic staining were withdrawn at the 8^{th}, 24^{th}, 48^{th} hours of the cultivation. After 48 hours samples were drawn for residual glucose, glycogen and PHB analysis at HPLC and GC, respectively. Microorganisms cultivated at 1000 mg/L NH_4Cl were centrifuged and washed 2 times with medium that did not contain any NH_4Cl. Resuspended pellets were inoculated into medium that did not contain NH_4Cl. OD_{600}, glycogen and PHB samples for microscopic staining were withdrawn at the 8^{th}, 24^{th}, 48^{th} hours of the cultivation. After 48 hours samples were drawn for residual glucose, glycogen and PHB analysis at HPLC and GC, respectively.

2.2.2. Bioreactor experiments

Aerobic batch cultivations in the bioreactor were performed in a 2 liter-bioreactor with 1.5 liters working volume. The cultivations were performed at 37°C and 250 rpm. Dissolved oxygen was measured by using an oxygen probe. Airflow was set to 3 l/min. The preculture cultivations were performed using two parallel 500 ml shaken flask sets with 150 ml of culture volume. They were incubated overnight at 37 °C 250 rpm in an orbital shaker (Certomat S-2, Germany) and centrifuged at 5000 rpm for 10 min. (Allegra 25R™ Centrifuge, Beckman Coulter, U.S.A). The resuspended cell pellet was then transferred into the bioreactor for inoculating 1500 ml of chemically defined medium (M9) including either glucose or glycerol as the carbon source. Different cultivation types in the bioreactor are described in detail in sections 2.2.2.1 - 2.2.2.3.

2.2.2.1. Batch cultivations

Zero hour (0 h) was defined to as time when the inoculation was applied, at which the first OD_{600} measurement and the first sampling were done. Batch cultivation continued until the $LnOD_{600}$ measurements displayed the late stationary phase of the growth profile. The glycogen, residual glucose, total protein, PHB and VFA measurements were done at 30 and/or 60 min. intervals.

2.2.2.2. Fed-batch cultivations

The Biostat® B bioreactor systems were operated as a fed-batch reactor with 1500 ml working volume. Previously described batch operation conditions were applied in the start-up of the fed-batch cultivation. The microorganisms were firstly cultured overnight with 4000 mg/L glucose as the sole carbon source until they consumed all the substrate in the medium, and afterwards a starvation stress of 11 h was applied. After this stress period, the 10x fold concentrated fresh medium (150 ml) containing the same concentration of the carbon source was added to the system. Samples were drawn from the reactor in short intervals in order to represent the profile of the system. Feeding was continued with 4000 mg/L glucose and the starvation period (11 h) was applied once more. After this stress period, 10-fold concentrated fresh medium (150) containing 400 mg/L glucose as the sole carbon source was added. Samples were drawn from the reactor in short intervals.

2.2.2.3. Sequencing-batch cultivations

The Biostat® B bioreactor systems was operated as a sequencing batch reactor (SBR) with a cycle length of 6 h and each cycle consisting of five process operation phases (Table 2.1).

Table 2.1. Different phases in the reactor cycle during the SBR run.

Description	Duration (min)	Time (min)	Aeration	Stirrer
Effluent discharge, V=75 mL	3	0-3	on	on
Culture is stirred and aerated, V=1425 mL	2	2-4	on	on
Addition of influent, V=75 mL	1	4-5	on	on
Culture is stirred and aerated, V=1500 mL	235	5-240	on	on

The stirrer speed was 250 rpm. Airflow of 3 l/min was maintained. The temperature was kept at 37°C by circulating water from a thermostat water bath around the reactor. The performance of the reactor was monitored on-line by MFCS/win Software (B. Braun Biotech International GmbH) connected to the bioreactor. Feeding and drawing were performed with two different parallel pumps connected to timers. The steady state was determined by on-line dissolved oxygen (DO) with the O_2 probe connected to the bioreactor and optical density (OD_{600}) measurements at regular intervals. Prior to steady-state microorganisms were fed with 300 ml per day of M9 containing 4000 mg/L glucose. When the microorganisms reached to the steady-state sampling was done in very short intervals for the analysis of intracellular and extracellular growth metabolites and the intracellular storage polymers in order to obtain the previous history of the cellular metabolites and the storage polymers of

E. coli under these conditions. Following these sampling, respiration analysis was performed for this steady-stated pure culture.

2.2.3. Determination of Cell Growth

Cell growth was monitored spectrophotometrically by optical density measurements at 600 nm (OD_{600}). Samples taken from the bioreactor were immediately treated according to the requirements of analytical protocols and frozen at –20°C. When ready for use, samples were thawn and used for the analysis of the metabolites.

2.2.4. Cellular Dry Weight (CDW) Determination

Cellular dry weights (CDW) were determined by filtration. Membrane filters (0.2μm) (Schleicher & Schuell, Germany) were dried in an oven at 105°C for 1 h, cooled in a dessicator for 30 min. and weighed. A volume of 10 ml culture sample was filtered through the membrane filters by using a vacuum pump. The filter was then heated in an oven at 105 °C for 1 h, and then cooled down in a dessicator for 30 min. prior to weighing. The net weight of the cells was determined by taking the difference between the two measurements. Dry weight versus optical density calibration line was constructed according to the data obtained. Biomass concentrations were calculated using OD_{600} values and the calibration equation.

2.2.5. Protein Determination From Whole Cells

The mostly used methods to quantify total protein are the colorimetric methods, where a portion of the protein solution is reacted with a reagent that produces a colored product. The amount of this colored product is then measured spectrophotometrically and a calibration line is made to determine the protein amounts. A standard calibration line is prepared by using known concentrations of bovine serum albumin (BSA), a low-cost, highly pure and readily available protein.

In this study, BCA Assay (Stoscheck, 1990) was performed with 1 ml of sample for determination of protein concentration. A working reagent was prepared by mixing 50 parts of Reagent A (1.0 % BCA-Na_2 (Bicinchoninic Acid), 2.0 % $Na_2CO_3.H_2O$, 0.16 % NaK tartrate, 0.4 % NaOH, 0.95 % $NaHCO_3$, pH to 11.25 with 50 % NaOH or $NaHCO_3$) with 1 part Reagent B (4 % $CuSO_4.5H_2O$). Ten μl of each standard, blank or unknown sample was put into the appropriate microtitre plate wells. 200 μl of working reagent was added to each well. Samples were mixed for 30 seconds on a

plate shaker. Microtitre plate was covered and incubated at 37°C for 30 minutes. With the microtitre plate reader (Biorad Model 3550 UV, USA) the absorbance was read at 570 nm. Using the calibration line, the protein concentration for each known sample was determined. All readings were carried out in duplicate.

2.2.6. Chromatography Techniques

Basically, all chromatography systems consist of the *stationary phase*, which may be a solid, gel, liquid, or a solid/liquid mixture that is immobilized, and the *mobile phase*, which may be liquid or gaseous and which flows over or through the stationary phase.

In the *elution development* mode, the sample is dissolved in a suitable solvent and applied to the stationary phase as a narrow, discrete band. Mobile phase, commonly referred to as the *eluent* and normally consisting of an organic solvent or a mixture of solvents is then allowed to flow continuously over the stationary phase, resulting in the progressive separation of the sample components (*analytes*). The analytes with the highest solubility in the mobile phase will move along the stationary phase fastest. When a particular analyte has been removed from the column by the mobile phase, it is said to have been *eluted.*

A chromatograph is the pictorial record of the detector response as a function of elution time or volume. It consists of a series of peaks, representing the elution of individual analytes. The *retention time* (t_R) for each analyte has two components. The first is the time it takes the analyte molecule to pass through the free spaces between the stationary phase matrixes. The second component is the time the analyte is retained by the stationary phase. This time is characteristic of the analyte.

The pure reference compounds (*standards)* are used to identify a particular analyte and to quantify it using previously obtained and stored data from standards. The new peak area calculation will give the amount of the analyte present by use of a calibration curve obtained by chromatographing, under identical conditions with the known amounts of the pure form of the analyte. Two types of standards are used; *internal* and *external standards*. The internal standard is a compound that has physical properties as similar as possible to the test analytes and which chromatographs near to, but distinct from them. It should have the same detector response as those of the analytes. A known amount of the standard is introduced into

the test sample as early as possible in the extraction, and is therefore taken through any preliminary procedures with it. Any loss of standard during the analysis will be identical with the loss of the test analytes. The external standard is the pure form of the analyte. It is independently chromatographed at different concentrations and a calibration curve is obtained. The results of the analytes are determined by using this calibration curve.

In this present research, basically two types of chromatography techniques are performed. Gas Chromatography for the investigation of volatile fatty acids (VFA) and polyhydroxyalkanoates (PHA) concentrations and High Performance Liquid Chromatography for glycogen and residual glucose analysis were used. The methods and the experimental protocols are explained in detail in the next section.

2.2.6.1. Gas Chromatography (GC)

Like all other chromatography techniques, gas chromatography separates mixtures by using the components' differential distribution between two phases -one stationary and the other moving across it. The most important feature of gas chromatography is the use of a gas as the mobile phase. The sample that will be separated is introduced into gas stream just before it encounters the stationary phase. The components are separated by elution and detected as they emerge in the gas at the other end of the column. They are distinguished by the different times, which they take to pass through the column, which is named as the retention time.

The most frequently used carrier gas is nitrogen. The second most popular is helium, which is more expensive, but essential for special applications. In our experiments, helium was used as the carrier gas for both PHA and VFA analysis.

In chromatography techniques, usually very small amounts of samples are used. Such small samples require special handling techniques, both for injecting them onto the column and monitoring them as they emerge from the column. Injection is usually achieved by means of a syringe inserted through a self-sealing silicone-rubber septum. In order to prevent the occurrence of ghost peaks we used an auto-injection system was used in this study.

Samples were analyzed with flame ionization detector (FID). It passes sample and carrier gas from the column through a hydrogen air flame. The hydrogen air flame creates few ions, but when an organic compound is burned there is an increase in the

ions produced. A polarizing voltage attracts these ions to a collector located near the flame. The current produced is proportional to the amount of sample being burned. This current is sensed by an electrometer, converted into digital form, and sent to an output device.

2.2.6.2. Volatile Fatty Acid (VFA) Analysis by Gas Chromatography (GC)

VFA concentration was determined using a simple method in GC system. A standard calibration line is prepared by using known concentrations (5/50/100/150/300/500/750/900 mg/L) of VFA Standard (Fatty Acid Solution 0.1 % in Water, FA-MIX-02, Alltech Associates Inc. U.S.A.).

One ml sample was derived from the bioreactor and centrifuged rapidly at 14000g in a bench top centrifuge (Microfuge® 18 Centrifuge, Beckman Coulter, U.S.A.) at room temperature. The supernatant was quickly filtered through 0,22μ filters (Millex®-GV, Millipore Corp. U.S.A.) and 0.30μl 3M H_3PO_4 (phosphoric acid) was added. Run was performed in GC system with the method described in the Table 2.2. Using the calibration line, the protein concentration for each known sample was determined. All readings were carried out in duplicate.

Agilent Technologies 125-3232 DB-FFAP (J&W Scientific, HP, USA) capillary 30.0m x 0.53mm x 1μm nominal column was used for the measurements. Data were analyzed by ChemStation Software, Agilent Technologies, HP. The injector (split-splitless) and flame ionization detector (FID) temperatures were set at 260°C and 300°C, respectively. The carrier gas was helium and the injection was performed in splitless mode. The column pressure was set to 21.07 psi and the flow rate in the column was set to 4.5 ml/min. In the detector; H_2 flow was set to 40 ml/min, airflow was set to 400 ml/min and the make up flow (He) was set to 30 ml/min.

Table 2.2. VFA analysis procedure in GC

Oven Ramp	°C/min	Next °C	Hold min.	Run Time
Initial		100	5.00	5.00
Ramp1	10.00	160	5.00	16.00
Ramp2	80.00	230	3.00	19.88
Ramp3	0.00			
Post Run		50	0.00	**19.88**

2.2.6.3. Polyhydroxybutyrate (PHB) Analysis by GC

PHB concentration was determined according to van Loosdrecht (Delft University of Technology, Netherlands) with slight modifications. Briefly, 10 ml sample was taken into glass tubes, each having a few drops of formaldehyde. The tubes were centrifuged at 5000 rpm for 7 min. (Allegra 25R™ Centrifuge, Beckman Coulter, U.S.A). After discharging the supernatant 4.5 ml phosphate buffer (0.58 g K_2HPO_4 and 0.23 g KH_2PO_4 per liter) was added to the pellet and shaken vigorously. After centrifugation at 5000 rpm for 7 min. (Allegra 25R™ Centrifuge, Beckman Coulter, U.S.A.) pellets were collected in a tube and freeze-dried at –20°C. Prior to measurements, the tubes were freeze dried at –50°C for 48 h. The freeze-dried pellets were crushed, weighed and the exact weights were recorded. The weighed samples were put in glass tubes with PTFE lined screw caps (Schott GL18 Max 200 °C). Benzoic acid (Riedel-de Haën, Sigma-Aldrich Laborchemikalien GmbH) was used as the internal standard and 50 µl of benzoic acid was added both to standards and the samples. 1.5 ml of the acid mixture (HCl/1-propanol mixture, 1:4 v/v) and 1.5 ml 1,2 dichloroethane was added onto each sample. Tubes were boiled at 100°C for 2 h by shaking every 15 min. After they have cooled down, 3 ml distilled water was added and the tubes were shaken for 10 min., centrifuged at 2500 rpm for 5 min. at room temperature and left for the phase separation. One ml was taken from the lower

organic phase and filtered by passing through a sodium sulfate ($NaSO_4$) column. For preparation of this column, 5 g $NaSO_4$ was dried at 105°C overnight. A small amount of glass wool was placed in the tip of one ml pipette and 1 mg $NaSO_4$ was added.

A standard calibration line is prepared by using known concentrations (1/2/3 mg/L) of five different PHA Standards (PHB Standard, Natural Origin, 36,350-2 Sigma-Aldrich; PHB/PHV Copolymer Powder, Natural Origin, 40,132-1, Sigma-Aldrich; PHV Standard, Synthesized at Gent University, personal communication; PHA copolymer, Delft, personal communication; PHB/PHV Copolymer Beads, Natural Origin, 40,312-1, Sigma-Aldrich). The PHA concentrations in the separated phase were determined using a GC system according to a method obtained from Van Loosdrecht (Delft University of Technology, Netherlands, personal communication) with slight modifications.(Table 2.3). Agilent Technologies 19091N-113E Innowax (J&W Scientific, HP, USA) capillary 30m x 0.32 mm x 0.25 μm nominal column was used. Data were analyzed by ChemStation Software, Agilent Technologies, HP. The injector (split-splitless) and flame ionization detector (FID) temperatures were set at 200°C and 250°C, respectively. The carrier gas was helium and the injection was performed in split mode at 10:1 ratio. The column pressure was set to 14.66 psi and the flow rate in the column was set to 3 ml/min. In the detector, H_2 flow was set to 30 ml/min, airflow was set to 400 ml/min and the make up flow (He) was set to 25 ml/min.

Table 2.3 PHB analysis procedures in GC

Oven Ramp	**°C/min**	**Next °C**	**Hold min.**	**Run Time**
Initial		80	0.00	0.00
Ramp1	25.00	130	0.00	2.00
Ramp2	15.00	210	12.00	19.33
Ramp3	0.00			
Post Run		80	0.00	**19.33**

The results were calculated as the percentage of the CDW and as mg/L according to Equations 2.2.6.3.1 and 2.2.6.3.2.

$$\text{PHB (\%)} = [\text{GC result (mg)} / \text{Pre-weighed Sample (mg)}] \times 100 \quad (2.2.6.3.1)$$

$$\text{PHB (mg/L)} = [\text{GC result (mg)} / \text{Pre-weighed Sample (mg)}] \times \text{CDW (mg/L)} \quad (2.2.6.3.2)$$

2.2.6.4. High Performance Liquid Chromatography (HPLC)

In liquid chromatography, the mobile phase is a liquid, while the stationary phase can be a solid or a liquid immobilized on a solid. An HPLC instrument requires a high pressure pump and a supply of mobile phase, a column containing a high efficiency stationary phase, an injection unit for introducing samples onto the column, an in-line detector and some method of displaying the detector signal.

The mobile phase in HPLC may be water, organic solvents or buffers either on their own or mixed with one another. In this present work 2.5 mM sulfuric acid (H_2SO_4) was used as the mobile phase for the glycogen and residual glucose analysis.

2.2.6.5. Residual Glucose Analysis by High Performance Liquid Chromatography (HPLC)

Residual glucose concentration in the growth medium was determined by HPLC analysis. BIORAD HPX87H column was used in Shimadzu® HPLC Systems, equipped with a Shimadzu SCL-10A vp system controller, a LC-10A vp pump, a DGU-14A degaser, a SPD-10A vp UV-Vis detector, RID-10A refractive index detector, SIL-10AD vp autoinjector, CTO-10AC vp oven, Class-VP software.

A standard calibration line was prepared by using known concentrations (0/200/400/1000/2000 mg/L) of D(+)-Glucose (Riedel-de Haën, Sigma-Aldrich Laborchemikalien GmbH). One ml of sample was derived from the bioreactor and centrifuged rapidly at 14000g in a bench top centrifuge (Microfuge® 18 Centrifuge, Beckman Coulter, U.S.A.) at room temperature. The supernatant is quickly filtered through 0.22μ filters (Millex®-GV, Millipore Corp. U.S.A.) and the run was

performed using an HPLC system with refractive index measurements. The mobile phase was 2.5 mM H_2SO_4 and the column temperature was 60°C.

2.2.6.6. Glycogen Analysis by HPLC

Glycogen concentration was determined using a BIORAD HPX87H column in Shimadzu HPLC Systems. A sample of 4.5 ml was withdrawn from the bioreactor and 0.5 ml 6M HCl was added for acid hydrolysis. Thus, intracellular glycogen was added to the supernatant and converted into glucose. The sample was incubated at 100 °C for 6 h. It was then quickly filtered through 0.22μ filters (Millex®-GV, Millipore Corp. U.S.A.) and the run was performed using an HPLC system as described in section 2.2.6.5.

The glycogen standards (Fluka-50571, Switzerland) at varying concentrations (0/200/400/1000/2000 mg/L) were prepared and they were run in the HPLC. Results were compared with the known standard concentrations. It was found that the yield of the hydrolysis of glycogen was 85%. The results were modified according to the obtained yield. Finally, the glycogen concentration was determined by the subtraction of residual glucose concentration from the glycogen analysis results.

2.2.7. Respiration Analysis

The bioreactor that is operated under SBR conditions was directly connected to Respirometer (RA-Combo Lab, Applitek, NV. SA). Respiration measurement was started prior to feeding of the following cycle of the SBR and waited until the culture showed a constant basic respiration rate. As soon as the basic rate has been measured for at least 15 min. with a variation less than or equal to 1 mg O_2/l.h., the culture was fed with 75 ml of M9 containing 4000 mg/L glucose as the sole carbon source. It was waited until the culture again showed a basic respiration rate for at least 15 min. with a variation of less than or equal to 1 mg O_2/l.h.

2.2.8. PHB Staining

PHB staining was performed according to the literature (Murray Ed.,R.G.E, 1981). Briefly a thin smear of cells were prepared on a microscopy slide and thoroughly dried in air. They were stained with Solution 1 (Sudan Black B (IV), 0.3% (w/v) in 60% (v/v) ethanol) for 10 min. The slides were rinsed with water for one second.

They were then stained with Solution II (Safranin O, 0.5 % (w/v) aqueous solution), rinsed well with water and blot dried. The slides were examined at 1000X magnification with oil immersion and direct illumination using an Olympus BX60 light microscope. The images were obtained by using the Spot Advanced Software. The PHB granules appear as intracellular, blue-black granules, while the cytoplasm is observed pink or clear.

2.2.9. Glycogen Staining

By using the Periodate-Schiff staining technique (Gerhardt *et al.*, 1994) glycogen-like inclusions could be observed. A slide with a heat-fixed cell smear was flooded with periodate solution (20 ml of 4% (w/v) aqueous periodic acid, 10 ml of 0.2 M aqueous sodium acetate, 70 ml of 95% (v/v) ethanol; light-protected solution) for 5 min. The slide was washed with 70% (v/v) ethanol and flooded for 5 min with a reducing solution containing 300 ml of ethanol, 5 ml of 2N hydrochloric acid, 10 g of potassium iodide, 5 ml of sodium thiosulfate, and 200 ml of distilled water. The slide was rewashed with 70% (w/v) ethanol and stained for 30 min with the Schiff reagent (2 g basic fuchsine was added to 400 ml boiling distilled water and cooled down to 50°C. It was filtered and 10 ml 2N HCl and 4g potassium metabisulfite was added to the solution. It was stoppered tightly and left at a cool and dark place for 12 h. Ten ml 2N HCl was added to the slide until the reagent, when dried on a glass slide, did not show a pink tint. It was stored in a dark place)

The smear was washed several times in a solution consisting of 2 g of potassium metabisulfite and 5 ml of concentrated hydrochloric acid in 500 ml of distilled water and washed with tap water, counterstained with a 0.002% (w/v) aqueous solution of malachite green for 5 s. Lastly, the slide was washed in tap water and blot dried. It was examined under the light microscope (Olympus BX60) with 1000x magnification with oil immersion. The images were obtained by using the Spot Advanced Software. Polysaccharides appear red, and other cytoplasmic components appear green.

2.3. Calibration Lines for Various Analyses

For determination of total protein and various metabolite concentrations of the cell samples of all cultivations, prior to analysis, the calibrations described in this section were made and used for all analysis throughout this study.

2.3.1. Total Protein Standard Curve

In order to determine the total protein concentration of the cells, a standard curve (Figure 2.1) was prepared using the following BSA concentrations: 0 mg/ml as blank, 0.5 mg/ml, 1.25 mg/ml, 2.5 mg/ml, 3.75 mg/ml, 5 mg/ml. Total protein contents of the cells were calculated by using the standard curve equation 2.1.1.

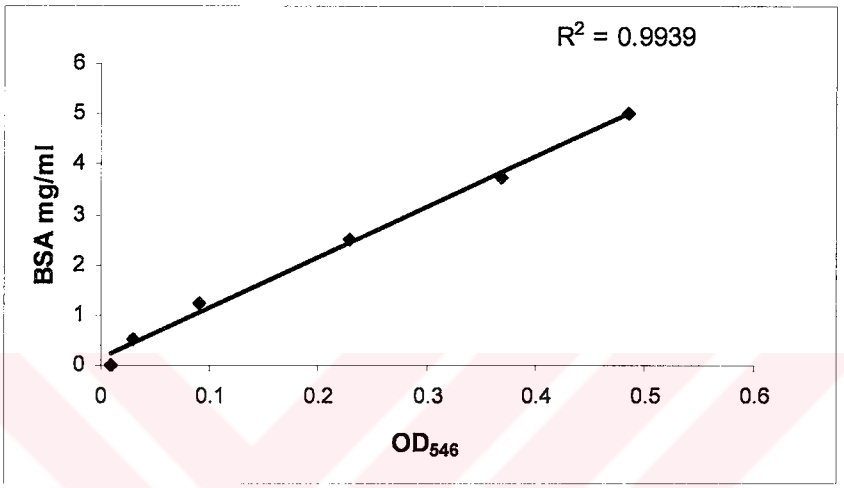


Figure 2.1 Calibration line of BSA, which is used for the determination of total protein concentration.

$$\textbf{BSA (mg/L)} = 9987(OD_{546}) + 1480 \qquad (2.1.1)$$

2.3.2. Volatile Fatty Acid Standard Curve

VFA standards were used as the external standards in order to obtain the calibration line with the following concentrations: 5 mg/L, 50 mg/L, 100 mg/L, 150 mg/L, 300 mg/L, 500 mg/L, 750 mg/L, 900 mg/L. Thus, a calibration line on GC was obtained by using the chromatograms of these concentrations of the standards. In Figure 2.2, the chromatogram of 500 mg/L VFA standard is shown.

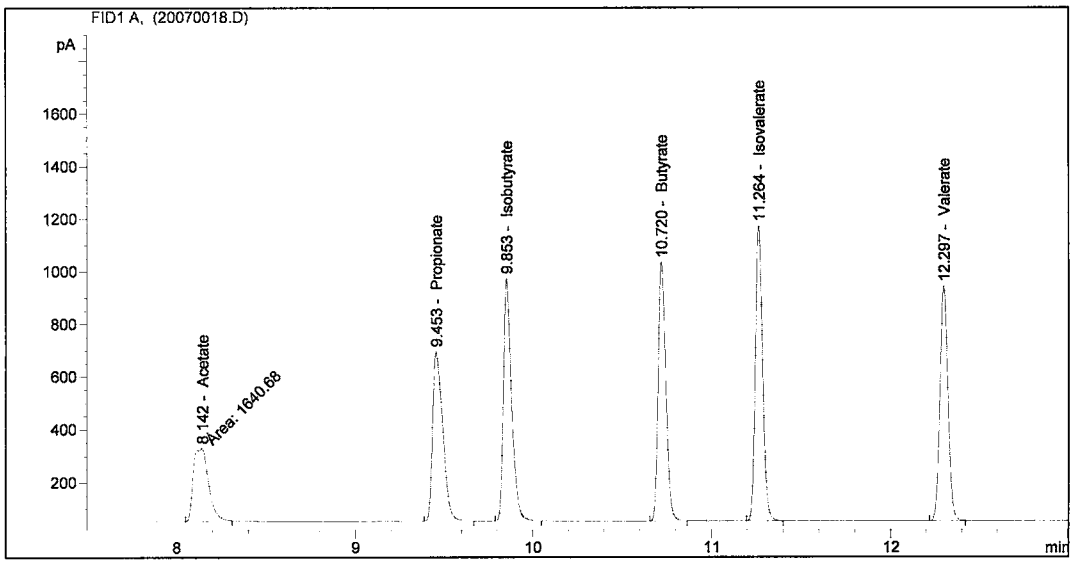
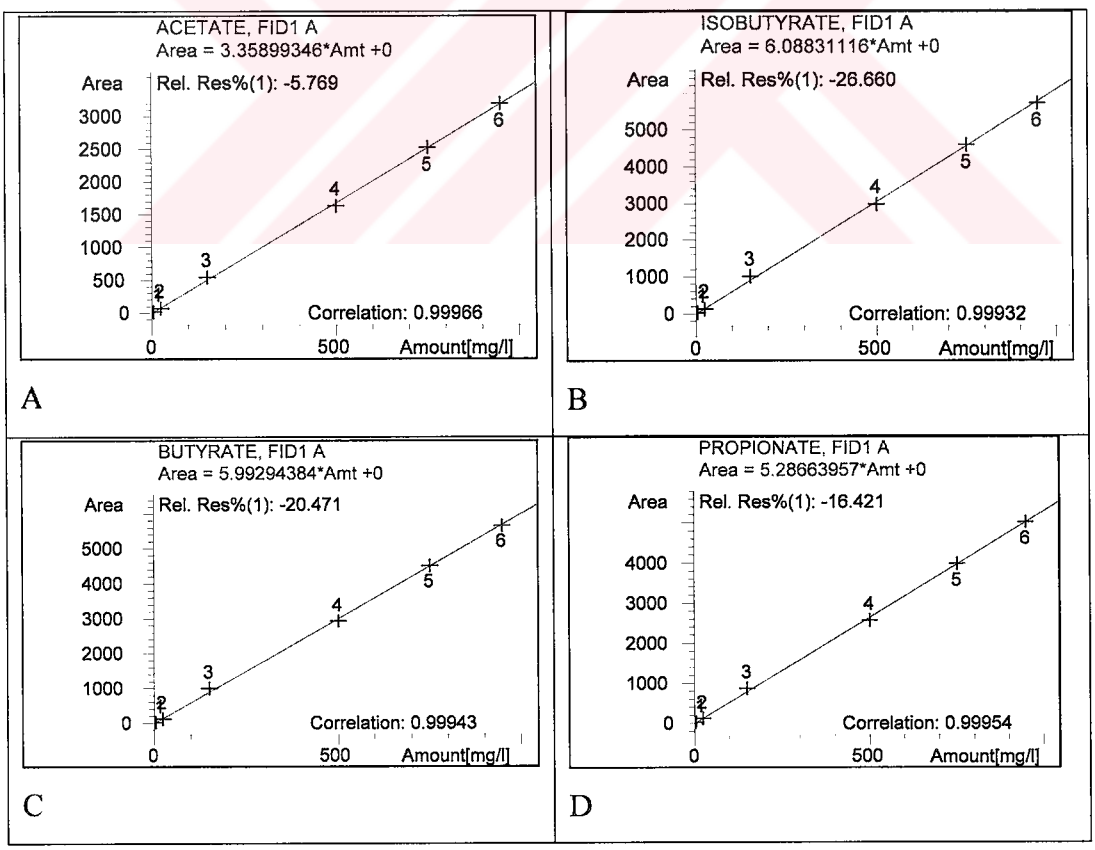


Figure 2.2 Chromatogram of 500 mg/L VFA standard analyzed by GC

The standard curves of acetate, isobutyrate, butyrate, propionate, isovalerate, and valerate on GC had very high regression coefficients, and correlations higher than 0.999 (Figure 2.3).



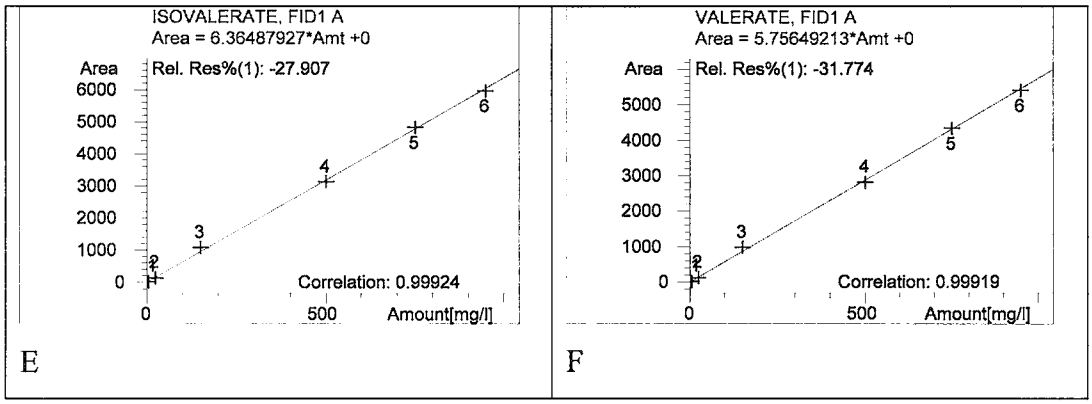


Figure 2.3 Calibration lines of VFA on GC; (A) Acetate, (B) Isobutyrate, (C) Butyrate, (D) Propionate, (E) Isovalerate, (F) Valerate.

VFA concentrations in the samples were calculated by ChemStation Software according to Equations 2.3.2.1 - 2.3.2.6 (Table 2.3).

Table 2.4 Equations of all VFAs obtained from GC

Acetate (mg/L) = 0.2987 x Area (2.3.2.1)	Propionate (mg/L) = 0.1892 x Area (2.3.2.4)
Isobutyrate (mg/L) = 0.1643 x Area (2.3.2.2)	Isovalerate (mg/L) = 0.1571 x Area (2.3.2.5)
Butyrate (mg/L) = 0.1669 x Area (2.3.2.3)	Valerate (mg/L) = 0.1737 x Area (2.3.2.6)

2.3.3. Residual Glucose Standard Curve

D (+)-Glucose was used as the external standard for the calibration of HPLC in residual glucose analysis. The concentrations were as follows: 0 mg/L, 200 mg/L, 400 mg/L, 1000 mg/L, and 2000 mg/L. In Figure 2.4, the chromatogram of 2000 mg/L glucose is shown. The first peak with 5.8 min retention time was the peak of water and the second peak with 8.479 min retention time belongs to glucose.

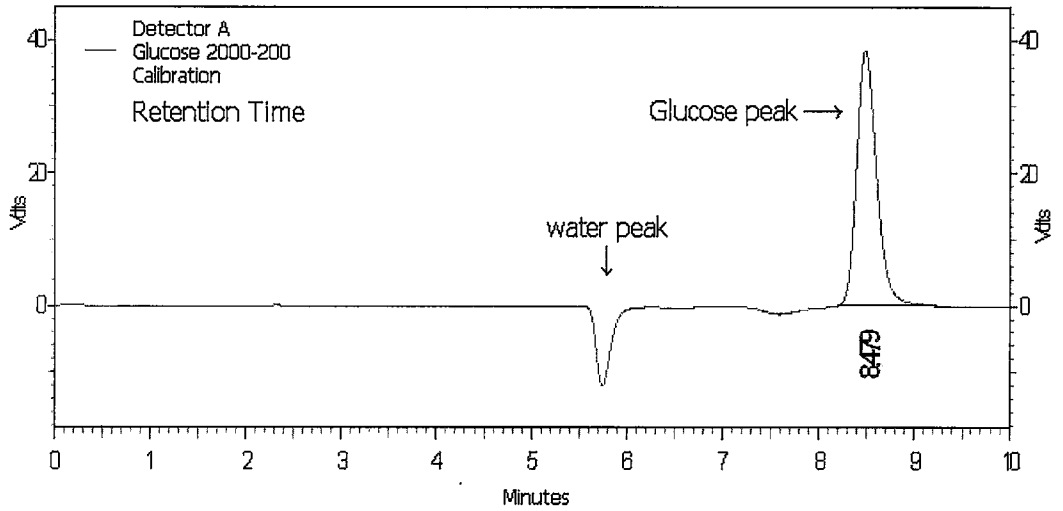


Figure 2.4 Chromatogram showing 2000 mg/L glucose concentration on HPLC.

A standard curve was drawn on HPLC by using the known amounts of D (+)-Glucose concentrations (Figure 2.5). Residual glucose concentrations were determined on HPLC by Class-VP Software according to Equation 2.3.3.1. The standard curve obtained on HPLC has a high regression coefficient, it shows a validity of 0.99993 probability level (Figure 2.5).

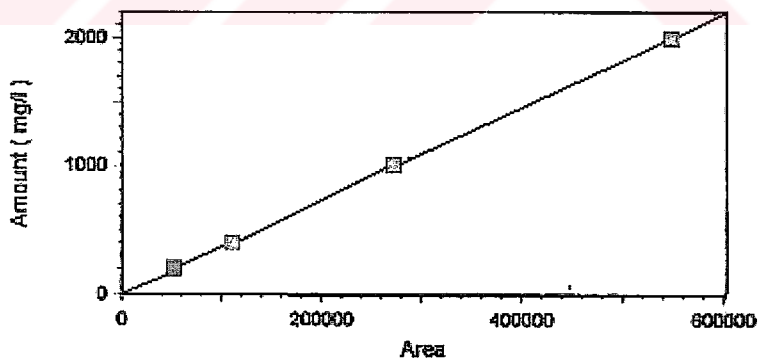


Figure 2.5 Calibration line of glucose standards analyzed by HPLC.

$$\text{Glucose (mg/L)} = 0.00365521 \times \text{Area} \qquad (2.3.3.1)$$

2.3.4. Glycogen Calibration on HPLC

Glycogen concentrations of culture samples (cell + medium) were measured following the acid hydrolysis procedure of the whole glycogen into glucose. Therefore, glucose determination protocol and the glucose calibration line on HPLC were used for glycogen analysis. In order to determine the amount lost during hydrolysis different concentrations of glycogen standard were prepared (200 mg/L – 2000 mg/L), hydrolyzed with 6M HCl and converted into glucose (G1). The glucose concentrations obtained were corrected with the hydrolysis yield by multiplying the results with 0.85 as described in section 2.2.6.6. The residual glucose concentration (G2) was subtracted from the result of the multiplication Equation (2.3.4.1).

$$\text{Glycogen Conc. (mg/L)} = (G1 \times 0.85) - G2 \qquad (2.3.4.1)$$

2.3.5. Polyhydroxyalkanoates Calibration on GC

Two different types of polyhydroxyalkaonates standards were used as the external standards for the calibration on GC. Both of the external standards contained polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV) copolymers. The first standard (Sigma-Aldrich, 40,132-1) was a powder whereas the second one (Sigma-Aldrich, 40,312-1) was a bead-shaped, hard plastic. Benzoic acid was added to both of the standards and the samples as the internal standard. The amounts of the external standards pre-weighed were 1 mg, 2 mg, and 3 mg. In Figure 2.6 the chromatogram of 1 mg polyhydroxybutyrate and polyhydroxyvalerate copolymers standard is shown. The first peak with 3.452 min retention time is polyhydroxybutyrate, the second peak with 3.838 min retention time is polyhydroxybutyrate and the third peak with 4.305 min retention time belongs to benzoic acid.

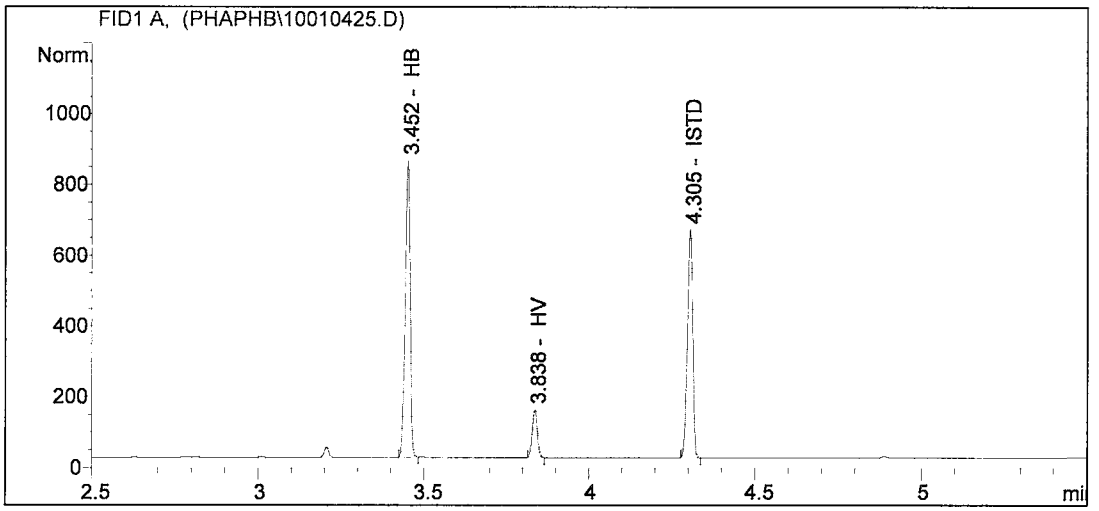


Figure 2.6 Chromatogram showing 1 mg polyhydroxybutyrate-polyhydroxyvalerate copolymers on GC

Areas obtained from the analysis of the two types of polyhydroxyalkanoate standards gave the response factor as 74 % of GC for that particular analysis. The amounts of the standards and the samples were corrected according to that response factor. The results were calculated according to the Equations 2.3.5.1 and 2.4.5.2 obtained by the calibration line of polyhydroxybutyrate-polyhydroxyvalerate copolymers on GC (Figure 2.7).

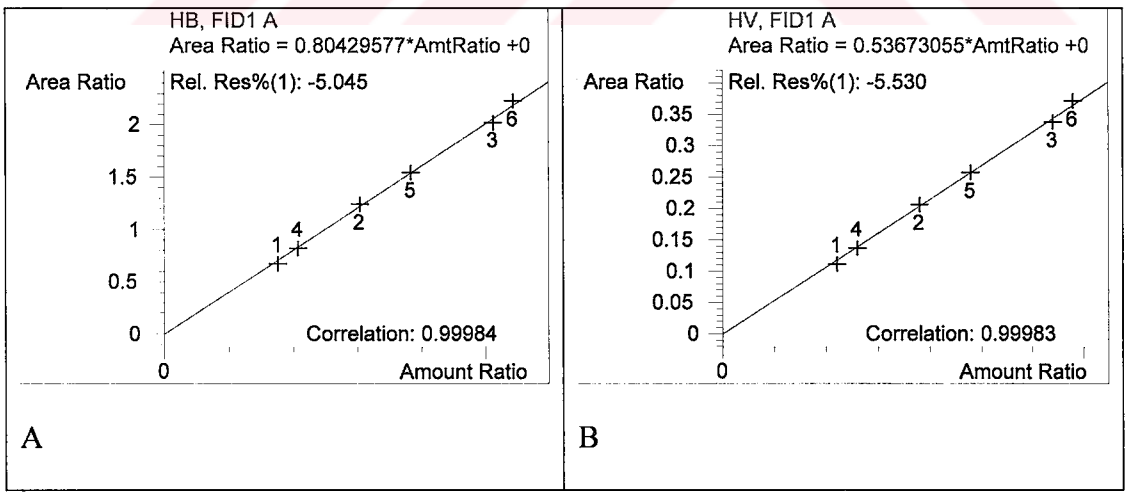


Figure 2.7 Calibration line of polyhydroxybutyrate-polyhydroxyvalerate copolymers on GC

Polyhydroxybutyrate (mg/L) = 1.243 x Area

(2.3.5.1)

Polyhydroxyvalerate (mg/L) = 1.863 x Area

(2.3.5.2)

3. RESULTS

3.1. Shake Flask Cultivations

Three different types of cultivations were performed in shake flasks. In the first type, glycerol was used in a minimal medium as the sole carbon source in varying concentrations between 30 mg/L, 50 mg/L, 200 mg/L, 500 mg/L and 1000 mg/L. In the second type of cultivation, varying concentrations of NH_4Cl were supplied in a minimal medium with 4000 mg /L glucose as the sole carbon source in order to investigate the effects of nitrogen source. And lastly, growth and storage metabolites of *E. coli* were analyzed in a minimal medium with 4000 mg/L glucose as the sole carbon source.

3.1.1. Batch Cultivations with Varying Concentrations of Glycerol as the Sole Carbon Source

Glycerol was chosen as one of the substrates for observing the possible effects of different substrate types to the physiology and biochemical storage mechanism of *E. coli*. Therefore, batch experiments in shake flasks were conducted as described in Section 2.2.1 to investigate whether glycogen and PHB are accumulated by the microorganisms. At the 8^{th} and 24^{th} hours of the cultivation, OD_{600} and microscopic staining samples were withdrawn from the shake flasks. In Figure 3.1, compared OD_{600} results of *E. coli* growing on various glycerol concentrations as the sole carbon source are demonstrated.

Microscopic staining methods were applied to detect possible glycogen and PHB accumulation during different cultivation hours of interest. No storage of glycogen and PHB was observed under these experimental conditions (data not shown).

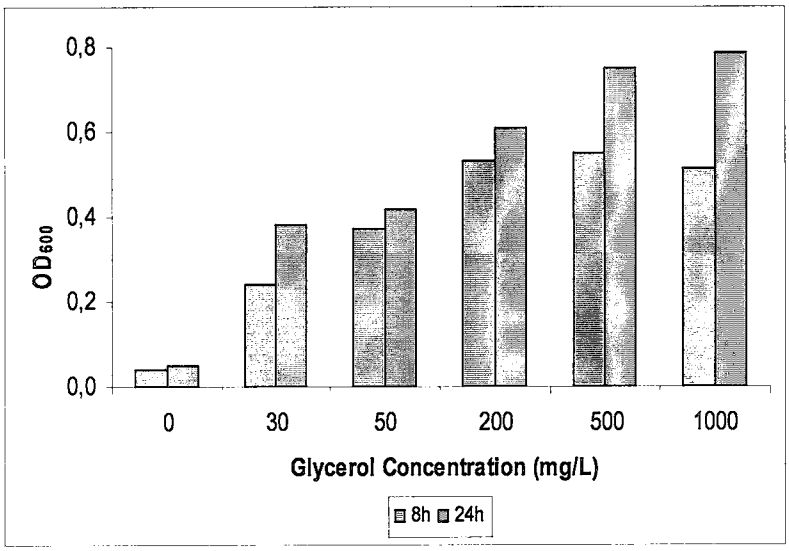


Figure 3.1 OD_{600} profiles of *E. coli* in a minimal medium with different initial concentrations of glycerol as the sole carbon source at 8^{th} and 24^{th} hours of batch cultivation.

3.1.2. Batch Cultivations with Varying Initial Concentrations of NH_4Cl as the Sole Nitrogen Source

NH_4Cl was the only nitrogen source in the growth medium. Thus, to investigate the effects of varying concentrations of nitrogen source on growth and storage metabolism cultivations with various initial concentrations of NH_4Cl were performed in the first step of this experiment. OD_{600} and microscopic staining samples were withdrawn from the shake flasks at 0^{th}, 8^{th}, 24^{th} and 48^{th} hours of the cultivation. OD_{600} profiles are shown in Figure 3.2. For cultivations at the 0^{th}, 8^{th}, and 24^{th} hours of this experimental set, the microscopic staining results for glycogen and PHB storage were negative (data not shown). Residual glucose, glycogen and PHB samples drawn at the 48^{th} hour were investigated by HPLC and GC systems, respectively. No PHB storage at the 48^{th} hour of this cultivation was observed by GC analysis. A significant amount of glycogen accumulation was detected at the 48^{th} hour of the cultivation (Figure 3.3) whereas no residual glucose peak was observed by HPLC.

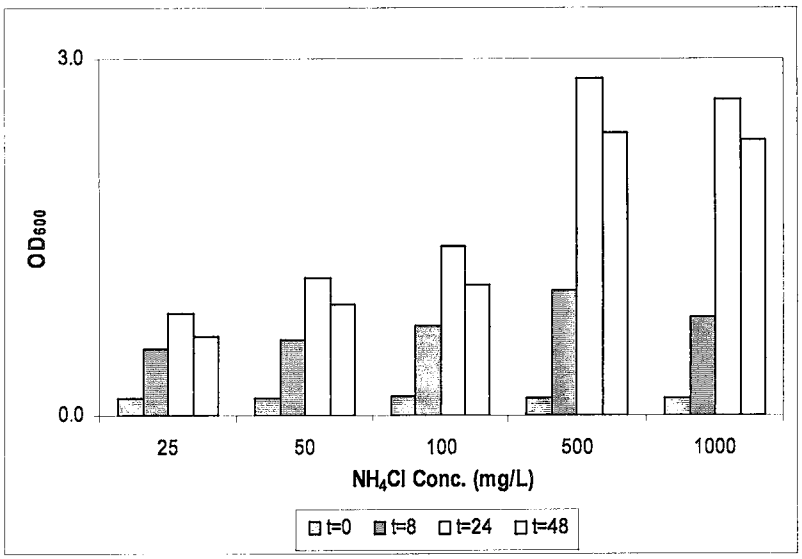


Figure 3.2 OD_{600} results of *E. coli* cultivated with different initial concentrations of NH_4Cl at 0^{th}, 8^{th}, 24^{th}, and 48^{th} hours of the batch cultivation in standard minimal medium.

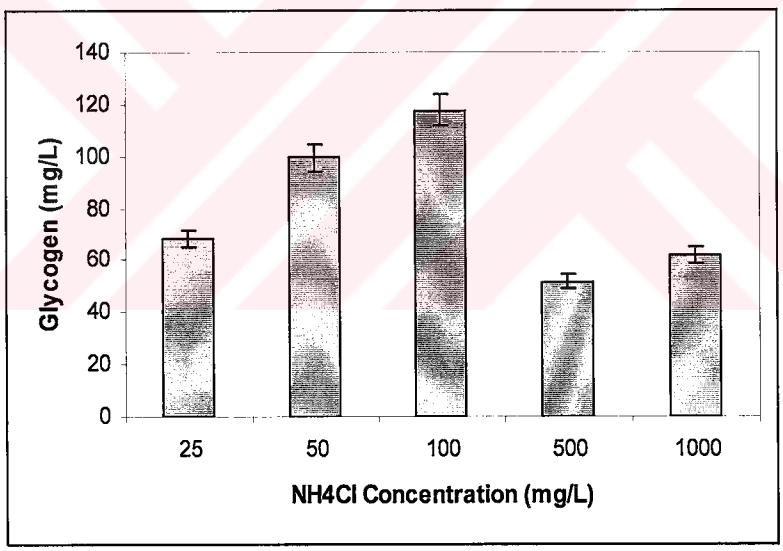


Figure 3.3 Glycogen results of *E. coli* cultivated with different initial concentrations of NH_4Cl at 48^{th} hours of the batch cultivation in standard minimal medium.

The experiment was continued with the transfer of *E. coli* -grown at 1000 mg/L NH_4Cl- in M9 in a new medium without any NH_4Cl in order to have nitrogen starvation. OD_{600} results of this culture were shown in Figure 3.4 at 0^{th}, 8^{th}, 24^{th} and 48^{th} hours of starvation in the medium.

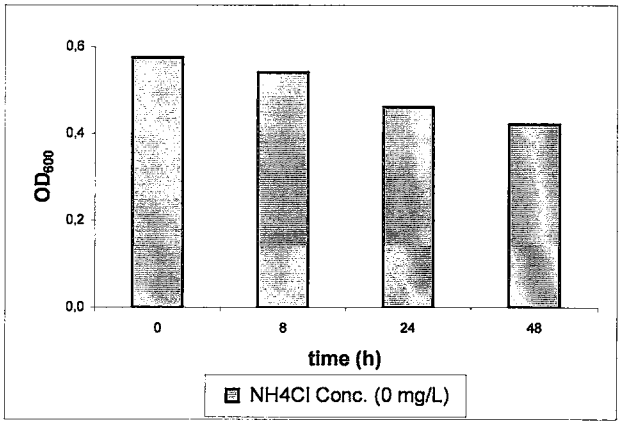


Figure 3.4 OD_{600} values of *E. coli* during nitrogen starvation up to 48 h.

During this nitrogen starvation experimental set, it is demonstrated by HPLC analyses that microorganisms did not consume residual glucose (data not shown). Thus, neither glycogen nor PHB storage was observed by HPLC and GC analyses, respectively.

3.1.3. Batch Cultivation in a Minimal Medium with 4000 mg/L Glucose as the Sole Carbon Source

During cultivation of *E. coli* in 1-liter baffled shake flasks in M9 containing 4000 mg/L glucose, samples were drawn at four different phases of growth. The growth profile was obtained by using OD_{600}, total protein, residual glucose and VFA data. According to the OD_{600} results 0, 4.5, 7 and 21st hours of growth were estimated to be the lag, early exponential, late exponential and stationary phases, respectively. Thus, samples for growth metabolites and glycogen -as the expected storage polymer- were withdrawn during these times of the cultivation, representing each growth phase.

3.1.3.1. Determination of Growth Behavior

Growth profile of *E. coli* in 1-L Baffled shake flasks was determined by using $LnOD_{600}$ values (Figure 3.5). Cellular dry weight analysis was also performed by using filtration method. As a result, equation 3.1.3.1.1 was obtained which correlates OD_{600} values with cellular dry weights for *E. coli* and is used in the next studies of shake flask experiments.

$$CDW\ (mg/L) = [(0.324 \times OD_{600}) - 0.00574] \times 1000 \qquad (3.1.3.1.1)$$

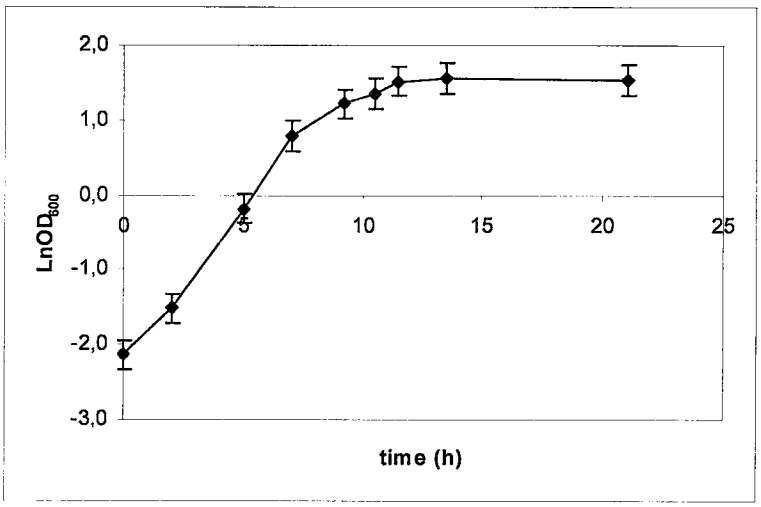


Figure 3.5 Growth profile of *E. coli* in 1-L baffled shake flasks.

Samples drawn from the shake flasks at four different phases of growth for the determination of the key metabolites of growth were analyzed according to the procedures. Total protein concentrations on these four phases were analyzed by BCA Assay. The concentrations were calculated by using the Equation 2.1.1. CDW concentrations were determined by using Equation 3.2.3.1.1. The results were shown in Figure 3.6 in parallel with the corresponding OD_{600} values of the four different phases of growth.

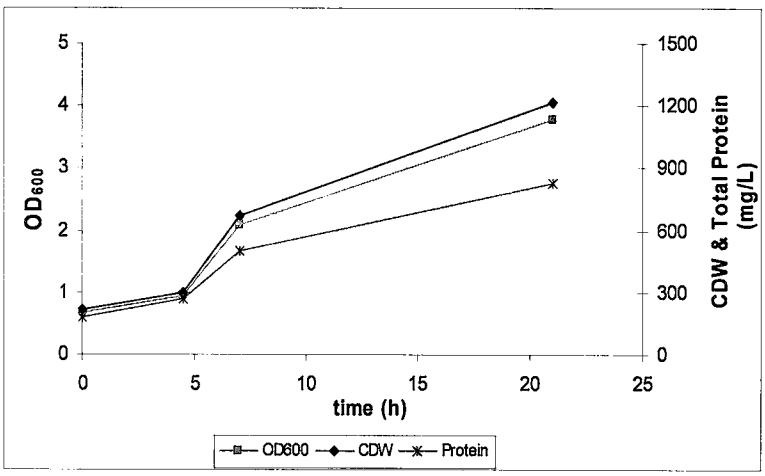


Figure 3.6 Growth behavior of *E. coli* in 1-L Baffled shake flasks with 4000 mg/L glucose.

Samples drawn from the shake flaks for residual glucose concentrations were analyzed by HPLC. Although GC was calibrated for acetate, isobutyrate, butyrate, propionate, isovalerate, and valerate, only acetate was produced at a detectable level during the cultivation. Thus, only the acetate concentrations at four different phases of growth are shown in Figure 3.7 by comparing data with residual glucose concentrations during these time intervals.

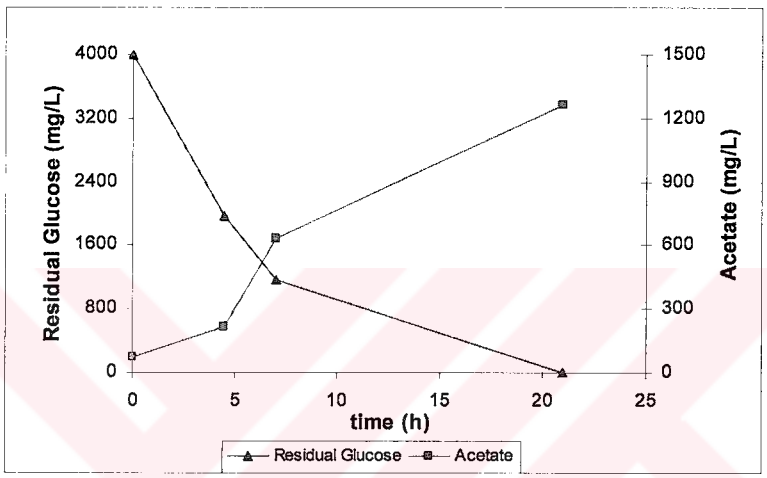


Figure 3.7 Residual glucose and acetate concentrations in 1-L Baffled shake flasks at four different phases of batch growth.

3.1.3.2. Analysis of Storage Polymers

Glycogen samples were withdrawn from shake flask cultures. Samples were hydrolyzed and converted into glucose prior to analysis as described in Section 2.2.6.6.

No storage of glycogen was observed during the batch growth of *E. coli* in shake flasks. According to microscopic staining results, no PHA accumulation was observed neither (data not shown).

3.2. Bioreactor Cultivations

Batch, fed-batch and sequencing batch (SBR) cultivations were performed using a bioreactor. Samples were withdrawn for the analysis of the intracellular and extracellular growth metabolites as well as the important storage polymers.

3.2.1. Batch Cultivation in a Bioreactor with 4000 mg/L Glucose as the Sole Carbon Source

3.2.1.1. Determination of Growth Behavior

The effects of high glucose content on the growth physiology and the storage response was investigated under batch cultivation conditions. Growth was monitored by OD_{600} values that were determined every 30 min. The growth profile is shown in Figure 3.8.

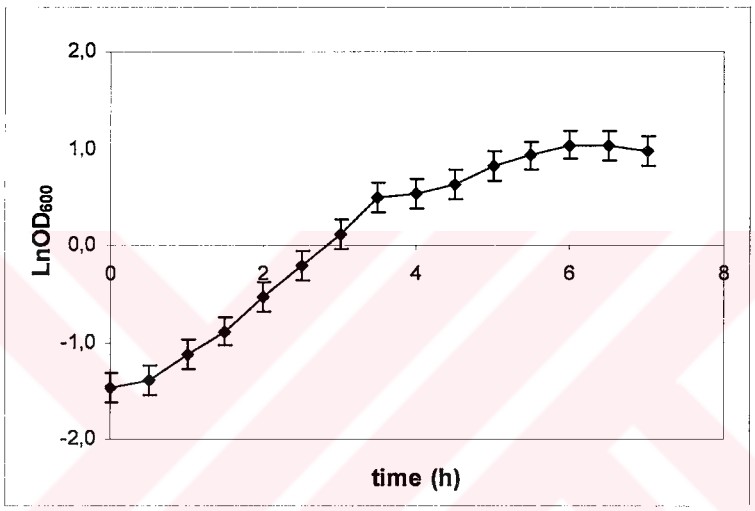


Figure 3.8 Growth profile of *E. coli* in a bioreactor during batch cultivation with 4000 mg/L glucose as the sole carbon source.

Total protein concentration -the most abundant intracellular macromolecule- of the biomass was compared with the cellular dry weight (CDW) content and also OD_{600} values of the culture (Figure 3.9). CDW was calculated according to Equation 3.2.1.1.1, obtained by filtration method.

$$CDW\ (mg/L) = [(0.3796 \times OD_{600}) + 0.1759] \times 1000 \qquad (3.2.1.1.1)$$

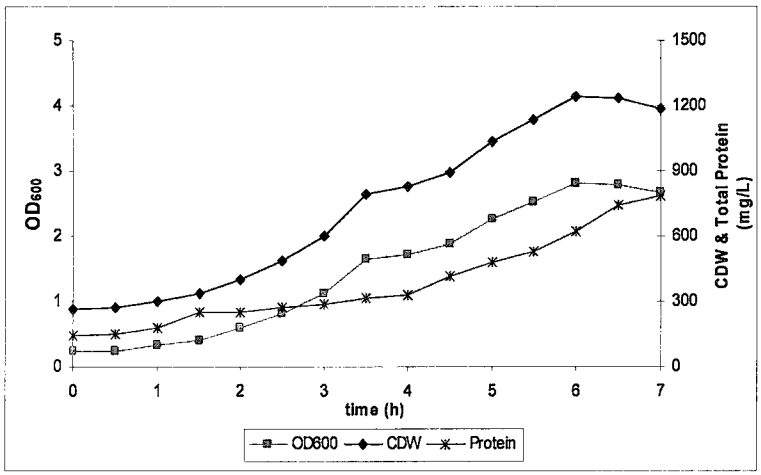


Figure 3.9 Growth behavior of *E. coli* in a bioreactor during batch cultivation with 4000 mg/L glucose as the sole carbon source.

Substrate utilization was monitored by extracellular analysis of residual glucose and acetate concentrations (Figure 3. 10).

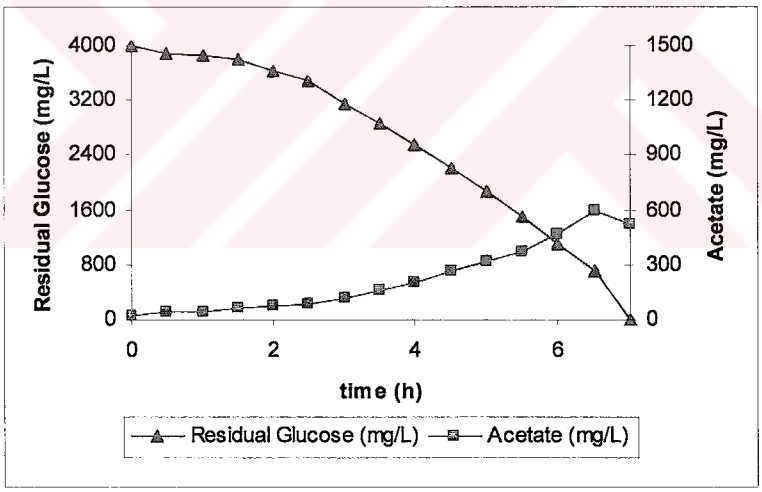


Figure 3.10 Residual glucose and acetate concentrations in a bioreactor during batch cultivation with 4000 mg/L glucose as the sole carbon source.

3.2.1.2. Analysis of Storage Polymers

PHB and glycogen analyses were performed as the important storage polymers under investigation. PHB analysis was done by GC and Glycogen analysis was performed by HPLC. No storage of these polymers was detected under these conditions (data not shown).

3.2.2. Batch Cultivation in a Bioreactor with 500 mg/L Glycerol as the Sole Carbon Source

3.2.2.1. Determination of Growth Behavior

The possible growth and storage effects of glycerol as the sole carbon source were investigated under batch cultivation conditions. Phases of growth under these conditions were monitored by OD_{600} measurements (Figure 3.11).

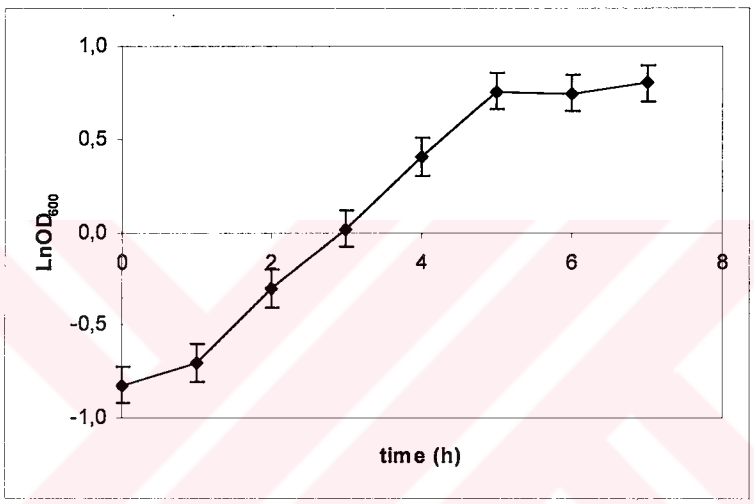


Figure 3.11 Growth profile of *E. coli* in a bioreactor during batch cultivation with 500 mg/L glycerol as the sole carbon source.

Biomass content was determined according to Equation 3.2.2.1.1 obtained by CDW analysis performed by filtering method. Total protein concentration and the biomass were compared in Figure 3.12.

$$CDW\ (mg/L) = [(0.447 \times OD_{600}) + 0.16913] \times 1000 \qquad (3.2.2.1.1)$$

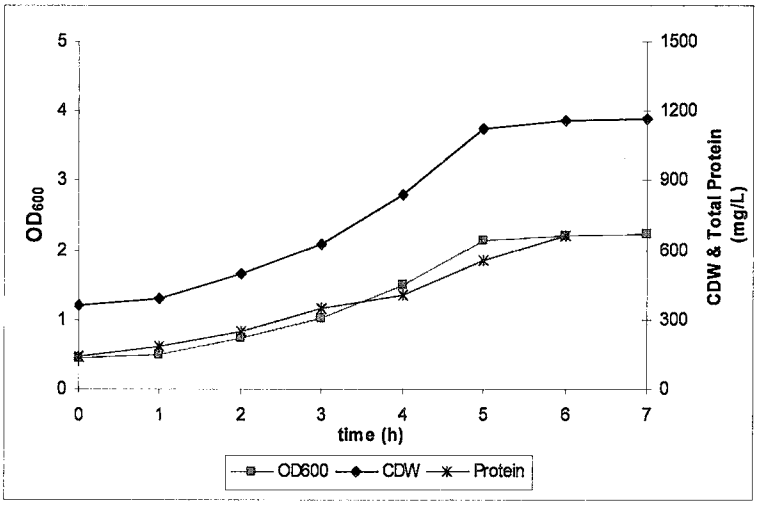


Figure 3.12 Growth behavior of *E. coli* in a bioreactor during batch cultivation with 500 mg/L glycerol as the sole carbon source.

Residual glucose and acetate concentrations were measured. No residual glucose was observed during the experiment (data not shown). Acetate profiles were shown on Figure 3.13.

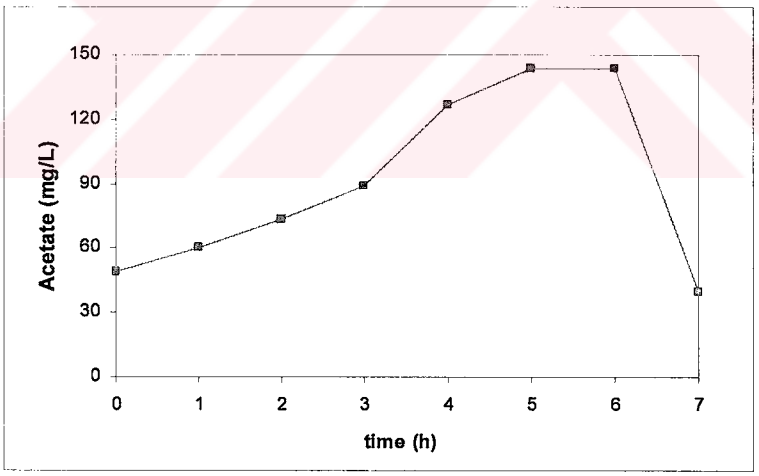


Figure 3.13 Acetate concentrations in a bioreactor during batch cultivation with 500 mg/L glycerol as the sole carbon source.

3.2.2.2. Analysis of Storage Polymers

Glycogen and PHB analysis were performed by HPLC and GC, respectively. No storage of both of the polymers was observed under these culturing conditions.

3.2.3. Fed-batch Cultivation in a Bioreactor with 4000 mg/L Glucose as the Sole Carbon Source

Ten-fold concentrated M9 containing 4000 mg/L glucose was added to overnight cultivated microorganisms in the bioreactor. Samples were drawn frequently after the pulse addition of the medium until the whole residual glucose content was consumed.

3.2.3.1. Determination of Growth Behavior

Growth behavior of *E. coli* was monitored using $LnOD_{600}$ data (Figure 3.14).

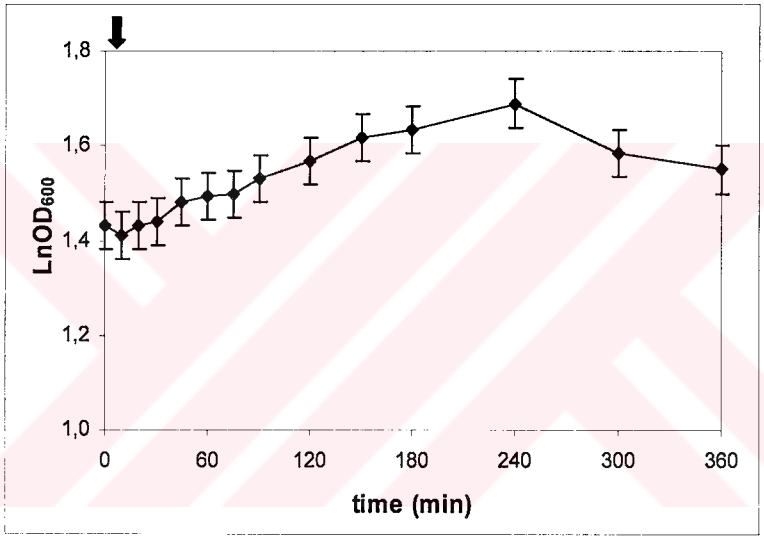


Figure 3.14 Growth profile of *E. coli* in a bioreactor during fed-batch cultivation with 4000 mg/L glucose as the sole carbon source. In the figure legend the arrow indicates the time point where the feed (M9 with 4000 mg/L glucose) was added.

Growth profile was monitored by measuring total protein, OD_{600} and CDW. CDW content was calculated according to Equation 3.2.2.1.1. Results were shown in Figure 3.15.

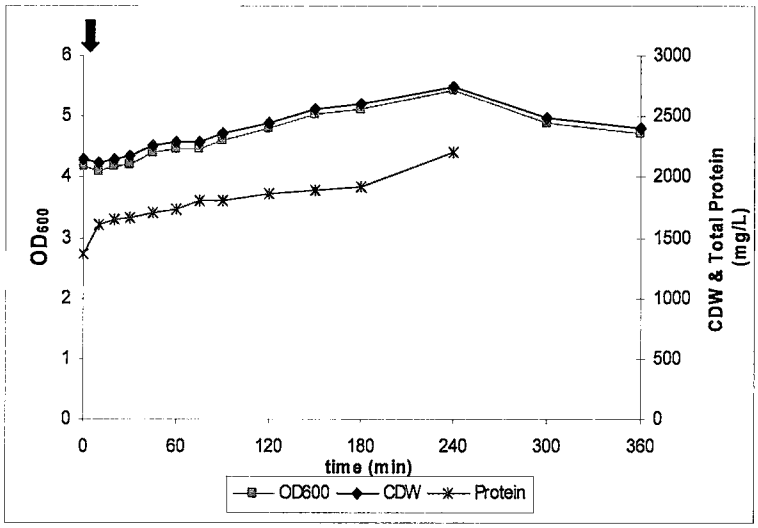


Figure 3.15 Growth behavior of *E. coli* in a bioreactor during fed-batch cultivation with 4000 mg/L glucose as the sole carbon source. In the figure legend the arrow indicates the time point where the feed (M9 with 4000 mg/L glucose) was added.

Residual glucose analysis was performed on-line while the experiment was continuing. Acetate concentrations are shown together with residual glucose results in Figure 3.16.

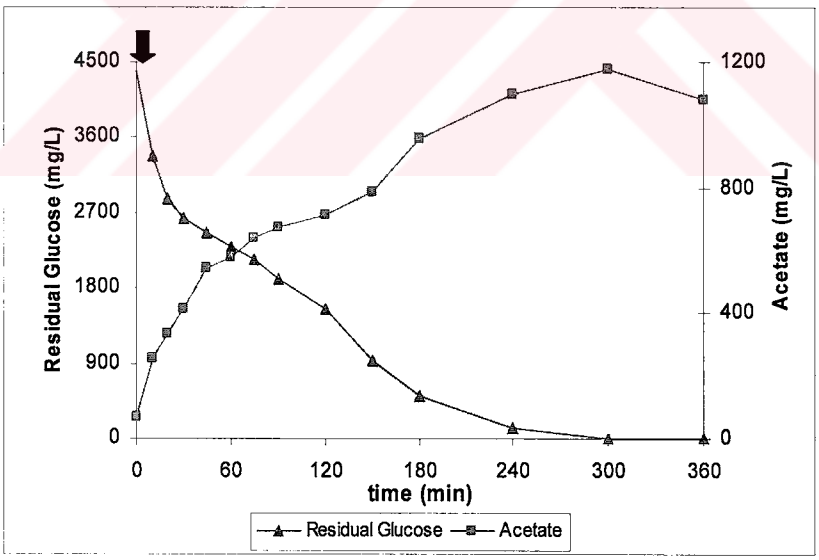


Figure 3.16 Residual glucose and acetate concentrations in a bioreactor during fed-batch cultivation with 4000 mg/L glucose as the sole carbon source. In the figure legend the arrow indicates the time point where the feed (M9 with 4000 mg/L glucose) was added.

3.2.3.2. Analysis of Storage Polymers

Under these fed-batch growth conditions, glycogen storage was detected in the culture. The time course of glycogen formation is shown in Figure 3.17.

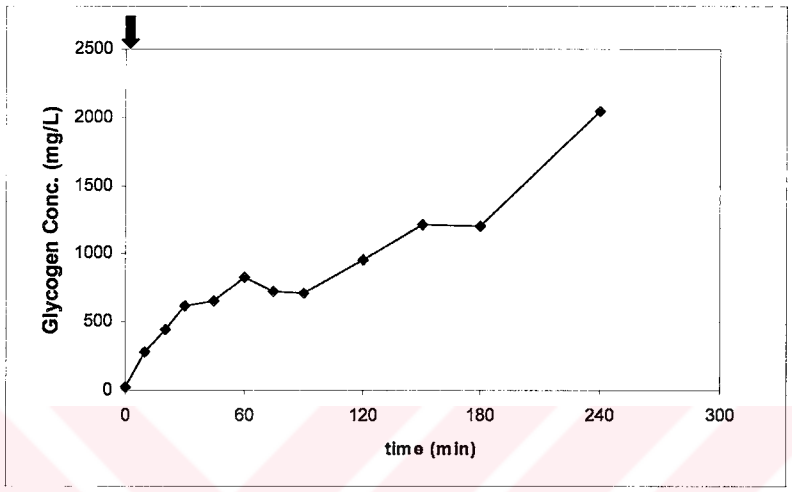


Figure 3.17 Glycogen concentrations in a bioreactor during fed-batch cultivation with 4000 mg/L glucose as the sole carbon source. In the figure legend the arrow indicates the time point where the feed (M9 with 4000 mg/L glucose) was added.

No PHB storage was observed under these conditions (data not shown).

3.2.4. Fed-batch Cultivation in a Bioreactor with 400 mg/L Glucose Feed

Following the fed-batch *E. coli* cultivation with 4000 mg/L glucose feed, the cultivation of *E. coli* was continued with the pulse addition of 150 ml of 10-fold concentrated M9 medium with 400 mg/L glucose. Sampling was done frequently.

3.2.4.1. Determination of Growth Behavior

$LnOD_{600}$ results were used to determine the growth profile of *E. coli* under these conditions (Figure 3.18).

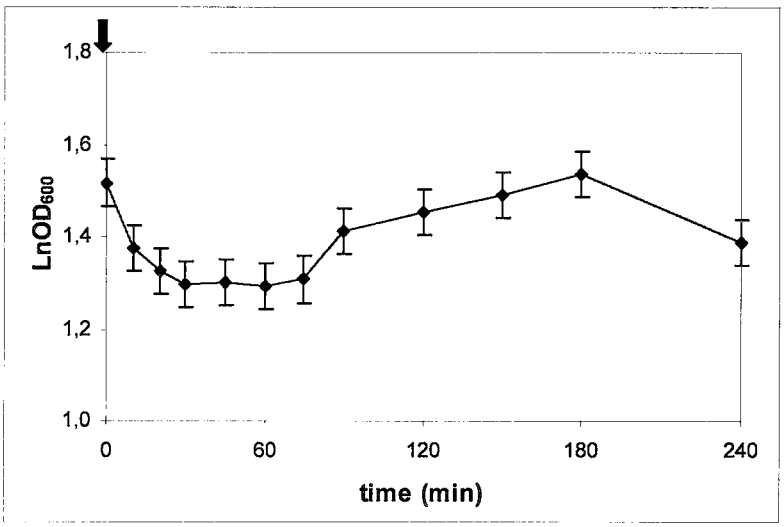


Figure 3.18 Growth profile of *E. coli* in a bioreactor during fed-batch cultivation with 400 mg/L glucose as the sole carbon source. In the figure legend the arrow indicates the time point where the feed (M9 with 400 mg/L glucose) was added.

CDW contents were calculated according to Equation 3.2.2.1.1. Total protein and OD_{600} results were compared with CDW (Figure 3.19).

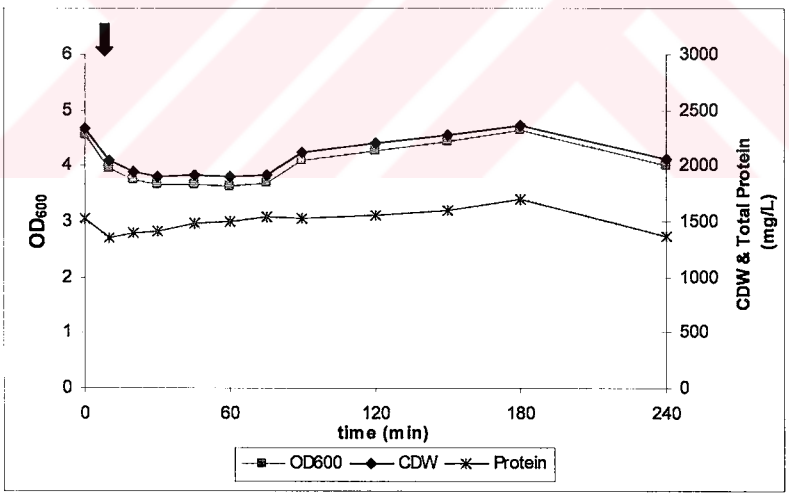


Figure 3.19 Growth behavior of *E. coli* in a bioreactor during fed-batch cultivation with 400 mg/L glucose as the sole carbon source. In the figure legend the arrow indicates the time point where the feed (M9 with 400 mg/L glucose) was added.

Acetate and residual glucose analyses were performed on GC and HPLC, respectively Figure 3.20).

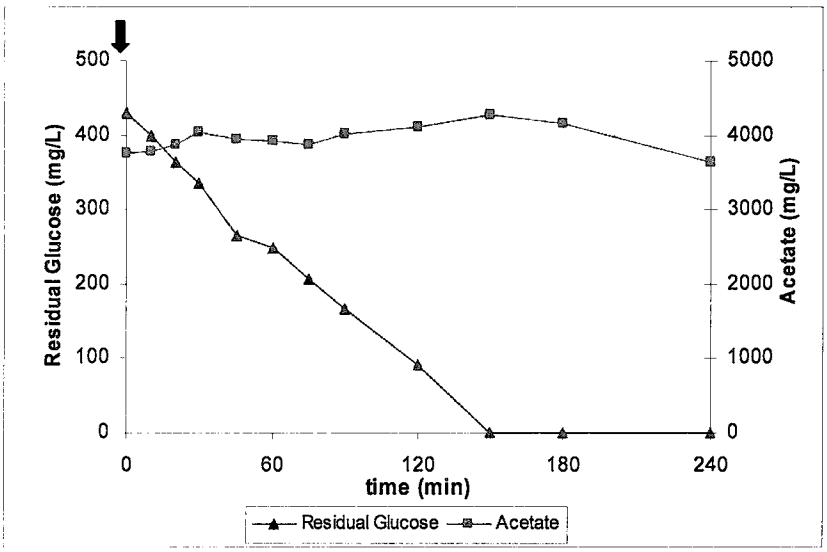


Figure 3.20 Residual glucose and acetate concentrations in a bioreactor during fed-batch cultivation with 400 mg/L glucose as the sole carbon source. In the figure legend the arrow indicates the time point where the feed (M9 with 400 mg/L glucose) was added.

3.2.4.2. Analysis of Storage Polymers

As a result of the HPLC analysis, glycogen was detected under these growth conditions (Figure 3.21). No storage of PHB was detected according to GC analysis.

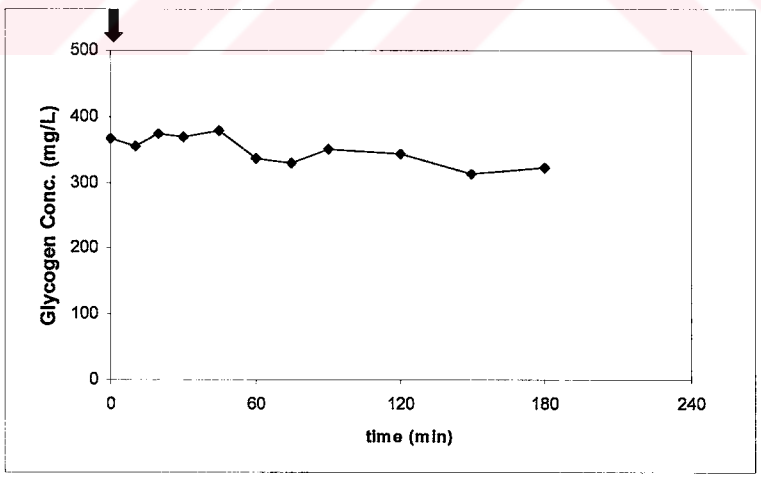


Figure 3.21 Glycogen concentrations in a bioreactor during fed-batch cultivation with 400 mg/L glucose as the sole carbon source. In the figure legend the arrow indicates the time point where the feed (M9 with 400 mg/L glucose) was added.

3.2.5. Sequencing Batch Reactor (SBR) Cultivation

E. coli was cultivated in a bioreactor, which was operated as fill and draw basis. Batch cultivation conditions were applied at the start-up of this system. Four cycles were run in a day where each cycle was 6 h. Feeding and drawing from the culture per day were 300 ml M9 minimal media with 4000 mg/L glucose and 300 ml from the whole culture (cell + medium), respectively. In-line dissolved oxygen (DO) and OD_{600} values were monitored until the system reached to steady-state. When the system reached to steady-state samples were withdrawn in very short intervals as soon as the addition of the feed (75ml M9 with 4000 mg/L glucose as the sole carbon source). In the next cycle of SBR respiration analysis was performed as soon as the addition of the same feed (75ml M9 with 4000 mg/L glucose as the sole carbon source).

3.2.5.1. Determination of Growth Behavior

OD_{600} values were monitored to calculate the CDW contents according to Equation 3.2.2.1.1. OD_{600} values were compared with CDW (Figure 3.22).

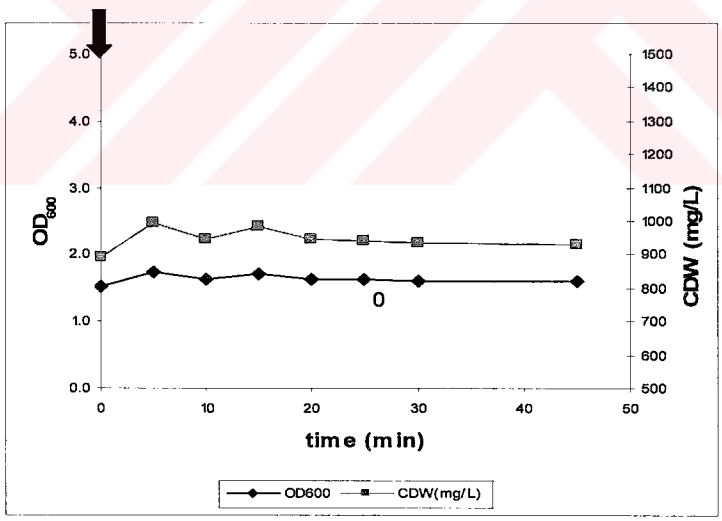


Figure 3.22 Growth behavior of *E. coli* in a bioreactor during SBR cultivation with 4000 mg/L glucose as the sole carbon source. In the figure legend the arrow indicates the time point where the feed (75 ml of 4000 mg/l glucose containing M9 per cycle) was added.

Residual glucose consumption and acetate production were also investigated (Figure 3.23). Acetate formation and consumption were shown with residual glucose consumption on Figure 3.23.

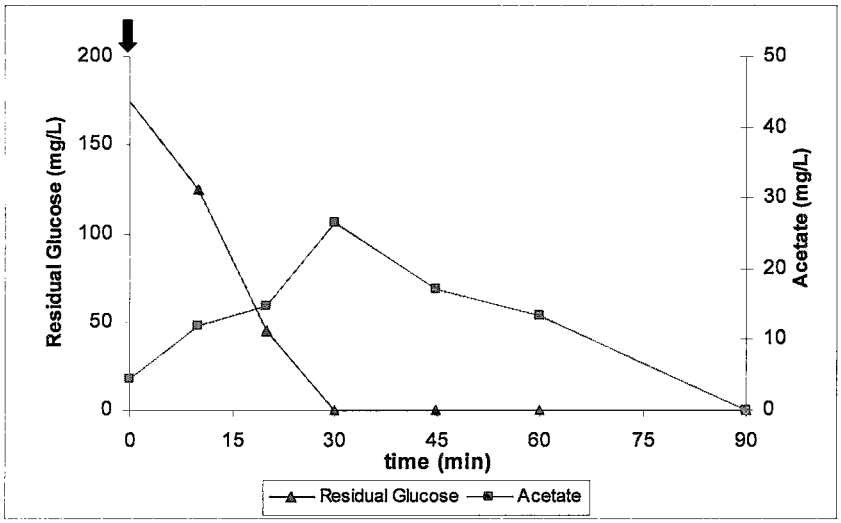


Figure 3.23 Residual glucose and acetate concentrations in a bioreactor during SBR cultivation with 4000 mg/L glucose as the sole carbon source. In the figure legend the arrow indicates the time point where the feed (75 ml of 4000 mg/l glucose containing M9 per cycle) was added.

3.2.5.2. Analysis of Storage Polymers

Glycogen accumulation was observed according to HPLC analyses of the samples withdrawn from the bioreactor. Data were compared with residual glucose values. (Figure 3.24)

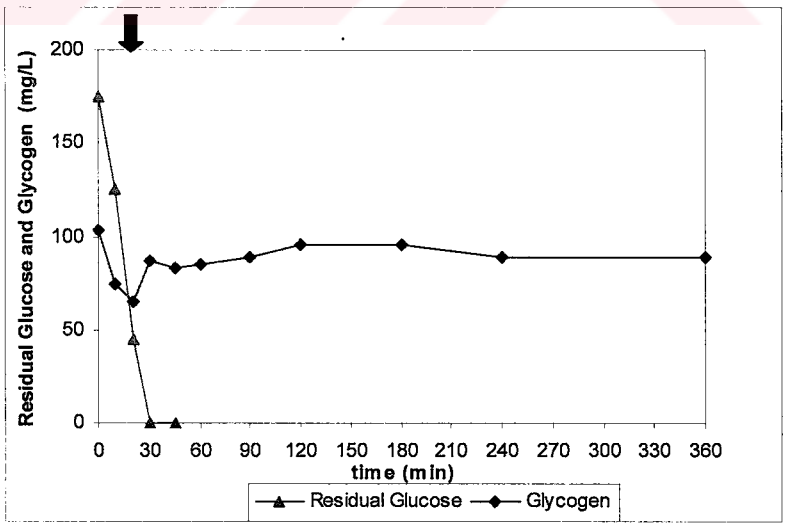


Figure 3.24 Glycogen concentrations in a bioreactor during SBR cultivation with 4000 mg/L glucose as the sole carbon source. In the figure legend the arrow indicates the time point where the feed (75 ml of 4000 mg/l glucose containing M9 per cycle) was added.

3.2.5.3. Respirometric Measurements

In the next cycle of the SBR cultivation, respirogram of the culture was also measured. (Figure 3.25)

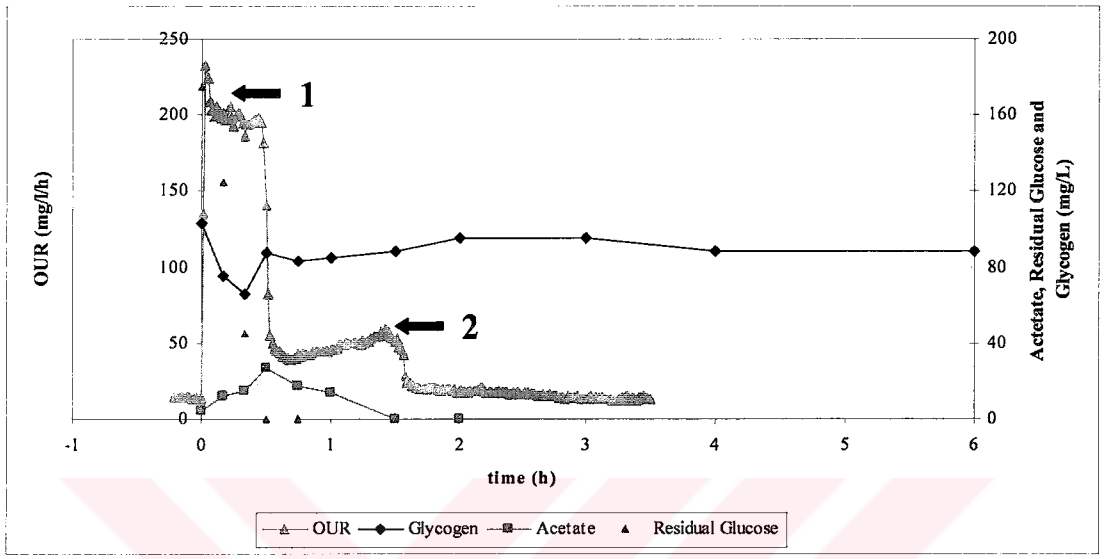


Figure 3.25 Respirogram analysis of SBR cultivation with 4000 mg/L glucose as the sole carbon source. In the figure legend arrow 1 indicates the time point and the oxygen consumption rate where the feed (75 ml of 4000 mg/l glucose containing M9 per cycle) was added. Arrow 2 is predicted to be the time point of the consumption of previously accumulated acetate and glycogen.

DISCUSSIONS AND CONCLUSIONS

In the present study, physiology and the biochemical storage characteristics of *E. coli* was investigated. Batch, fed-batch and sequencing batch cultivations were performed along with respirometric measurements to compare the effects of different cultivation conditions on growth and storage mechanisms. Various concentrations of either glucose or glycerol were used as the sole carbon source of the standard minimal medium (M9) to investigate the effects of different carbon sources on growth and storage. Batch cultivations with M9 without any nitrogen source and with varying amounts of NH_4Cl as the sole nitrogen source were conducted to analyze the effects of nitrogen limitation on the storage and growth behavior of *E. coli*. GC and HPLC systems were used as the analytical methods to determine the expected storage polymers. To characterize the culture behavior and the oxygen uptake rate (OUR), simple and short-time aerobic respirometric batch tests were performed with a steady-state pure culture.

Methods for residual glucose and glycogen analyses in HPLC and volatile fatty acid (VFA) and polyhydroxybutyrate (PHB) analyses in GC have been firstly established for the first time in our laboratory during the course of this study.

In batch and fed-batch cultivations of *E. coli* a significant amount of acetate formation was observed when the sole carbon source was glucose (4 g/L). In literature, it is stated that in aerobic glucose-based cultures of *E. coli* acetate overflow occurs when the specific glucose uptake is greater than the conversion of glucose to biomass and carbon dioxide (Lin *et al.*, 2001). Under conditions of excess glucose or even in glucose-limited cultures, *E. coli* produces acetate as an overflow waste product. Since it has been reported to inhibit growth and protein production, acetate production is often considered to be a problem in *E. coli* processes (Luli and Strohl, 1990; Shimizu *et al.*, 1988).

Polyhydroxyalkanoates were one of the storage polymers under interest in this study. No storage of PHB was observed under any of the cultivation conditions with neither glycerol nor glucose as the sole carbon source. As a biodegradable and biocompatible polymer, polyhydroxybutyrate (PHB) –the best characterized PHA- has found a widespread application especially in biomedical field. However, no PHB accumulation in *E. coli* was observed in any of the cultivations under the conditions investigated. In literature, it is reported that alternative strategies for PHB production have been investigated. In general, PHB production has been studied mostly in recombinant *E. coli* cells harboring PHA synthesizing genes from other microorganisms. Manishi *et al.*, (2003) have studied the effects of different carbon and nitrogen sources on PHB accumulation by recombinant *E. coli* harboring PHA synthesizing genes from *Streptomyces aureofaciens* NRRL 2209. They have concluded that glucose supports PHB accumulation up to only 40% of cell dry weight compared with 60% cell dry weight with glycerol as the carbon source. They have also observed an increasing cumulative effect on PHB accumulation by the recombinant *E. coli* cells with a combination of yeast extract and peptone in the medium as the nitrogen source.

The use of biodegradable plastics such as PHAs, which are the important products of bacterial fermentation, will solve the environmental problems of the plastic disposal. Therefore, production of bacterial PHAs on industrial scale has been conducted since 1990 (Page, 1995). However, widespread acceptance and use of PHA in the marketplace has not been achieved due to its high cost. As a result, new biotechnological approaches with recombinant *E. coli* are in progress to produce cheaper and more effective polymers in bacteria (Van Wegen *et al.*, 2001). Efforts in this direction have lead to inadequate understanding of metabolism during PHB production. In general, it is observed that PHB production in recombinant *E. coli* generally follows a two-phase profile (Wang and Lee, 1997; Wong *et al.*, 1999): a "growth" phase characterized by rapid cellular growth with little PHB production; followed by a "PHB accumulation" phase, exhibiting little cellular growth and rapid PHB production. The environmental and metabolic changes that cause this transition are still unknown.

In our study during fed-batch and SBR cultivations of *E. coli* with glucose as the sole carbon source, glycogen storage and consumption was observed. Studies on glycogen metabolism in aerobic mixed cultures fed with glucose showed that storage and subsequent growth on glycogen occur without a significant loss of energy (Dircks *et al.*, 2001). The metabolic pathway analysis of glycogen storage in pure cultures of activated sludge microorganisms is not yet studied in detail. Despite some recent debates on glycogen accumulation of glycerol-fed *E. coli*, we could not observe glycogen formation with various concentrations of glycerol in batch shake flask and bioreactor cultivations. In batch shake flask experiments performed with different NH_4Cl concentrations as the sole nitrogen source in M9, various levels of glycogen accumulation was observed. Thus, glycogen accumulation in *E. coli* seems to be related with nitrogen starvation conditions. Additionally, in nitrogen starvation cultivations performed with M9 that does not contain any nitrogen source, the growth was inhibited.

During fed-batch cultivation with 400 mg/L glucose as the sole carbon source it is observed that $LnOD_{600}$ results showed that less amount of glucose was rapidly consumed and used only for survival. Acetate and glycogen levels did not decrease while the residual glucose was decreasing.

Studies on SBR systems provide the advantage of identifying the previous behavior of microorganisms up to the steady-state, which we cannot observe with traditional batch cultivations. Therefore, we have also conducted SBR studies on *E. coli* growing on glucose as the carbon source. In general, this system is performed on mixed cultures of activated sludge. This study will be an example for the application of SBR systems on pure cultures.

Since the determination of the steady-state oxygen requirement is a valuable tool to obtain more information on biological processes, we have also measured the oxygen uptake rate of SBR-cultivated *E. coli* in the respirometer. The direct measurement of the oxygen and the substrate consumption rates of the culture gave us an insight into the metabolism of the microorganisms. The OUR results of the steady-state *E. coli* culture also supported our findings of a storage polymer. As the readily biodegradable substrate, glucose, level decreases oxygen utilization rate also decreases. But an increase was

observed in the OUR graph while the glycogen and acetate, which are detected by HPLC and GC measurements, was utilized by the culture. Thus, we will be studying the storage mechanisms of activated sludge organisms individually. Additionally, modeling approaches of activated sludge systems will be applied on pure cultures with the slight modifications that were gained through this study.

Although *E. coli* has been a well known and a well characterized microorganism, its role and importance in activated sludge systems from the storage point of view is not yet identified. Individual studies on the characterization of physiology and biochemical storage properties of *E. coli* will help us answer the questions related to the storage metabolism of activated sludge microorganisms. The future works of this study will be the examination of growth and storage profiles of *E. coli* with various substrates such as starch and casein and cultivation with different feeding regimes. Recombinants will help us to understand the kinetics and metabolic pathways of different storage polymers. Studies on genes regulating the synthesis and enzymes will show the reasons of metabolic shift from growth to storage.

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