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ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

PHYSIOLOGY AND BIOCHEMICAL STORAGE
CHARACTERISTICS OF
MICROLUNATUS PHOSPHOVORUS

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MICROLUNATUS PHOSPHOVORUS'UN FİZYOLOJİSİ VE
BİYOKİMYASAL DEPOLAMA ÖZELLİKLERİ

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ABBREVIATIONS

ADP : Adenosine Diphosphate
AMP : Adenosine Monophosphate
ATP : Adenosine Triphosphate
CDW : Cellular Dry Weight
EBPR : Enhanced Biological Phosphorus Removal
ETS : Electron Transport System
GAOs : Glycogen Accumulating Organisms
GC : Gas Chromatography
HPLC : High Performance Liquid Chromatography
NADH : Nicotinamide Adenine Dinucleotide
PAB : Polyphosphate Accumulating Bacterium
PHA : Polyhydroxyalkanoate
PHAs : Polyhydroxyalkanoates
PHB : Polyhydroxybutyrate
PHV : Polyhydroxyvalerate
P$_i$: Ortophosphate
Poly-P : Polyphosphate
PPAT : Polyphosphate AMP phosphotransferase
PPK : Polyphosphate kinase
PPX : Exopolyphosphatase
RID : Refractive Index Detector
RNA : Ribonucleic Acid
RPM : Revolutions Per Minute

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SUMMARY

Microlunatus phosphovorus is a microorganism found in activated sludge systems. It belongs to the group of polyphosphate-accumulating organisms. Under anaerobic conditions, these organisms utilize the energy released by the hydrolysis of polyphosphate, for substrate uptake and conversion to internal carbon sources. Under aerobic conditions, however, the previously stored polyhydroxyalkanoate (PHA) is utilized for growth. Due to these properties, *M. phosphovorus* is a member of wastewater treatment plants during biological phosphorus removal.

In this study, the growth physiology and biochemical storage metabolism of *M. phosphovorus* was investigated. Pure culture studies in batch growth systems were conducted using chemically defined media. PHB, polyphosphate and glycogen staining was applied to microscopy samples to detect the presence of these metabolites. PHB and glycogen biopolymers were also detected by GC and HPLC, respectively. Other key metabolites like total protein, acetate were determined during different phases of growth in addition to cell dry weight and optical density measurements.

It was observed that *M.phosphovorus* already accumulates intracellular PHB (polyhydroxybutyrate) during fully aerobic bioreactor conditions with glucose as the sole C-source. A batch growth system with anaerobic-aerobic cycles was also employed to examine the effect of this different growth condition on PHB storage and metabolism.

To summarize, it was observed that *M.phosphovorus* stores high amounts of PHB under various batch growth conditions and without a significant limitation. Glucose seems to be a suitable C-source for PHB production of *M.phosphovorus*, although our preliminary experiment with acetate as the sole C-source resulted in PHB accumulation.

Keywords: PHB storage, *Microlunatus phosphovorus*, batch growth systems, M9

ÖZET

Microlunatus phosphovorus aktif çamur sistemlerinde bulunan bir mikroorganizmadır. Polifosfat depolayan organizmalar grubuna aittir. Bu organizmalar, anaerobik koşullar altında substrat alımı ve bu substratın içsel karbon kaynağına dönüşümü için polifosfat hidrolizinden ortaya çıkardığı enerjiyi kullanır. Aerobik ortama geçince ise karbon kaynağı yokluğunda anaerobik koşullarda depoladığı polihidroksialkonatı (PHA) bölünmek için kullanır. Bu özelliklere bağlı olarak *M. phosphovorus* atık su arıtım tesislerinde fosfor giderimi amacıyla kullanılan mikroorganizmalardandır.

Bu çalışmada *M. phosphovorus*'un büyüme fizyolojisi ve biyokimyasal depolama metabolizması araştırılmıştır. Kapalı sistemlerde yapılan saf kültür çalışmaları kimyasal içeriği tanımlanmış besiyeriyle birleştirilmiştir. Deneyler esnasında mikroskopta incelemek üzere alınan PHB, polifosfat ve glikojen numunelerine bu depolama metabolitlerinin varlığını saptamak üzere boyama yöntemleri uygulanmıştır. PHB ve glikojen polimerlerinin varlığına sırasıyla GC ve HPLC cihazlarıyla da bakılmıştır. Optik yoğunluk ve kuru hücre ağırlığı ölçümlerine ek olarak büyümenin her safhasında toplam protein ve asetat gibi kilit metabolitlerin varlığı da araştırılmıştır.

M.phosphovorus'un aerobik koşullarda ve tek karbon kaynağı olarak glikoz kullanılan bioreaktörde bile hücre içi polihidroksibütirat (PHB) biriktirdiği saptanmıştır. Anaerobik-aerobik döngüler şeklindeki deney düzeneğinde ise farklı büyüme koşullarının *M.phosphovorus*'un PHB depolaması ve metabolizması üzerine etkileri araştırılmıştır.

Özetle, değişik kapalı sistem şartlarında ve çok önemli bir limitasyona gerek olmaksızın *M.phosphovorus*'un yüksek miktarda PHB biriktirdiği gözlemlenmiştir. Tek karbon kaynağı olarak asetatın kullanıldığı çalışmalarımızda PHB birikimine rastlansa da, *M.phosphovorus*'un PHB üretmesi için en uygun karbon kaynağının glikoz olduğu düşünülmektedir.

Anahtar Sözcükler: PHB depolama, *Microlunatus phosphovorus*, Kapalı büyüme sistemleri, minimal besiyeri M9

1. INTRODUCTION

1.1. The Phosphate-Accumulating Bacterium *Microlunatus phosphovorus*: a Brief Introduction

Phosphorus contamination in wastewater causes eutrophication, a worldwide water pollution problem (Torien *et al.*, 1990). Alga (e.g. Cyanobacteria) proliferation leading to the death of other organisms in surface waters cannot be stopped simply by minimizing the amount of phosphate (and nitrate) which is discharged into wastewater by industrial and agriculture activities. Therefore, it is essential to remove phosphorus efficiently from wastewater. (Santos *et al.*, 1999)

Activated sludge processes with alternating anaerobic and aerobic conditions have been widely used for elimination of excess phosphorus from sewage and wastewater. Phosphorus removal in these processes is assumed to be achieved by the dominant growth of polyphosphate-accumulating bacteria in activated sludge.

It is confirmed by many researchers that *Microlunatus phosphovorus* is a polyphosphate-accumulating bacterium (PAB). PAB exhibit high phosphate removal activity under various environmental stress conditions. Thus, they were studied taxonomically and phylogenetically in order to identify their specific characteristics (Nakamura et al., 1995).

Phylogenetic analysis based on 16S rDNA sequence data is currently one of the most effective methods for the delineation of bacteria. The suborder *Propionibacterineae* (Stackebrandt et al.,1997) of the order *Actinomycetales* (Stackebrandt et al.,1997) encompasses the type family *Propionibacteriaceae* (Stackebrandt et al.,1997). The family *Propionibacteriaceae* contains the *Luteococcus* (Tamura *et al.*, 1994) , *Microlunatus* (Nakamura *et al.*, 1995), *Propioniferax* (Yokota *et al.*, 1994) and *Propionibacterium* (Cummins *et al.*, 1986).

A phylogenetic analysis based on 16S rRNA sequences showed that this isolate differ from other genera belonging to the high G+C-content gram-positive group. Accordingly, it is concluded that the strain NM-1T (T= type strain) should be

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assigned to a new genus and species, for which the name is proposed as *Microlunatus phosphovorus* (Nakamura et al., 1995).

1.2. General Characteristics of *Microlunatus phosphovorus*

These organisms are gram- positive, coccus-shaped *Actinobacteria*. Gram-positive bacteria contain peptidoglycan as the major component of the cell wall (Fig.1.2). In many of these microorganisms it accounts for 80 to 90% of the cell wall components (Gottschalk 1986; Nakamura *et al*. 1995). They are also aerobic chemoorganotrophs that have a strictly respiratory type of metabolism in which oxygen is a terminal electron acceptor (Nakamura et al., 1995). They accumulate large amounts of polyphosphate under aerobic conditions and degrade polyphosphate (poly-P) under anaerobic conditions along with an uptake of glucose.
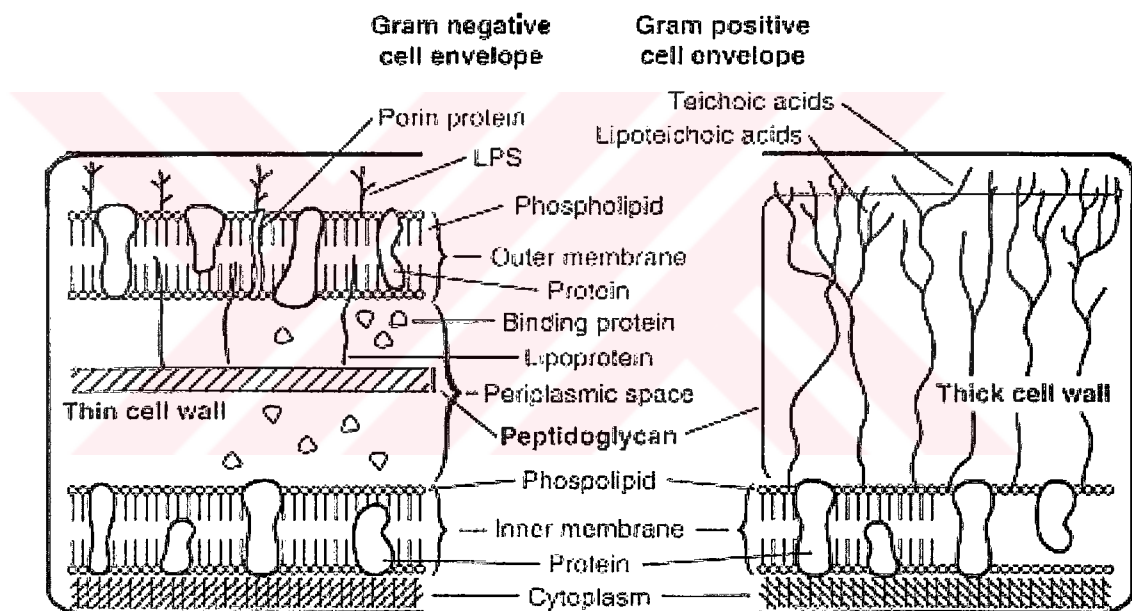
Fig.1.2. Comparison of gram(+) and gram (-) cell wall structure. (Metzenberg, 2002)

The most interesting study has been performed on gram (+) cocci obtained from activated sludge. One isolate tentatively assigned to "Micrococcus" was capable of accumulating 16% of the dry cell mass as phosphorus (Nakamura *et al.*, 1991).

Microlunatus phosphovorus is unable to accumulate acetate and propionate in the form of polyhydroxyalkanoate (PHA) (Mino, 2000). Instead, the organism anaerobically sequesters glucose. This indicates that *M. phosphovorus* is different from the much more common polyP-accumulating organisms in enhanced biological phosphorus removal (EBPR) plants that are able to accumulate PHA from acetate

and propionate. On the other hand, *Microlunatus phosphovorus* may well be involved in EBPR in plants receiving glucose-rich wastewater.

To yield an effective process of phosphorus uptake, the microorganisms should possess enzyme systems providing accumulation and regulation of polyphosphate content (Lichko *et al.*, 2002). Poly-P is consumed as the energy source by the help of the enzyme Poly-P: AMP phosphotransferase. Poly-P kinase can combine with adenylate kinase to catalyze the formation of ADP from AMP and poly (Pi). This polyP:AMP phosphotransferase activity requires both enzymes acting together.

Poly-P kinase catalyzes the synthesis of poly(P_i) through the transfer of a phosphoryl group of ATP to a poly(P_i) polymer. The reaction is readily reversible. Poly-P kinase is most active with poly(P_i) substrates of chain lengths greater than 132 phosphoryl units. Activity decreases with decreasing chain length. In the reverse reaction ATP is synthesized from poly(P_i) (Kuroda *et al.*, 1999).

1.3. Biotechnological Importance of *Microlunatus phosphovorus* as a Member of EBPR in Activated Sludge Systems

In order to control eutrophication, phosphate removal from wastewater is often required before wastewater is discharged to the receiving water bodies (seas, lakes...). Activated sludge processes with alternating anaerobic and aerobic conditions have been successfully used for EBPR from wastewater (Fuhs *et al.*, 1975; Kulaev *et al.*, 1979). This anaerobic-aerobic alternation can be achieved either by spatial configuration of anaerobic and aerobic zones in series in continuous flow systems with sludge recycle or by temporal arrangement of anaerobic and aerobic periods in sequence batch reactors. It has been shown in investigations (Fuhs *et al.*, 1975, Mino *et al.*, 1985) that PAB play an essential role for EBPR in the anaerobic-aerobic process. To achieve high and stable EBPR performance, it is essential to maintain PAB in the system (Mino *et al.*, 2000).

Acinetobacter spp., *Microlunatus phosphovorus*, *Lampropedia* spp., and the *Rhodocyclus* group have been reported as candidates of PAB. PAB may not be composed of a few limited generas, but involve phylogenetically and taxonomically diverse groups of bacteria. To define microbial community structure of EBPR processes, it is needed to look more closely into the occurrence and behavior of each species of PAB in EBPR process. (Mino, 2000)

In the case of understanding the structure and function of the microbial communities in these activated sludge systems, pure culture studies should be studied for each genera by providing the same environmental conditions as in the activated sludge systems. Equally important is the need to understand how the biochemistry of EBPR is regulated. For example, what is known about the enzymes involved in the synthesis and degradation of poly-P, PHA and glycogen in PAB, and what affects their synthesis and levels of activity? It is known very little, and interpretation of what is known is much more complicated.

In EBPR mechanism, it is assumed that all PAB behave identically, which seems increasingly unlikely, bearing in mind the evidence that PAB populations are probably phylogenetically diverse (Seviour *et al.*, 2003).

It is accepted that PAO take up short-chain fatty acids and store them as polyhydroxyalkanoates (PHAs) (Santos *et. al.*, 1999). PHA is another important storage biopolymer for the microorganisms involved in EBPR process. PHAs are good candidates for completely biodegradable polymers (they are alternative to petroleum-based polymers like plastics). Due to this property, they are biotechnologically important polymers, but the high production cost of PHAs is a big disadvantage for their commercial application. Therefore, much effort has been devoted to reduce the production cost of PHAs by developing more efficient processes for the production and recovery of PHA (Han-Qing Yu, 2000).

1.4. Metabolic Pathways in EBPR Process

The process has been investigated by many research groups since the first description of the EBPR in the 1960s (Van Loosdrecht *et al.*, 1997). Today the process is well established in sewage treatment plants, although a fundamental understanding of microbiological and biochemical perspectives is still lacking. As a common feature, all EBPR processes share an operational feature in which the biomass is cycled through alternating anaerobic/aerobic regimes. These conditions are considered necessary to provide polyphosphate-accumulating bacteria with a selective advantage based on their ability to assimilate substrates anaerobically into intracellular storage compounds that can then be used as energy sources under aerobic conditions (Mino *et al.*, 1998).

In the EBPR process, microorganisms are exposed to alternate carbon-rich anaerobic environments and carbon-poor aerobic environments. By this alternation, synthesis and degradation of three kinds of biopolymers (poly-P, PHA, and

glycogen) are induced and metabolic cycling through these biopolymers is established in microorganisms. Such metabolic cycling is energy consuming and not favorable for microorganisms in terms of energy utilization efficiency. Ecologically, however, this metabolic cycling enables PAB to win in the microbial selection in the anaerobic-aerobic process (Mino *et al.*, 1998). To explain how this metabolic cycling is regulated in the cell, a metabolic flux model was developed by Pramanik et al.,1999. This model contains a complete set of metabolic pathways involved in biosynthesis and energy production and accounts for energy requirements for macromolecule synthesis and metabolite transport across the cell membrane. The model not only supports the hypothesis that the biopolymer metabolism provides a means for PAB to balance intracellular energy supplies, but also suggests pathways at which metabolic regulation should occur (Pramanik *et al.*, 1999).

In the basic configuration of an EBPR activated-sludge plant, the influent wastewater flows into an anaerobic zone where it is mixed with the returned microbial biomass from the clarifier to form the so-called mixed liquor. This mixed liquor then flows into an aerobic zone, after which the biomass is separated from the treated wastewater in the clarifier. In the aerobic zones, excessive phosphate accumulation occurs. Removal of a portion of the growing biomass (waste-activated sludge) results in the net removal of P from the wastewater (Fig. 1.4.a).
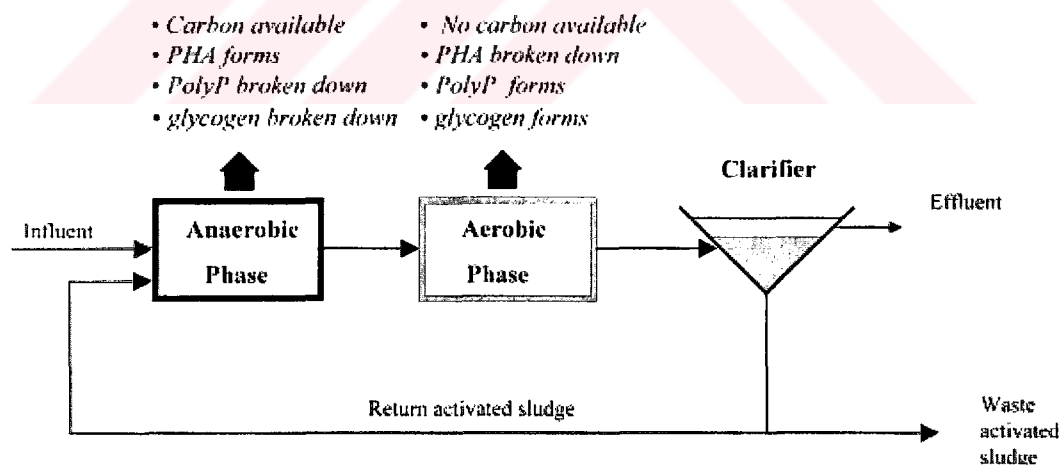
Fig. 1.4.a. Basic concept of anaerobic-aerobic process for EBPR (Pramanik *et al.*, 1999).

In the anaerobic zone, short-chain fatty acids (volatile fatty acids, VFAs) such as acetate are consumed and polymerized into PHAs, orthophosphate (P_i) generated from poly-P depolymerization is secreted into the medium, and glycogen is

degraded. In the subsequent aerobic stage, PHAs are degraded, glycogen is synthesized, and P$_i$ is taken up and polymerized into poly-P (Fig.1.4.b).
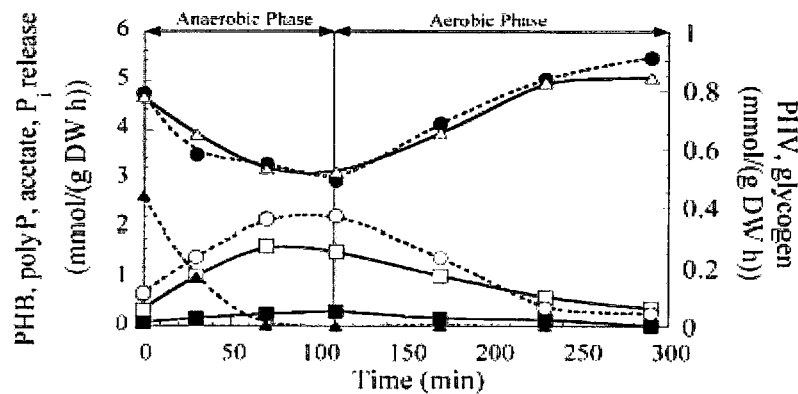
Fig.1.4.b. Experimental data for the EBPR culture over one complete anaerobic/aerobic cycle. Open squares, PHB; filled squares, PHV; open triangles, polyP (calculated from Pi release data); filled triangles, acetate; open circles, Pi released; filled circles, glycogen (Pramanik et al., 1999).

Several models have been developed to capture the dynamics of biopolymer synthesis and degradation during EBPR. Significant amounts of the energy-rich molecules NADH and ATP are generated or consumed in the production of biomass and the metabolism of glycogen, these pathways can significantly impact the production and consumption of PHAs and poly-P, which themselves require investments in NADH and ATP, respectively. A full understanding of the pathways used in EBPR metabolism will help elucidate the mechanisms involved. Therefore, the balances of redox, energy, and precursors in cells performing EBPR were investigated (Fig.1.4.c.).
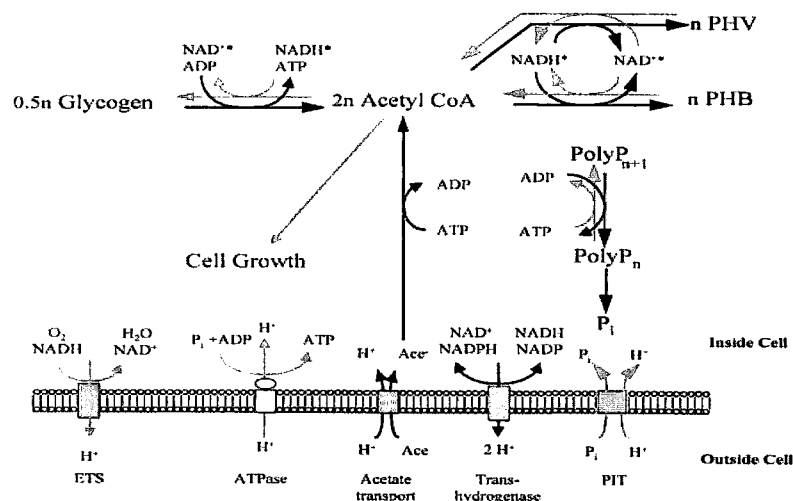
Fig.1.4.c. Biochemical pathway modeling of an EBPR organism (Pramanik et al., 1999).

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Even though the phenomenon is quite general, type and extent of transient response depend on the microorganisms and on the previous growth conditions (the so-called "history", usually different dilution rates or starvation).

However, the type of such dependence is quite different for different microorganisms, thus showing that there is not a single pattern that links transient response to previous growth condition. Moreover, this indicates that the previous history of the biomass can be the key factor in the kinetic selection of different microorganisms in mixed cultures (Tomei, 1999).

1.5. Intracellular Storage Compounds of Activated Sludge Organisms

The major PAB storage polymers are PHA, glycogen and poly-P (Satoh *et al.*, 1992; Pereira *et al.*, 1996). According to investigations achieved by researchers, poly-P accumulating bacteria, such as *M. phosphovorus*, store trehalose, poly-P and glycogen, but not PHA (Santos *et al.*, 1999).

1.5.1. Poly-P Storage

Poly-P is a polymer consisting of condensed inorganic phosphates with the tetrahadral phosphate groups linked together by oxygen bridges (Dawes *et al.*, 1992). In biological systems, poly-P is a polyanion stabilized by metal cations or basic proteins and the degree of polymerization varies within and between microorganisms (Kulaev *et al.*, 1979). Such variations in the polymer length have also been detected in activated sludge samples (Seviour *et al.*, 1999).

Polyphosphate (poly-P) accumulation is widespread among bacteria. It functions as a phosphorus storage material and is utilized for nucleic acid and phospholipid synthesis under conditions of phosphorus starvation or by limitation of some other nutrient. These events effect 'overplus' or 'luxury' metabolism, respectively (Harold *et al.*, 1966). Microbiologists have investigated sludge and isolates for the presence of poly-P metabolizing enzymes in attempts to better describe the EBPR metabolic picture.

Polyphosphate kinase (PPK) activity has been observed in bacteria and eukarya (Kulaev *et al.*, 1979). Poly-P is a high-energy compound and it is assumed to form in conditions when energy is in excess. Its synthesis has been linked to the ATP-utilizing enzyme polyphosphate kinase. PPK catalyses the following reaction:

$$ATP + (poly\text{-}P)_n \rightleftharpoons ADP + (poly\text{-}P)_{n+1}$$
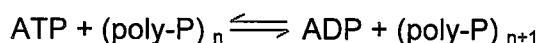
PPK may act to catalyse the above reaction towards the left. Thus, poly-P functions as an energy reserve polymer, and poly-P degradation produces ATP (Kulaev *et al.*, 1979; Dawes *et al.*, 1973; Kornberg *et al.*, 1957).

Polyphosphate glucokinase catalyzes the phosphorylation of glucose:

$$(\text{poly-P})_n + \text{glucose} \rightarrow (\text{poly-P})_{n-1} + \text{glucose-6-phosphate}$$
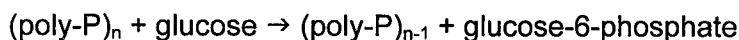
In this reaction the high-energy bonds of the polymer are transferred without the involvement of adenylates. This activity would therefore conserve the energy stored in poly-P (Seviour *et al.*, 1999).

Polyphosphate AMP phosphotransferase (PPAT) catalyses the reversible reaction:

$$(\text{poly-P})_n + \text{AMP} \rightleftharpoons \text{ADP} + (\text{poly-P})_{n-1}$$
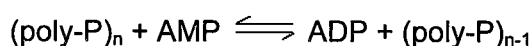
It is suggested that the degradation of poly-P may then be linked to the production of ATP in combination with the reaction catalyzed by adenylate kinase.

$$2\text{ADP} \rightarrow \text{ATP} + \text{AMP}$$

Polyphosphatases can hydrolyze phosphoanhydride bonds in polyphosphates, without direct production of ATP or a source of phosphorylation. These enzymes include exo- and endopolyphosphatase (Wood *et al.*, 1988). Bacterial exopolyphosphatase (PPX) is a major enzyme involved in poly-P degradation. The general reaction is:

$$(\text{poly-P})_n + \text{H}_2\text{O} \rightarrow (\text{poly-P})_{n-1} + \text{phosphate}$$
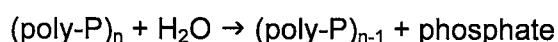
Physiological functions that poly-P hydrolysis by PPX may include the regulation of intracellular phosphate levels.

Localization of poly-P in activated sludge cells has been observed and suggested to be in: (i) volutin granules in cytoplasm; (ii) the cell membranes, complexed with cytoplasmic RNA; and (iii) the periplasm (Seviour *et al.*, 1999). Poly-P is also known as "volutin granules" because they were first found in *Spirillum volutans*.

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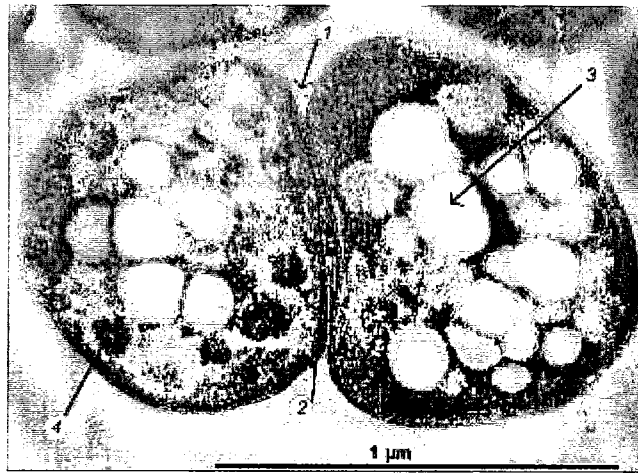
Fig.1.5.1. Transmission electron micrograph of EBPR organisms. Arrows indicate: septa (1), exopolymeric substance (2), PHB granule (3), and dark polyphosphate granule (4) (Kortstee *et al.*, 2000).

Currently accepted EBPR models assume that P release and PHA accumulation are metabolically linked and that part of the energy to anaerobically convert carbon substrate into PHA comes from intracellular poly-P breakdown (Satoh *et al.*, 1992).

1.5.2. Glycogen Metabolism

Glycogen is a carbohydrate store in mammalian cells and some species of bacteria. It is a branched polymer of glucose, synthesized mainly by muscle and liver (Wilson, 2002). Bacterial glycogen accumulation is seen in many bacterial species (Lemos *et al.*, 1998). It occurs when carbon is in excess and growth is limited by the supply of nitrogen or some other nutrient (Stryer, 1995).

As well as the intracellular storage of the reserve molecules poly-P and PHA, it seems that metabolism of a glycogen-like reserve polymer is also important to EBPR. A glucose-based storage molecule is observed in EBPR system with metabolic transformations opposite to those involving PHA. This glycogen-like molecule is accumulated in the aerobic period (Fig.1.5.2.a) and degraded in the anaerobic stage (Satoh *et al.*, 1992; Kortstee *et al.*, 2000 and Satoh *et al.*, 1996).

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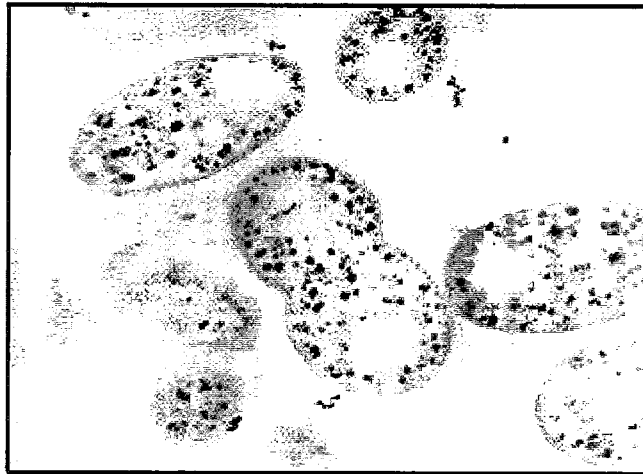
Fig.1.5.2.a. Electron micrograph of EBPR sludge at the end of the aerobic period (Smolders *et al.*, 1994). The stained dark spots are glycogen granules.

Glycogen, a readily mobilized fuel which is stored in the cytoplasm in the form of hydrated granules, is a branched polymer of glucose residues (Fig.1.5.2.b). Most of the glucose units in glycogen are linked by α-1,4 glycosidic bonds. Branching occurs after a number of glucosyl residues are joined in α-1,4 linkage by glycogen synthase. Glycogen synthase catalyzes only the synthesis of α-1,4 linkages. Another enzyme forms the α-1,6 linkages that make glycogen a branched polymer.

Branching is important because it increases the solubility of glycogen. Furthermore, branching creates a large number of terminal residues, the sites of action of glycogen synthase and phosphorylase. Thus, branching increases the rate of glycogen synthesis and degradation (Stryer, 1995).
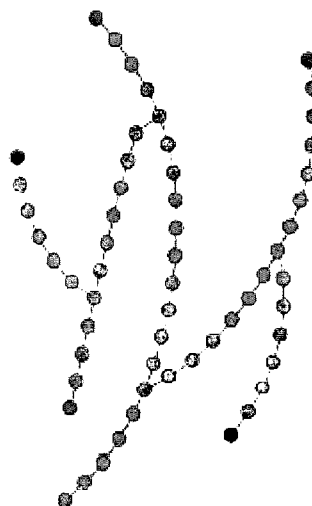
Fig.1.5.2.b. Chemical structure of glycogen molecule. The nonreducing ends are shown in red. The residue that starts a branch is shown in green (Miles, 2003).

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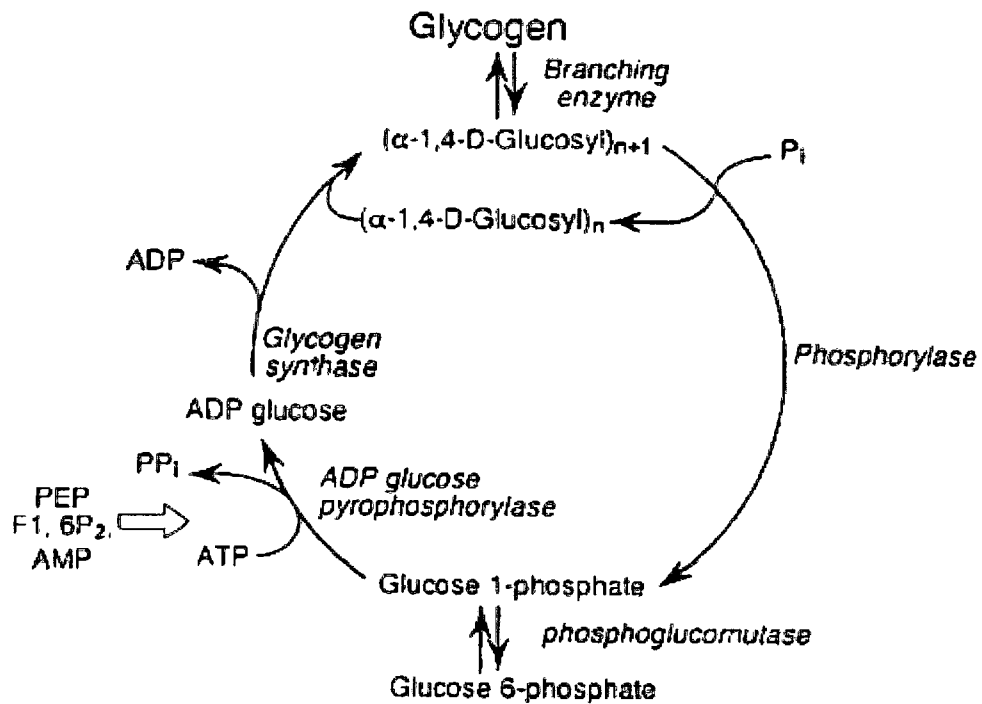
Fig.1.5.2.c. Scheme for glycogen synthesis and degradation in bacteria (Seviour *et al.*, 1999).

During glycogen synthesis, ATP and glycosyl units are required to form ADP-glucose, a reaction which is mediated by ADP-glucose pyrophosphorylase. The glucosyl units are then added to the glycogen chain. In the degradation of glycogen, the enzyme glycogen phosphorylase utilizes the glucosyl chain and the phosphate to produce glucose-1-phosphate. This product may then be converted to glucose-6-phosphate, which may then enter catabolic pathways like glycolysis for ATP generation (Fig.1.5.2.c).

There have been recent reports of bacteria inhibiting EBPR in laboratory-scale activated-sludge systems designed for P removal. The microbial transformations in these systems were investigated, and a biochemical model describing the bacterial inhibition of EBPR was proposed (Satoh *et al.*, 1994). Microorganisms in these systems in which deterioration of P removal is evident have been labeled glycogen-accumulating non-polyphosphate organisms, or GAOs. As with PAOs, there is little known about the ecological details of GAOs and how they affect EBPR. For example, if GAOs compete with PAOs, their presence could partially explain why optimal performance is not always attained in full-scale EBPR systems. However, there is also the possibility that the PAO and the GAO are the same organism. In that case, variable P removal could result from an alteration in the phosphate-accumulating capabilities of that particular bacterium. If more were known about

PAOs and GAOs, the development of strategies to improve the P removal performance of a system would be more focused (Bond *et al.*, 1999).

1.5.3. Polyhydroxyalkanoate Storage

Polyhydroxyalkanoates (PHAs) are intracellular energy and carbon storage material synthesized by numerous microorganisms. Bacteria that are used for the production of PHAs can be divided into two groups based on the culture conditions required for PHA synthesis. The first group which includes *Alcaligenes eutrophus*, methylotrophs and Pseudomonads requires the limitation of an essential nutritional element in the presence of an excess carbon source for efficient synthesis of PHA. The second group which includes *Alcaligenes latus*, *Azotobacter vinelandii* and recombinant *Escherichia coli*, does not require nutrient limitation for PHA synthesis and can accumulate PHA during growth (Han-Qing Yu, 2000).

$$HO - CH - H_2C - C - \left[-O - CH - CH_2 - C - \right]_n -O - CH - CH_2 - COOH$$

with the substituents:

$$\underset{CH_3}{|} \quad \underset{O}{\|} \quad \underset{CH_3}{|} \quad \underset{O}{\|} \quad \underset{CH_3}{|}$$
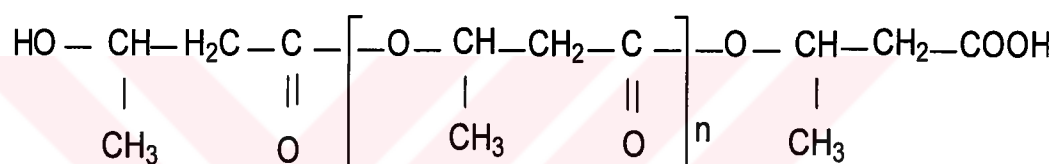
Fig.1.5.3.a. The structure of PHB polymer (Gottschalk, 1986).

PHAs exist in the cell cytoplasm as 0.2 to 0.5 μm granules surrounded by a membrane (Sudesh *et al.*, 2000). Polyhydroxybutyrate (PHB), a lipid-like polymer of 3-hydroxybutyrate, is the most common PHA stored by bacteria, but polymers of other hydroxyacids can also be found (Madigan *et al.*, 2000). In PAOs the stored PHA is typically a copolymer consisting of mostly PHB and polyhydroxyvalerate (PHV) units with smaller amounts 3- hydroxy-2-methylbutyrate (3H-2-MB) and 3-hydroxy- 2-methylvalerate (3H-2-MV) (Liu *et al.*, 1996). The polymer composition depends on the nature and concentration of the carbon source assimilated by the organism (Fig.1.5.3.b). In a research it was discovered that propionate yields essentially a polymer of 3-hydroxyvalerate (HB/HV molar ratio = 0.39); with butyrate small quantities of both are formed (HB/HV= 1.31) while with acetate, 3-hydroxybutyrate is mainly produced (HB/HV = 3.04) (Lemos *et al.*, 1998).

Based on ^{13}C-NMR results, when the Entner-Doudoroff pathway was used for glycolysis, which yields 2 ATP per glycogen converted to pyruvate. Pyruvate may lead to acetyl-CoA (via the pyruvate dehydrogenase reaction) or to propionyl-CoA (via the modified succinate-propionate pathway). Therefore, some glycogen may be

transformed to PHB and some to PHV. It was assumed that all PHV formed originates from glycogen. The glycogen transformed into PHB did not supply sufficient reducing equivalents for the PHB formation from acetate. Therefore it was assumed in accordance to Pereira et al. (Pereira *et al.*, 1996) that some glycogen was oxidized to CO_2 via the TCA cycle to supply [H^+] (Fig.1.5.3.b.).

Glycogen

Entner–Doudoroff pathway

Pyruvate — 2H / CO_2 — Acetate — Acetyl-CoA

Oxaloacetate — Citrate

2H — Malate — 2H / CO_2 — 2-Oxoglutarate

Fumarate — 2H / CO_2

2H — ATP

ADP

Succinate — ADP — Succinyl-CoA

Methylmalonyl-CoA

CO_2

Propionyl-CoA

2H — PHV — 2H — PHB
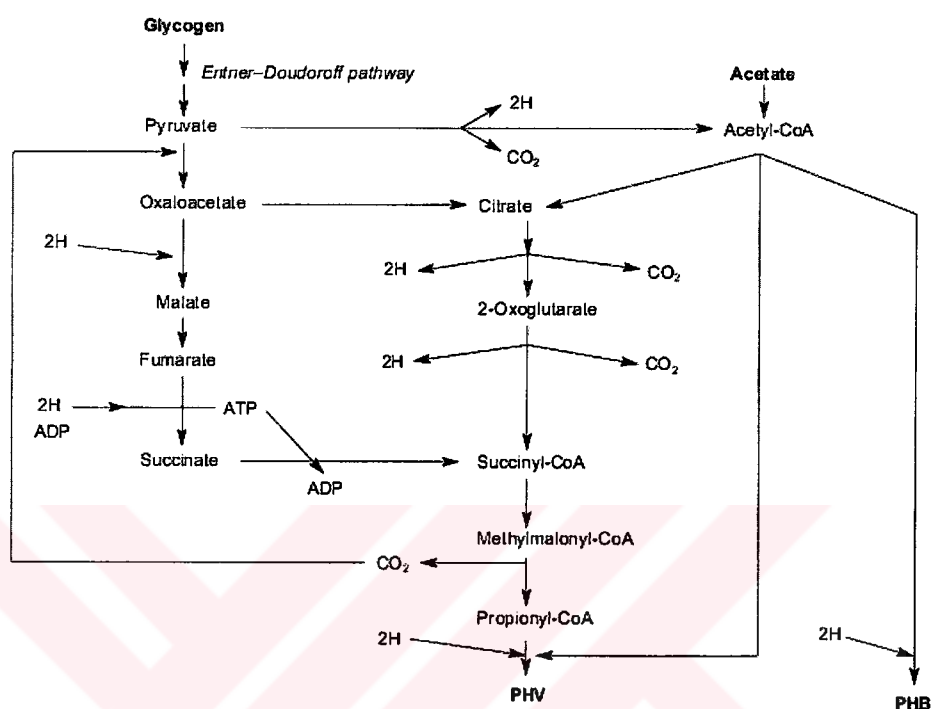
Fig.1.5.3.b. Pathways of PHB and PHV synthesis during anaerobic assimilation of different carbon sources (Hesselmann *et al.*, 2000).

During PHB synthesis, two acetyl-CoA molecules derived from substrates like glucose condense to produce acetoacetyl-CoA, which is then reduced to hydroxybutyryl-CoA. This then undergoes polymerization to PHB. The enzymes involved in the synthesis are 3-ketothiolase, which is regulated, acetocetyl-CoA reductase and PHB synthetase, respectively. Variations of this PHB synthesis and degradation pathway can be observed for other bacteria species. Oxygen limitation is an initiating factor in PHB formation. In these conditions, NADH accumulates, inhibition of TCA cycle enzymes occur and acetyl-CoA accumulates. These events stimulate enzymes involved in PHB synthesis. When NADH levels are lowered by the presence of oxygen, the synthesis of PHB is inhibited and enzymes in the TCA cycle are activated (Fig.1.5.3.c).
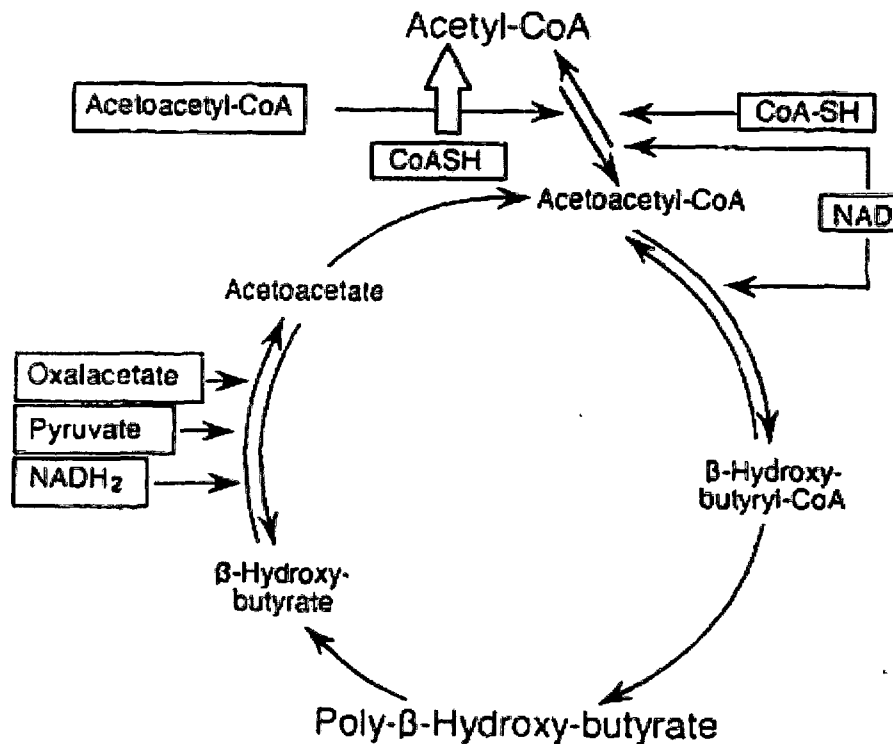
Fig.1.5.3.c. Cyclic scheme for the metabolism of PHB in *Alcaligenes eutrophus* (Seviour *et al.*, 1996).

PHA biosynthesis requires a source of reducing power without direct involvement of ATP, and occurs in bacteria during unbalanced aerobic growth. In EBPR biomass, PHA synthesis occurs in anaerobic conditions, where it is thought that PHA serves as electron sink. According to Comeau model, the source of reducing power is suggested to be the TCA cycle (Comeau *et al.*, 1986).

1.6. Role of Activated Sludge System in Wastewater Treatment Plants

Wastewater treatment plants worldwide are required to more efficiently remove nutrients such as phosphorus (P). The two main P removal approaches are chemical precipitation and biological accumulation of phosphate. Knowledge of the biological process known as EBPR has advanced over the last 20 years. Full-scale activated-sludge plants now operate for efficient P removal without the use of chemical precipitation (Barnard *et al.*, 1974; Barnard *et al.*, 1976; Davelaar *et al.*, 1978; Toerien *et al.*, 1990).

Wastewater coming into the plant undergoes a series of treatments, including the following:

- Preliminary treatment: large sticks, rocks, and rags are removed

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- Primary treatment: skimming and settling that removes about 60 percent of the solids and pollutants

- Secondary treatment: biological treatment that consumes and removes more than 90 percent of the pollutants

- Disinfection, then release through an outfall to receiving body (Gray, 2003).

Wastewater Treatment Plants employ an activated sludge treatment process. This activated sludge or biomass is a mixture of waste and microbiological cultures. Billions upon billions of bacteria consume the organic material converting it into biosolids. These bacteria or biomass is closely monitored to keep the bacteria in perfect balance to perform the aeration process effectively and efficiently. There are many types of activated sludge treatment in the wastewater industry (i.e. single-stage nitrification. Not only is organic material converted, but also ammonia nitrogen is removed.)

The highly mixed wastewater and biomass flows into the Secondary Clarifiers, where biomass in separated from the water. The biomass that settles to the bottom is returned to the aeration tanks to begin the aeration process all over again. The treated water then flows to the disinfection process. The average removal rate of our Aeration Process and Secondary Clarifiers is around 93% to 95% of the incoming flow.

Treated water, although clean, may still contain pathogenic organisms, which could cause disease in the receiving stream. These pathogenic organisms are commonly referred to as *Escherichia coli*. Because of this, every gallon of effluent is disinfected with ultraviolet light. Ultraviolet disinfection is much safer to the environment, and takes place by the water passing over 576 UV-bulbs, where the water is completely disinfected within seconds. After disinfection, the water is discharged into the receiving stream (Gray, 2003).

1.7. Aim of the Present Research

In order to obtain more effective EBPR process, biochemical pathways of each organism that is involved in the system should be understood clearly. At this point, pure culture studies are required in terms of manipulating the key factors that play important roles during the synthesis and degradation of biopolymers. When we shift to larger scale with mixed culture, it will be easier to direct the storage of the desired biopolymer.

In this study, the influence of different growth conditions and carbon sources on growth physiology, metabolic activities and storage behavior of *Microlunatus phosphovorus*, a model organism for activated sludge systems, was investigated.

Pure culture studies in batch, aerobic growth systems were conducted using chemically defined minimal media. The effects of substrate composition on biochemical storage were investigated by using various carbon sources such as glucose and acetate. A batch growth system with anaerobic-aerobic cycles was also employed. PHB, polyphosphate and glycogen staining was applied to microscopy samples to detect the possible presence of these storage compounds. Other key metabolites like total protein and volatile fatty acids were analyzed during different phases of growth in addition to cell dry weight and optical density measurements.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Bacterial Strain

Microlunatus phosphovorus (strain no: 10555) was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) GmbH, Braunschweig, Germany.

2.1.2 Culture Medium of *M.phosphovorus*

All experiments were performed with M9 minimal media, which does not involve enrichment compounds (e.g. vitamins, yeast extract...). Either glucose or acetate was used as the sole carbon source in M9 medium.

2.1.2.1. Composition of M9 Minimal Medium

Sodium hydrogen phosphate ($Na_2HPO_4.7H_2O$)	8.421 g
Potassium dihydrogen phosphate (KH_2PO_4)	3 g
Sodium chloride (NaCl),	0.5 g
Ammonium chloride (NH_4Cl)	1 g
Magnesium sulphate, 1M ($MgSO_4$)	1 ml
Calcium chloride, 1M ($CaCl_2$)	1 ml
Glucose (or another carbon source) at varying concentrations	4, 2, 1, 0.5 g
Sodium acetate (CH_3COONa)	2 g

Per liter of distilled water.

2.1.3. Chemicals

Ethanol (absolute) was purchased from J.T. Baker, Holland.

Hydrochloric acid (HCl), fuming 37% (v/v) was purchased from Merck, Germany.

Formaldehyde (HCHO), extra pure, was purchased from Merck, Germany.

Sulphiric Acid (H_2SO_4), extra pure 97-98% (v/v) was purchased from Merck, Germany.

2.1.4. Solutions

2.1.4.1. Total protein reagents

Reagent A:

1.0% (w/v) BCA-Na_2 (Bicinchoninic Acid)

2.0% (w/v) $Na_2CO_3.H_2O$

0.16% (w/v) NaK tartarate

0.4% (w/v) NaOH

0.95% (w/v) $NaHCO_3$

pH was adjusted to 11.25 by 50% NaOH or $NaHCO_3$

Reagent B:

4% $CuSO_4.5H_2O$

2.1.4.2. PHB Staining Reagents

Solution 1:

Sudan Black B (IV), 0.3% (w/v) in 60% (v/v) ethanol.

Solution 2:

Safranin O, 0.5% (w/v) aqueous solution.

2.1.4.3. Poly-P Staining Reagents

Solution 1:

Part A

Methylene Blue (0.1 g), 95% (v/v) ethanol (5 ml), glacial acetic acid (5 ml) and distilled water (100 ml).

Part B

Crystal violet [10% (w/v) in 95% (v/v) ethanol] 95% (v/v) ethanol (6.7 ml) and distilled water (100 ml)

2 volumes of Part A were mixed with 1 volume of Part B.

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Solution 2:

Bismarck Brown [1%, (w/v) aqueous] (33.3 ml) and completed with distilled water up to 100 ml.

2.1.4.4. Glycogen Staining Reagents

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

Sodium thiosulphate pentahydrate ($Na_2SO_3.5H_2O$) solution:

Sodium thiosulphate pentahydrate, 0.173 g, was dissolved in 100 ml distilled water.

Reducing Solution:

Ethanol (absolute)	300 ml
2N HCl	5 ml
Potassium iodide (KI)	10 g
Sodium thiosulphate pentahydrate solution	5 ml
Distilled water	200 ml

HCl was added to the mixture containing KI and $Na_2SO_3.5H_2O$. After a while, sulphur precipitated. The supernatant was used for staining.

Schiff Reagent:

Basic fuchsin (dissolved in 400 ml boiling distilled water), 2 g, was cooled to 50°C and filtered through a filter paper.

Ten ml of 2N HCl and 4 g of potassium metabisulfite ($K_2S_2O_5$) were added to the basic fuchsin filtrate. It was left in a cool, dark place for 12 hours. At the end, 10 ml of 2N HCl was added again.

2.1.4.5. VFA standard solution

0.1% (w/v) VFA standard, in double-distilled water, 5 ml, was purchased from Alltech Associates Inc., USA

2.1.5. Laboratory Equipment

The High Performance Liquid Chromatography (HPLC) system, Shimadzu (Japan), consisted of the following subunits:

- System Controller SCL-10A, Japan

- Liquid Chromatograph LC-10AD, FCV-10AL, Japan

- Degasser DGU-14A, Japan

- UV-Visible Detector SPD-10A, Japan

- Auto-injector SIL-10AD, Japan

- Column Oven CTO-10AC, Japan

Gas Chromatography (GC)	Agilent Technologies, 6890N, USA
Spectrophotometer	Beckman DU 530, USA
Bioreactor	Braun, Biostat B, Germany
Light Microscope	Olympus BX60, Japan
Centrifuges	Beckman Coulter, Allegra 25R, USA
	Beckman Coulter, Microfuge 18, USA
	Beckman Coulter, Avanti J-30I Centrifuge, USA
Vacuum freeze Dryers	Biotron, CleanVac 8B, Turkey
Incubators	Memmert, Switzerland
	Nüve EN400, Turkey
Orbital Shaker Incubators	Certomat S-2, Germany
Autoclaves	Tuttnauer Systec 2450 ml, Switzerland
	Nüve OT 4060, Turkey
Balances	Precisa XB 220A, Switzerland
	Precisa BJ 610C, Germany
pH meters	Mettler Toledo MP220, Switzerland
Vortexes	Nüve NM 110, Turkey
Magnetic Stirrers	Age, Italy

Deep Freezers and refrigerators	-80°C Heto Ultrafreeze 4410, Denmark
	-20°C Arçelik, Turkey
	+4°C Arçelik, Turkey
Laminar Flow	Özge, Turkey
Micropipettes	Mettler Toledo, Switzerland, (1000µl, 200 µl,100µl).
Filters	Schleiche & Schuell, Germany, 0.22µ
Flasks and Beakers	Simax Glass, Czech Republic
Glass tubes	Schott, Germany
Graduated Cylinders	Teknik Cam, Turkey

2.2. Methods

2.2.1. Batch Cultivation of *Microlunatus phosphovorus*

2.2.1.1. Cultivation of *Microlunatus phosphovorus* with Aerobic-Anaerobic Cycles

An aerobic-anaerobic cultivation system was constructed in 500 ml flasks and 50 ml Falcon tubes, respectively, with the working volume of 50 ml. During aerobic phase, flasks were shaken at 250 rpm in a shaker for 15 hours. At the end of the aerobic phase, the culture medium was centrifuged and the pellet, which contains *M.phosphovorus* cells, was supplied with fresh medium. Nitrogen gas was pumped into the medium for 2 minutes in order to deplete the soluble oxygen existing in the medium. At the end of this procedure, cells were switched to anaerobic phase for 9 hours in falcon tubes with 100 rpm rotation speed to prevent agglutination.

Each aerobic-anaerobic cycle lasts for 24 hours. At least 8 cycles were carried out to observe if *M.phosphovorus* cells accumulate any of the storage compounds such as PHB, poly-P and glycogen. Different staining techniques were employed for each of the storage compounds to observe the cells under the light microscope.

Five sets of aerobic-anaerobic cultivation system were performed with different types and quantities of carbon sources present in M9 minimal medium.

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2.2.1.2. Batch Cultivation in baffled shake flasks

One ml from the frozen stock samples of the strains kept at -20°C in 50% (v/v) glycerol were inoculated into 50 ml of M9 and the culture was incubated overnight at 30°C, 250 rpm. It was used as the preculture to inoculate 1000 ml baffled-flasks (Sigma) with a working volume of 250 ml, thus an inoculum size of 4%. Overnight grown cells were utilized for inoculation. Four g/l of glucose was supplied to M9 minimal medium as the sole carbon source. Sampling periods and volumes are shown in Table 2.2.1.2.

Table 2.2.1.2. Sampling periods and volumes in baffled-flasks.

	OD$_{600}$	VFA	Total Protein	Residual Glucose	Staining (poly-P, PHB, glycogen)
t=0	1 ml	1 ml	1 ml	1 ml	smear on slide
Early log phase	1 ml	1 ml	1 ml	1 ml	smear on slide
Mid-log phase	1 ml	1 ml	1 ml	1 ml	smear on slide
Late log phase	1 ml	1 ml	1 ml	1 ml	smear on slide
Stationary phase	1 ml	1 ml	1 ml	1 ml	smear on slide

2.2.1.3. Batch Cultivation in Bioreactor

For culture preparatrations, one ml of frozen culture stock samples kept at -20°C in 50% (v/v) glycerol was used. Two parallel shake flasks (500 ml) with the total working volume of 300 ml, containing M9 with 4 g/l glucose as the sole carbon source, were inoculated with frozen stock samples of *M.phosphovorus*, and the culture was incubated overnight at 30°C, 250 rpm. Precultures were centrifuged in a Beckman Coulter, Avanti J-30I, USA centrifuge at 5000 rpm for 10 minutes to collect the cells as a pellet. In the next step, the pellet was utilized to inoculate the medium in the bioreactor. Temperature, stirrer speed values of the bioreactor were set as: 30°C, 250 rpm respectively and the system was fully aerobic.

After inoculation, first sample at time zero was drawn in order to examine optic density, VFA, residual glucose, total protein and PHB. Sampling period was determined by the OD$_{600}$ measurements of the culture.

2.2.2. Optical Density Measurements

Optical density of the culture medium was measured at 600 nm using a spectrophotometer. Optical density measurements were taken immediately after inoculation with the overnight grown preculture until stationary phase. Samples from

the culture medium were drawn in order to determine the absorbance value at different phases of growth including lag, exponential, and stationary phases.

2.2.3. Total Protein Determination

Protein standards of known concentration (0 mg/l, 1 mg/l, 2.5 mg/l, 5 mg/l, 7.5 mg/l, and 10 mg/l) were prepared from the BSA standard solution (1 mg/ml). For working reagent preparation, 50 parts of Reagent A was mixed with 1 part of Reagent B. Ten µl of each standard, blank or sample was transferred into the appropriate microtiter plates. Top right two wells were used for standards. All determinations were performed in duplicate. Two hundred µl of working reagent was added to each well. Samples were shaken for 30 seconds. The lid was covered on the microtiter plate before incubation at 37°C for 30 minutes. Absorbance was determined by the ELISA plate reader at 570 nm. Protein concentration for each sample was determined by the plate reader in accordance with the calibration curve of BSA standards with known concentration (Table 2.2.3). (Stoscheck, 1990)

Table 2.2.3. Optical density (at 570 nm) values of BSA standards.

BSA Standard (mg/ml)	OD_{570}
0	0,125
0,1	0,171
0,25	0,235
0,5	0,314
0,75	0,410
1	0,472

The calibration curve and the equation 2.2.3, which were used to calculate the amount of total protein content in the whole study, was obtained with the trend line of "BSA concentration vs. OD_{570}"graph (Figure 2.2.3).
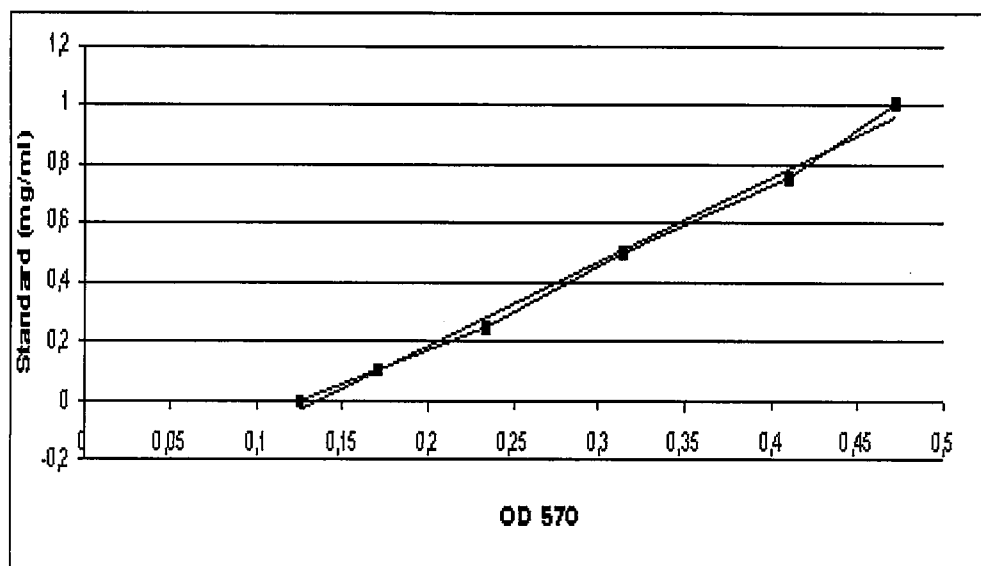
Figure 2.2.3. Calibration line of BSA standards.

$$\text{Total protein (mg/L)} = 2856.2 \times OD_{570} - 387.6$$

<div align="right">(2.2.3)</div>

2.2.4. Volatile Fatty Acid (VFA) Determination by Gas Chromatography (GC)

VFA concentrations of each culture sample, which were obtained during different phases of growth, were determined by GC. Preparation of the analyte required the following steps:

- Sample (1 ml) was centrifuged for 2 minutes at 14000 rpm.

- The supernatant was immediately filtered through filters having 0,22μ pore size to make sure that there were no particles or cells present in the analyte.

- 0.30μl, 3M, phosphoric acid (H_3PO_4) was added in order to prevent the loss of fatty acids.

The following method was performed by GC to determine the VFA concentration of each sample (Table 2.2.4). Flow rate of the samples and standards in the GC column was set as 4.5 μl/min.

Table 2.2.4. The GC protocol for VFA determination.

Oven Ramp	°C/min	Next °C	Hold (min)	Run Time (min)
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

GC was calibrated using various concentrations of VFA standards between 5 and 950 mg/l.

VFA standard was run in GC and five peaks belonging to different components of VFA standard were observed calibration chromatograms (Figure 2.2.4).
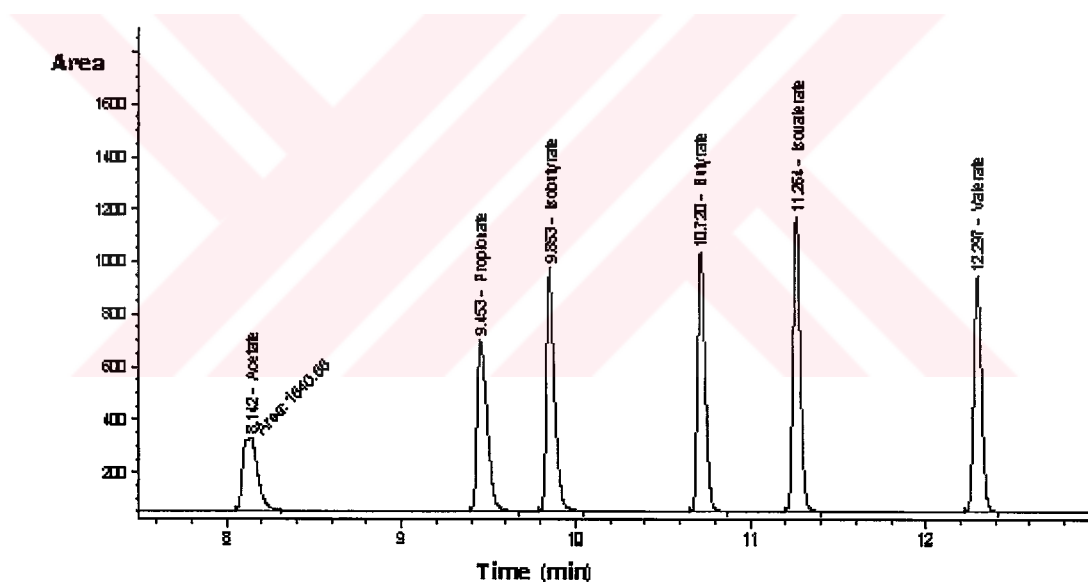
Figure 2.2.4. Chromatogram graph of 250 mg/l VFA standard.

Trend line (Figure 2.2.4.a) of the various concentrations of VFA standard was used for the derivation of trend line equation (Equation 2.2). Acetate concentrations of the samples and standards were determined with this equation.
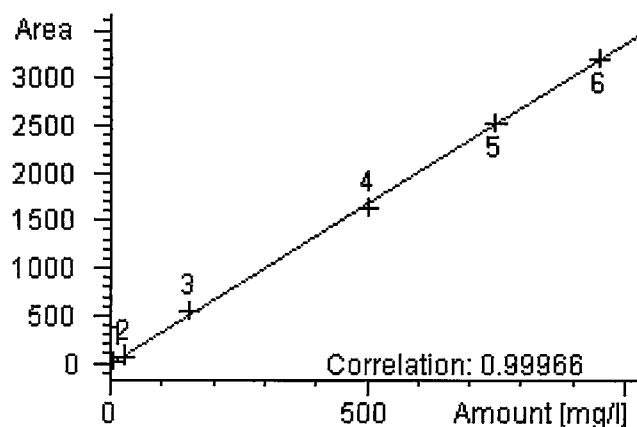
Figure 2.2.4.a. Calibration line for various acetate concentrations.

$$\text{Acetate (mg/l)} = 0.2987 \times \text{Area}$$

(2.2.4)

2.2.5. PHB Determination by GC

Firstly, GC was calibrated using the following concentrations of the PHB standard: 1.0, 2.0, 3.0 mg/l. PHA standard was consisting of 87% of PHB and 13% of PHV. Internal standards were prepared by dissolving 20 mg benzoic acid in 1 ml 1-propanol. It was considered that internal standards were added as 10% of PHB content. 50 μl of internal standards were transferred to each sample and standard.

Samples, which were drawn with the volume of 25-30 ml, were immediately subjected to a few drops of formaldehyde to stop the cell metabolism. The samples were centrifuged (5000 rpm, 7 minutes) and the supernatant was discarded. For phosphate buffer preparation K_2HPO_4 (0.58 g/l) and KH_2PO_4 (0.23 g/l) were mixed in 1 liter of distilled water. Five ml of phosphate buffer was added to pellet containing *M.phosphovorus* cells and vortexed. After the second centrifugation (5000 rpm, 7 minutes), phosphate buffer was discarded and the pellets were freeze-dried at − 50°C for 48 hours. The pellets were weighed and the exact amount was recorded. Weighed amounts of samples were put in glass tubes with PTFE lined screw caps. In the extraction step, 50 μl of internal standard was transferred to each sample and standard. 1.5 ml of the acid mixture (HCl and 1-propanol were mixed in 1:4 ratio, respectively.) and 1.5 ml 1,2-dichloro ethane were added to each sample and standard. Glass tubes, containing samples and standards were boiled at 100°C for 2 hours and shaken every 15 minutes.

In the next step, after 3 ml of distilled water addition and 10 minutes of shaking period, they were centrifuged at 3000 rpm for 5 minutes. After a few minutes, phase separation occured and 1 ml of the lower (organic) phase was drawn. It was filtered through a filter, containing glass fiber and sodium sulphate, into GC vials (Van Loosdrecht *et al.*, 1997). PHB concentration of the samples and standards were determined by GC with the following modified method:

Table 2.2.5. The GC protocol for PHB determination.

Oven Ramp	°C/min	Next °C	Hold (min)	Run Time (min)
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

2.2.6. Residual Glucose Determination by HPLC

Culture sample (1 ml) obtained at different time intervals, was centrifuged for 2 minutes at 14000 rpm and the supernatant was sterile filtered through 0,22 μm filters.

During residual glucose analysis, column temperature of HPLC was kept at 65°C and the flow rate of the mobile phase (5 mM H_2SO_4) -which circulates the column- was adjusted to 0.6 ml/min. The amount of sample in the column was set as 20 μl. Pure glucose with the following concentrations: 0, 0.2, 0.4, 1, 2 g/l was used to calibrate the system and refractive index detector (RID) was utilized for residual glucose determination. The chromatogram, which is indicated in Figure 2.2.6, belongs to 2 g/l pure glucose standard.
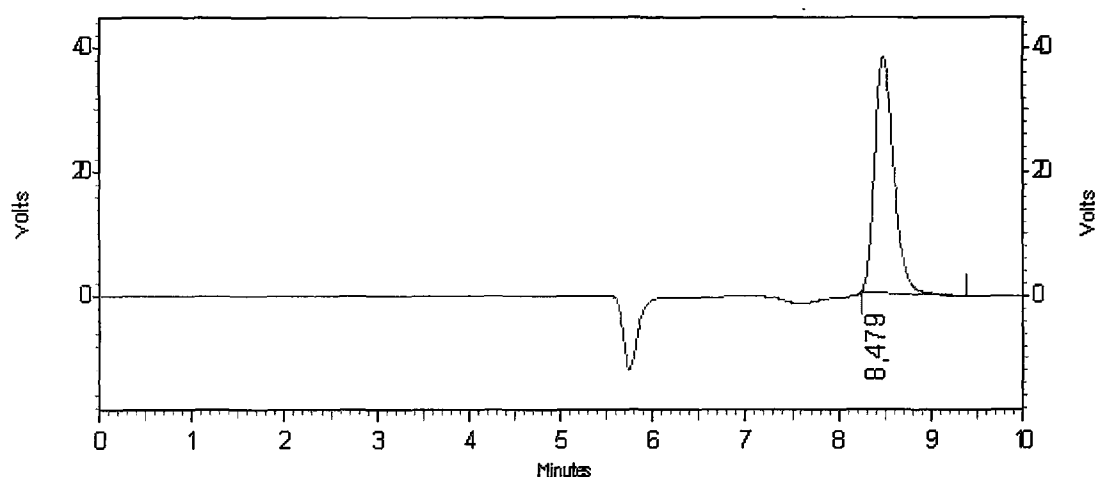
Figure 2.2.6. Chromatogram graph of glucose standard with 2 g/l concentration.

Retention time of the glucose (2000 mg/l) peak was measured as 8.479 minutes and residual glucose concentration was calculated by using the area of the calibration peak.
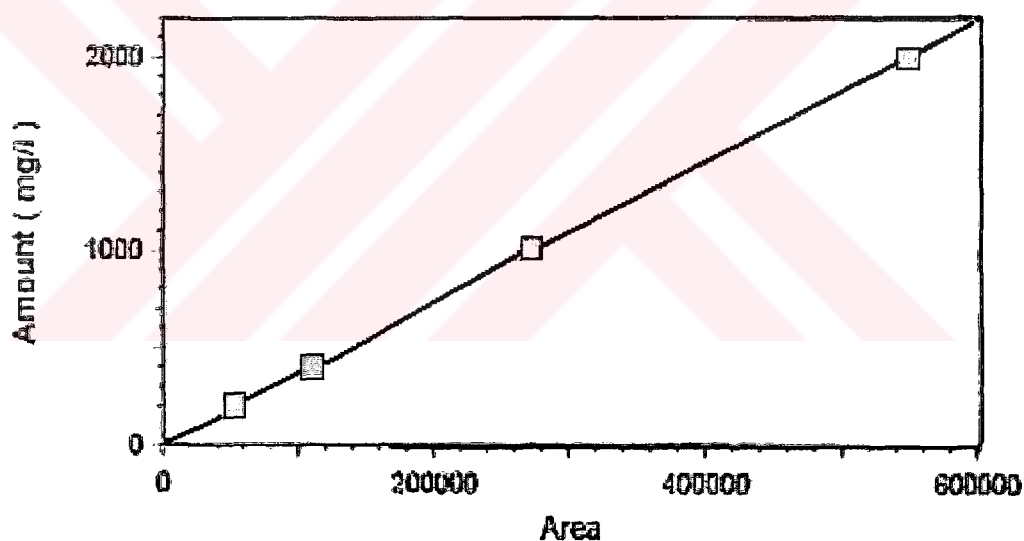
Figure 2.2.6.a. Calibration line of glucose standards with various concentrations.

Trend line of the calibration curve (Figure 2.2.6.a) and the trend line equation (Equation 2.2.6) were derived from the areas of the peaks belonging to different glucose concentrations.

$$Glucose \ (mg/l) = 0.00365521 \times Area$$

$$(2.2.6)$$

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2.2.7. Glycogen Determination by HPLC

Both glycogen and residual glucose measurements were made according to the same method in HPLC as described in the previous section (2.2.6).

During bioreactor experiments, samples of 4.5 ml volume were withdrawn and transferred in glass tubes containing 0.5 ml 6M HCl. They were boiled for 5 hours and filtered through filters of 0.22μm pore size. They were then analyzed by HPLC for glycogen and residual glucose determination.

2.2.8. Cellular Dry Weight (CDW) Determination

Filters with 0.22μm pore size were kept in an oven at 105°C for 1 hour. They were then cooled down in a dessicator for 30 minutes to minimize the moist adsorbed on filters. The filters were then weighed. During the experiments, 5 ml samples were filtered through the vacuum pump in order to obtain the biomass on the filter surface. Filters were again subjected to same drying procedure. Cell dry weight was calculated as the difference between the weights of the filters with and without dry cell mass.

2.2.9. Intracellular Poly-P Staining

The presence of energy storage granules (e.g. polyphosphate volutins or beads) in some bacterial species was not visible without treatment by a stain, which selectively reacts with polyphosphate.

Solution 1 stained the air-dried smear for 30 seconds and the stain was rinsed with water for one second. Excess water was poured off and solution 2 was applied to microscopy samples for 1 minute. It should be rinsed well with water and dried prior to examination. Poly-P appears as blue-black granules and the cytoplasm of the cell appears in brown (Murray, 1981).

2.2.10. Intracellular PHB Staining

Thin smears were prepared on a microscopy slide and air-dried thoroughly. Smears were firstly stained with solution 1 for 10 minutes; more stain was added if the slide started to dry out. It was rinsed with water for one second. In the next step, the slide was stained with solution 2 for 10 seconds and rinsed well with water. For examination, at 1000x magnification with direct illumination, immersion oil was applied and the sample was observed under the light microscope. When the preparation was PHB (+), the PHB granules then appeared as intracellular, blue-black granules, while the cytoplasm was observed pink or colorless (Murray, 1981).

2.2.11. Intracellular Glycogen Staining

A heat-fixed smear of the specimen was prepared on a microscopy slide. The slide was flood for 5 minutes with periodate solution. This solution was protected from light. The slide was washed with 70% (v/v) ethanol. In the next step, the slide was flood with the reducing solution for 5 minutes and then washed with 70% (v/v) ethanol. The slides were stained with Schiff-reagent for 30 minutes. The smear was washed several times in a solution consisting of 2 g of potassium metabisulfite and 5 ml of concentrated HCl in 500 ml of distilled water. The slide was washed with tap water, and stained with a 0.002% (w/v) aqueous solution of malachite green for 2 to 5 seconds. The slide was then washed with tap water. Last step of the glycogen determination procedure was the light microscope examinations with 1000x magnification, under oil immersion. Polysaccharides appear in red and the cytoplasm appears in green (Gerthardt *et.al.*, 1994).

3. RESULTS

3.1. Batch Cultivation of *Microlunatus phosphovorus* with anaerobic-aerobic cycles

Glucose was used as the sole carbon source at varying concentrations: 4 g/l, 2 g/l, 1 g/l and 0.5 g/l. Three parallel cultures were run for each concentration. Samples were drawn at the end of each phase for optical density and microscopy analysis of PHB, poly-P and glycogen staining. The cultures were analyzed in GC for PHB determination at the end of the experiment.

3.1.1. The Growth Behavior of *M. phosphovorus*

3.1.1.1. Optical Density Determination

Average OD_{600} values of the grown in three parallel sets with 4 g/l glucose are shown in Figure 3.1.1. Eight cycles were run in order to observe the presence of storage biopolymers.

At the end of the fourth cycle the average cell density reached its highest value, and in the following cycles it decreased.
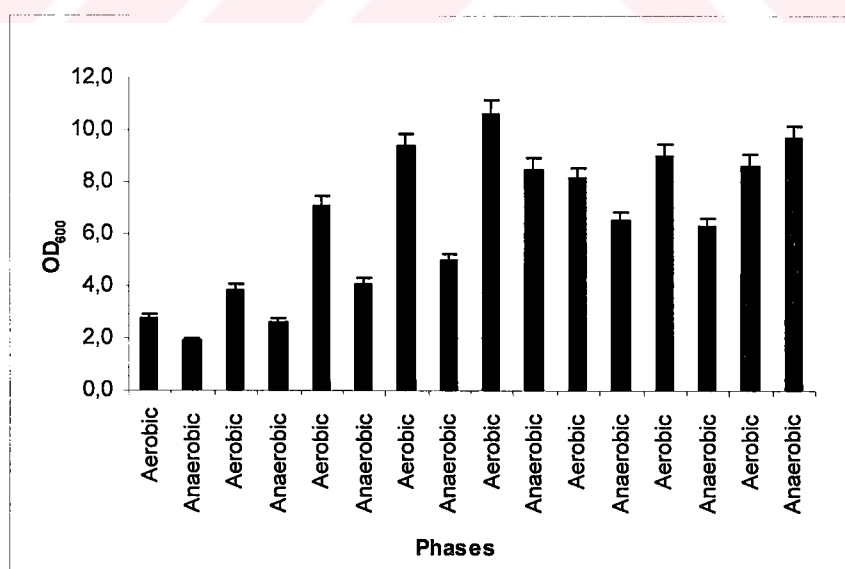
Fig. 3.1.1.1. Average OD_{600} values of three parallel culture sets during anaerobic-aerobic phases of each of the eight successive cycles. Cells were grown with 4 g/l glucose as the sole C source.

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Average OD_{600} values of three parallel cultures with 2 g/l glucose are shown in Figure 3.1.1.1.a. Nine cycles were run in order to observe the presence of storage biopolymers.

Average optical density reaches up to 4.636 after 6 cycles in the presence of 2 g/l glucose as the sole C source.
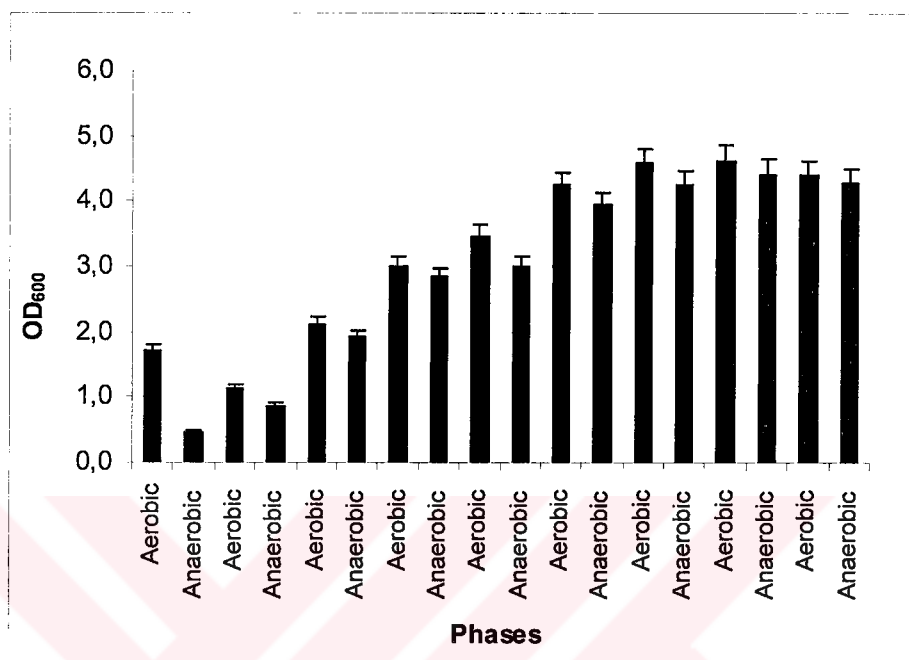
Figure 3.1.1.1.a. Average OD_{600} values of three parallel culture sets during anaerobic-aerobic phases of each of the eight successive cycles. Cells were grown with 2 g/l glucose as the sole C source.

The average OD_{600} values of three cultures grown with 1 g/l glucose are shown in Figure 3.1.1.b. Nine cycles were run in order to observe the presence of storage biopolymers.

Cell densities reach their maximum at the end of 8 cycles, when grown in the presence of 1 g/l glucose as the sole C source in the medium. (Figure 3.1.1.b)
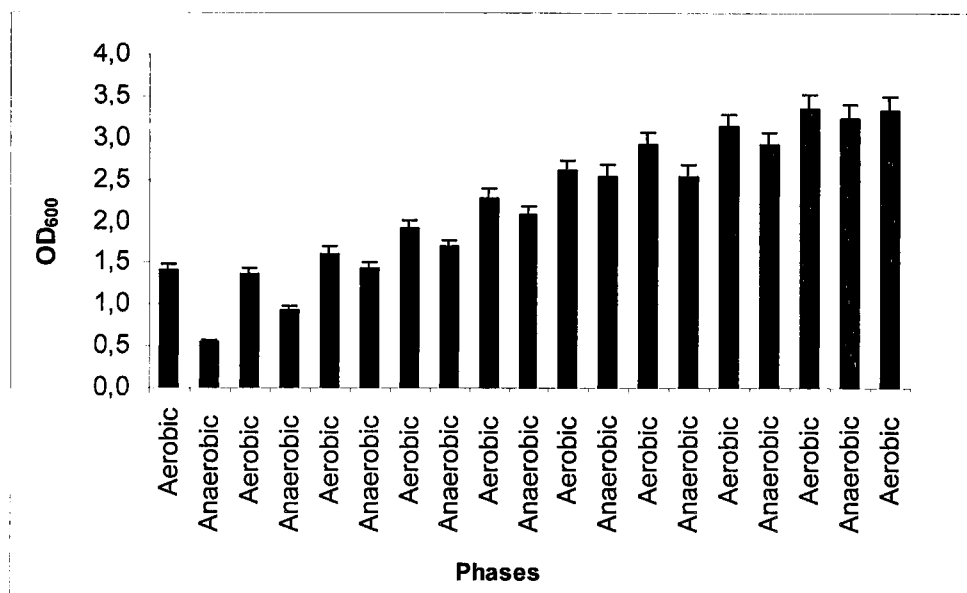
Figure 3.1.1.1.b. Average of OD_{600} values belonging to three parallel culture sets during anaerobic-aerobic phases of each of the eight successive cycles. Cells were grown with 1 g/l glucose as the sole C source.

The average OD_{600} values of three cultures containing 0.5 g/l glucose as the sole C source, are shown in Figure 3.1.1.1.c. Nine cycles were run in order to observe the presence of storage biopolymers.

After 7 cycles the optical density reaches its maximum when grown in the presence of 0.5 g/l glucose as the sole C source (Figure 3.1.1.1.c).
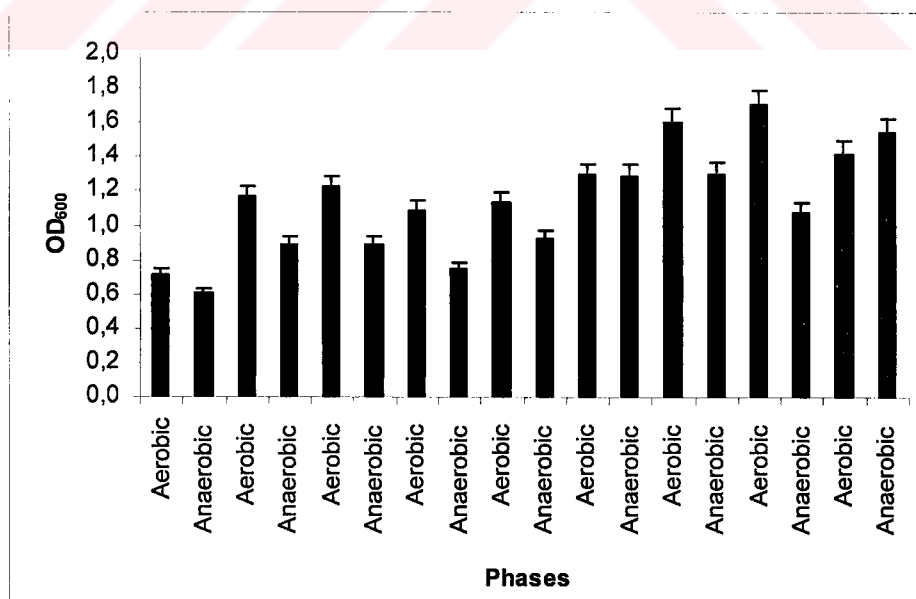

Figure 3.1.1.1.c. Average of OD_{600} values belonging to three parallel culture sets during anaerobic-aerobic phases of each of the eight successive cycles. Cells were grown with 1 g/l glucose as the sole C source.

3.1.1.2. Residual Glucose Determination

Residual glucose concentrations were determined for one cycle of each experimental set at the end of both aerobic and anaerobic phases of the cycle. Glucose consumption was higher in aerobic phase than in anaerobic phase (Table 3.1.1.2). Under cultivation conditions with 1 and 0.5 g/l glucose, the whole C-source was consumed at the end of the cycle.

Table 3.1.1.2. Residual glucose concentrations of three parallel cultures for various initial glucose concentrations.

Initial Glucose (mg/l)	Phase	Res. Gluc. (mg/l) Culture 1	Res. Gluc. (mg/l) Culture 2	Res. Gluc. (mg/l) Culture 3	Res. Gluc. (mg/l) Average
4000	Aerobic End	2542	2613	2440	2532±87
	Anaerobic End	3512	3608	3464	3528±73
2000	Aerobic End	1109	1171	1026	1102±73
	Anaerobic End	1643	1514	1606	1588±66
1000	Aerobic End	0	0	0	0
	Anaerobic End	565	444	567	525±70
500	Aerobic End	0	0	0	0
	Anaerobic End	78	55	58	64±13

3.1.2. Analysis of cellular storage metabolites

Cellular storage biopolymers (PHB, poly-P and glycogen) were analyzed by using microscopic staining procedures. Microscopy samples were then examined by light microscope under 1000x magnification. Intracellular PHB concentrations were also measured by GC analysis.

3.1.2.1. PHB Determination

The cultures were used for quantitative determination of PHB by GC analysis at the end of the cultivations. PHB and PHV concentrations of the cultures, fed with different initial glucose quantities as the sole C source, are shown in Table 3.1.2.1.

Table 3.1.2.1. PHB and PHV contents of the medium.

C-source concentration	PHB (mg/l)	PHV (mg/l)
4 g/l glucose	1496,45	0
2 g/l glucose	913,98	0,008
1 g/l glucose	324,81	0
2 g/l acetate	709,20	0,005

Maximum intracellular PHB and PHV contents were obtained when 2 g/l glucose was supplied in the medium as the sole C-source.

3.1.3. Microscopic Analysis of Intracellular Storage Compounds

Under anaerobic-aerobic growth conditions, when initial glucose concentrations were 4 and 2 g/l, PHB storage was observed upon PHB (+) staining under light microscopy at 1000x magnification with oil immersion; the cells appeared in blue-black color (Figures 3.1.3 and 3.1.3.a), indicating PHB storage.
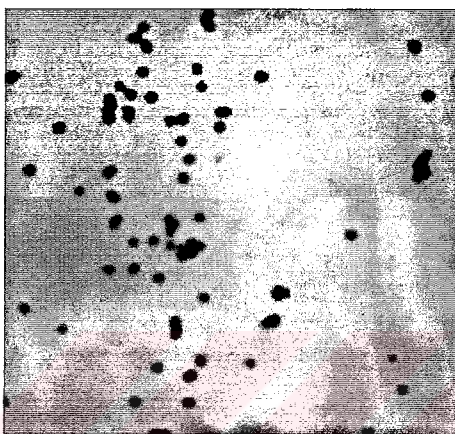
Figure 3.1.3. PHB storage of *M.phosphovorus* with 4 g/l glucose as the sole C source in M9 minimal medium.
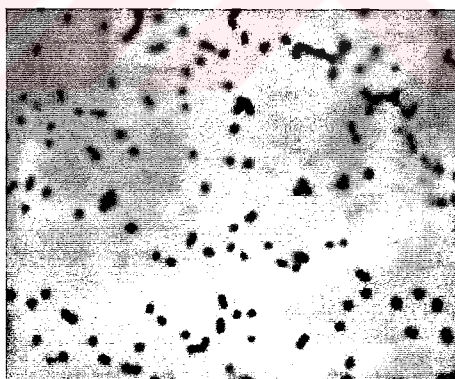
Figure 3.1.3.a. PHB storage of *M.phosphovorus* with 2 g/l glucose as the sole C source in M9 minimal medium.

After applying polyphosphate staining and glycogen staining procedures to microscopy samples, neither polyphosphate nor glycogen storage was observed upon light microscopic analysis at 1000x magnification with oil immersion. The cells were observed in brown color instead of blue-black color (data not shown).

3.2. Batch Cultivation of *M.phosphovorus* in baffled shake flasks

M.phosphovorus was cultivated in batch shake flasks for 24 hours. Optical density, VFA, residual glucose, total protein and microscopy samples were taken during the experiment in order to determine the growth physiology, cellular metabolites and storage compounds of *M.phosphovorus*.

3.2.1. The Growth Behavior of *M.phosphovorus*

Regular optical density samples representing different phases of growth were taken during cultivation. Various metabolite samples were withdrawn in accordance with the optical density measurements.

3.2.1.1. Optical Density Determination

Average optical density values of three parallel flasks are shown in Figure 3.2.1.1 as a function of the cultivation time.
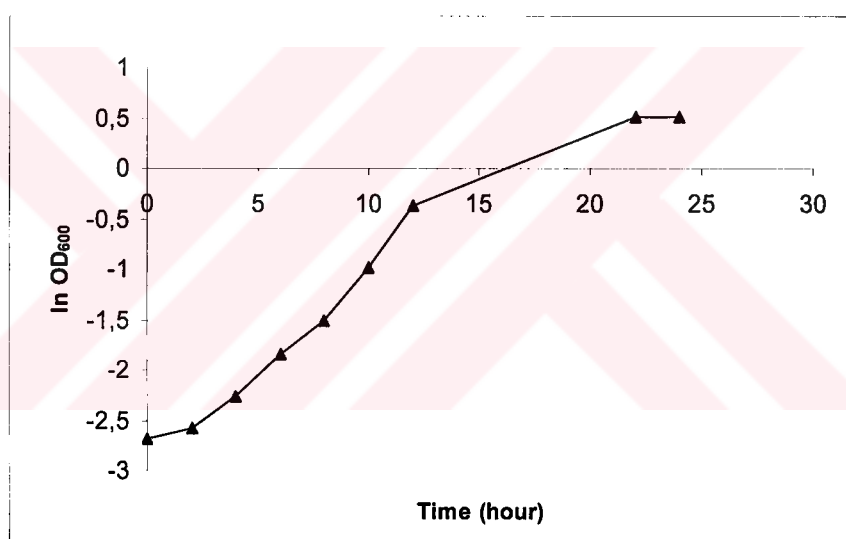
Figure 3.2.1.1. Average $lnOD_{600}$ vs. time graph of the three parallel shake flask cultures.

3.2.1.2. Determination of the Total Protein Concentrations

Samples were taken for the determination of total protein concentration of the culture during growth. The total protein concentrations of the samples were calculated according to a calibration curve and an equation, which were described in Section 2.2.3.

Table 3.2.1.2. Total protein concentration of the three flasks.

Time (hour)	Culture 1 (mg/l)	Culture 2 (mg/l)	Culture 3 (mg/l)	Average value (mg/l)
0	437,84	412,14	526,38	458,79±60
8	514,96	510,67	584,94	536,86±42
10	559,23	784,87	717,75	687,28±116
14	750,6	874,84	816,29	813,91±62
22	1001,94	939,1	930,54	957,19±39
24	1004,8	999,09	976,24	993,38±15

In Figure 3.2.1.2, the changes in the total protein concentration of the samples were compared with the growth behavior of *M.phosphovorus*.
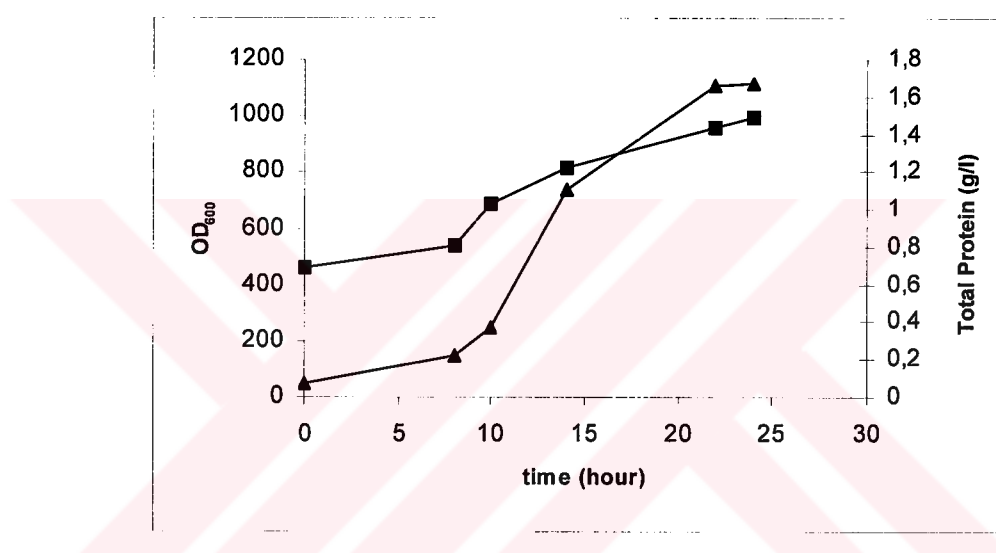
Figure 3.2.1.2. Average optical density and total protein vs. time graph for batch shake flask cultivations of *M.phosphovorus*. Full triangles indicate optical density; full squares indicate total protein values.

Total protein concentration and cell density of the culture increases in parallel during the experiment. The slope of the curves (Figure 3.2.1.2) start to decrease after 15 hours of cultivation, which means that the total protein concentration becomes constant and the organisms shift to stationary phase.

3.2.1.3. Residual Glucose Determination

Concentration of the residual glucose in the medium during bacterial growth was calculated by using a calibration curve and the equation described in Section 2.2.6.
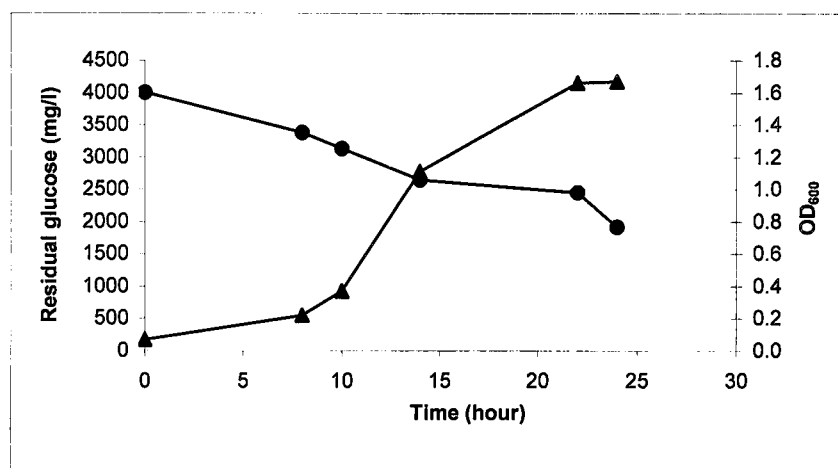
Figure 3.2.1.3. Average OD_{600} and residual glucose vs. time values of three parallel shake flask cultivations. Full triangles indicate optical density, full circles indicate residual glucose.

It is shown in Figure 3.2.1.3 that the residual glucose concentration of the medium decreases during growth, but glucose is not completely depleted.

3.2.2. Analysis of Cellular Metabolites

In order to estimate the concentrations of the metabolites produced by *M.phosphovorus*, the presence of VFA was investigated during baffled shake flask experiments. However, only small acetate peaks were observed during the analysis of all the VFA samples belonging to batch experiments.

3.2.2.1. VFA Determination

Samples were taken for the determination of VFA concentration during different phases of growth at indicated time periods (Figure 3.2.2.1).
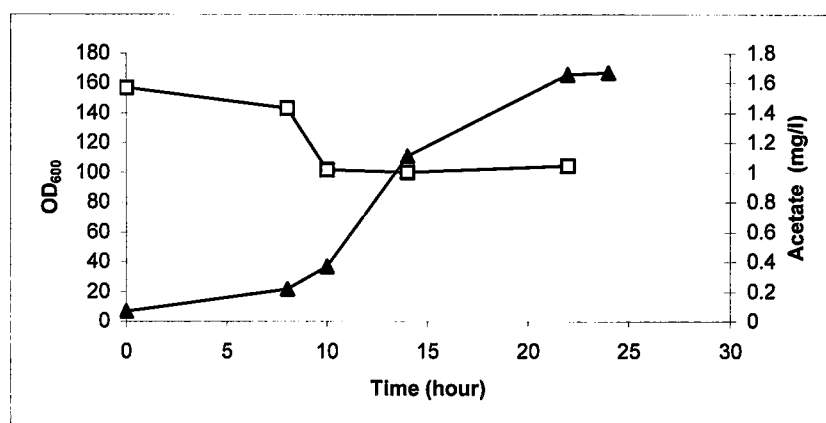
Figure 3.2.2.1. Optical density and acetate concentration vs. time graph of *M.phosphovorus*. Full triangles indicate optical density, open squares indicate acetate concentration.

Acetate concentration of the medium decreased during growth and there was a rapid decrease at the end of 22 hours of cultivation, which corresponds to the stationary phase of growth.

3.2.3. Microscopic Analysis of Intracellular Storage Compounds

Microscopy samples withdrawn during different phases of growth for three intracellular storage biopolymers (poly-P, glycogen and PHB) showed no storage under the light microscope at 1000x magnification with oil immersion. (data not shown)

3.3. Batch Cultivations in a Bioreactor

Batch cultivations were also performed in bioreactor under controlled conditions. Optical density, cellular dry weight, VFA, total protein, residual glucose, PHB (GC analysis) and microscopy samples were taken during 24 hours of cultivation period.

3.3.1. The Growth Behavior of *Microlunatus phosphovorus*

In order to understand the growth behavior of *M.phosphovorus*, firstly, a correlation between optical density and cellular dry weight concentrations was made. This correlation was also used for other metabolite calculations.

3.3.1.1. Optical Density vs. Cellular Dry Weight Correlation

Metabolite contents of the cells (e.g. PHB) were determined by using optical density vs. cellular dry weight correlation. Cellular dry weight vs. optical density graph was obtained during the experiments.
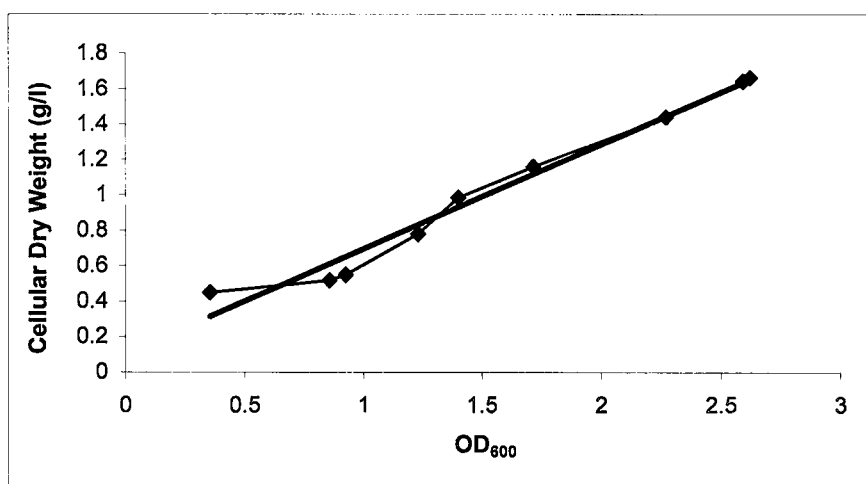
Figure 3.3.1.1. Optical density and cellular dry weight correlation graph of the culture medium during growth.

$$CDW \ (g/l) = 0.5892 \ x \ OD_{600} + 0.1073$$

<div align="right">(3.3.1.1)</div>

Equation 3.3.1.1 was derived from the trend line of the optical density vs. cellular dry weight graph (Figure 3.3.1.1). Additionally, the resulting biomass in the medium was calculated as 1.44 g/l according to Equation 3.3.1.1.

3.3.1.2. Total Protein Determination

Samples were taken for the determination of total protein concentration according to the calibration curve and equation described in Section 2.2.3.

Figure 3.3.1.2 shows the changes in cell density and total protein concentrations with respect to cultivation time. Total protein concentration increases in parallel with the growth.
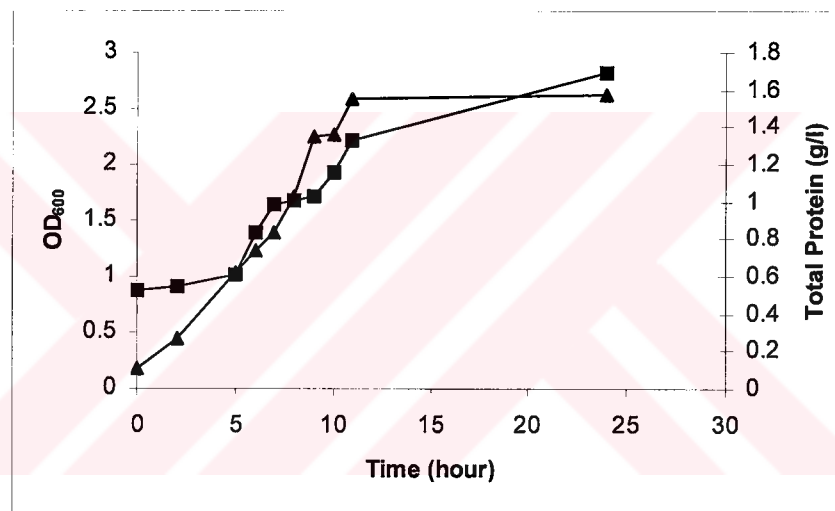
Figure 3.3.1.2. Optical density and total protein concentration vs. time graph. Full triangles indicate optical density; full squares indicate total protein values.

3.3.1.3. Residual Glucose Determination

Residual glucose concentration of the medium was determined during bioreactor experiments using the calibration equation described in Section 2.2.6.

Figure 3.3.1.3 shows that nearly 2.1 g/l glucose was used for growth during bioreactor conditions.
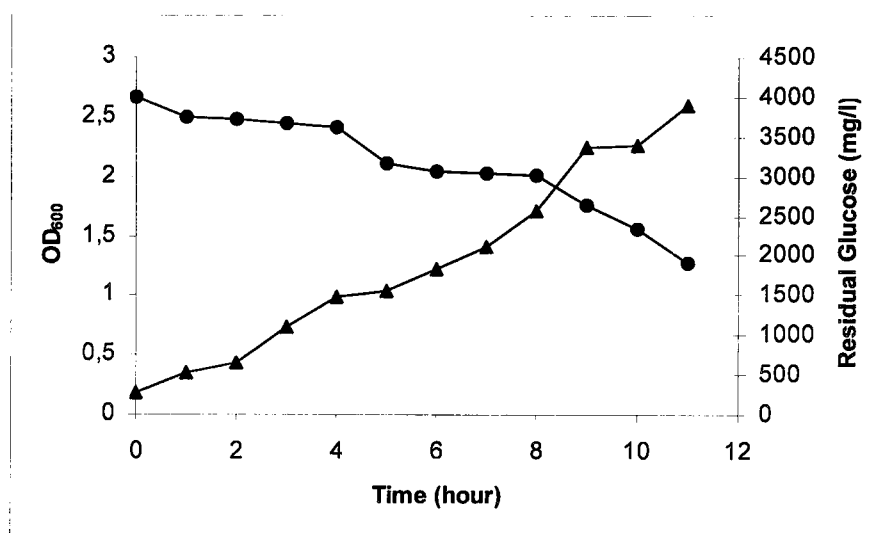
Figure 3.3.1.3. Optical density and residual glucose vs. time graph. Full triangles indicate optical density, full circles indicate residual glucose.

3.3.2. Analysis of cellular metabolites

Acetate concentrations in the medium was determined with the calibration equation which was described in Section 2.2.4.

3.3.2.1. VFA Determination

VFA samples were taken during growth at indicated time periods (Table 3.3.2.1.). Among all VFA measured, only acetate was at detectable levels which were, however, still very low and thus, probably, fluctuating.

Table 3.3.2.1. Acetate concentration of the medium during growth.

Time (h)	Acetate (mg/l)
0	84,95
2	78,65
5	23,74
6	54,93
7	45,845
8	69,1
9	54,53
10	51,355
11	43,535
24	17,655

3.3.2.2. PHB Determination

PHB and PHV concentrations of the samples were determined by using the calibration equation described in Section 2.2.5. PHB samples were withdrawn after 6 hours of batch cultivation under bioreactor conditions.

Table 3.3.2.2. PHB and PHV concentrations of the culture medium during growth.

Time (hour)	CDW (mg/l)	PHB (mg/l)	PHV (mg/l)
6	832.02	164.57	0
7	932.18	203.1	0
8	1117.78	260.87	0
9	1429.46	293.08	0
10	1444.78	390.3	0
11	1633.33	366.37	0.01
24	1651	395.79	0.02

Figure 3.3.2.2 shows that PHB concentration increases until the end of the cultivation without any consumption. Maximum amount of cellular dry weight, which was obtained at the end of the growth, was measured as 1136,58 mg/l with the PHB content of 311,62 mg/l. There seems to be also a PHV storage, which starts after 10 hours of growth (Table 3.3.2.2), but it's at very low levels.
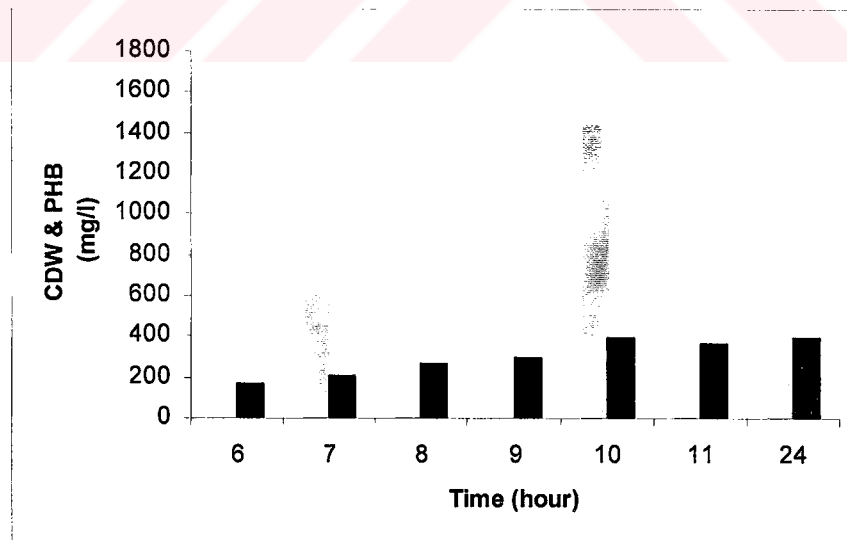
Figure 3.3.2.2. PHB concentration vs. OD_{600} graph of *M.phosphovorus.* Gray columns indicate CDW, black columns indicate PHB.

3.3.2.3. Glycogen Determination

Glycogen peak wasn't observed during HPLC analysis of the samples implying that there wasn't any glycogen storage under the bioreactor conditions investigated (data not shown).

3.3.3. Microscopic Analysis of Intracellular Storage Compounds

No intracellular storage compounds were observed by using staining techniques for light microscopic examinations. PHB storage was observed as shown in the previous section (3.3.2.2) during GC analysis of the bioreactor samples although the PHB staining results were negative.

Microscopic staining results of poly-P and glycogen biopolymers were also negative. Cytoplasm of the cells, after applying poly-P and glycogen staining procedures, appeared in brown and green color, respectively.

4. DISCUSSION AND CONCLUSIONS

In this study, the influence of different growth conditions and carbon sources on growth physiology, metabolic activities and storage behavior of *Microlunatus phosphovorus*, a model organism for activated sludge systems, was investigated. For this purpose, *M.phosphovorus* was cultivated either under aerobic batch or under anaerobic-aerobic sequential batch conditions.

Activated sludge systems include many different species that grow in a chemically undefined medium with different types and concentrations of C-sources. These systems are being used during EBPR process. *M.phosphovorus* is a member of this process. It is known that *M.phosphovorus* stores polyphosphate under EBPR conditions (Nakamura *et al.*, 1991). It is also indicated in the literature that *M.phosphovorus* cannot store PHA polymer either in the form of PHB or PHV (Mino, 2000). Almost all the studies that are focusing on the storage characteristics of *M.phosphovorus* base on activated sludge conditions. In this research, it was observed that these bacteria could mostly store PHB under the defined environmental conditions in which there's a chemically defined medium involving excess glucose or acetate as the sole C-source.

Due to adaptation of the organisms to environmental conditions and start up the intracellular storage mechanism, at least 8 cycles were run during the sequential aerobic-anaerobic cultivation experiments. In order to yield maximum amount of storage material either glucose (4, 2, 1, 0.5 g/l) or acetate (2 g/l) was used in the medium as the sole carbon source at varying concentrations in the chemically defined media M9. The results have shown that when 4 g/l of glucose was supplied, PHB was stored at the highest concentration (1496,45 mg/l) by *M.phosphovorus*. Also, PHB staining method have shown that the cells, which were subjected to 4 g/l glucose were observed in darker blue-black color than the cells with 2 g/l glucose, which is due to higher amount of PHB storage. PHB storage characteristic of the organisms was observed at the end of both anaerobic and aerobic phases within the microscopy samples, which were taken from the medium involving 4 and 2 g/l glucose. This result can be interpreted as; microorganisms do not need to consume the anaerobically stored PHB during the aerobic phase due to high C-source

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concentration. PHB storage was observable under light microscope at the fifth cycle for both of the glucose concentrations. On the other hand, observations of the microscopy samples belonging to medium involving 1 g/l glucose showed that PHB storage occured only at the end of anaerobic phase. It can be concluded that lack of C-source directs organisms to utilize the anaerobically stored PHB. No PHB storage was observed at the end of both phases after staining the samples of 0.5 g/l medium. When 2 g/l acetate was supplied to M9 minimal medium PHB staining results have shown that intracellular PHB was present at the end of both phases. Also GC analysis of the sample, which was taken at the end of anaerobic phase, has shown that a significant amount of PHB was present in the medium.

PHB and PHV biopolymers were also detected by GC during the batch experiment, which was performed in bioreactor under fully aerobic conditions with M9 involving 4 g/l glucose as the sole C source. At the end of 24 hours of cultivation, maximum amount of PHB and PHV was obtained as 260,47 mg/l and 0.03 mg/l, respectively. PHV storage occured after 10 hours of cultivation, but 258,19 mg/l PHB was already present at the end of the sixth hour of growth. PHB is known as the most common type of PHA stored by bacteria (Madigan *et al.*, 2000).

Polyphosphate-staining procedure was applied to samples, which belong to all the batch conditions, but no polyphosphate storage was observed during light microscopic analysis at 1000x magnification. More quantitative and sensitive techniques should be applied for the detection of polyphosphate. In the presence of trace amount of polyphosphate, qualitative methods such as staining may not be responsive to pure culture studies.

Since the oxygen concentration was higher in the bioreactor, total protein and cellular dry weight concentrations of the culture grown in the bioreactor were higher, and the residual glucose concentration was lower than that of the baffled flask conditions.

It was concluded that *M.phosphovorus* utilizes the C-source for growth and PHA storage instead of producing secondary metabolites (e.g. acetate) under the studied conditions of this research.

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