HYDROGEN PRODUCTION BY MICROORGANISMS IN SOLAR BIOREACTOR

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

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IN SOLAR BIOREACTOR

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The main objective of this study is exploring the parameters affecting photobiological hydrogen production and developing anaerobic photobioreactor for efficient photofermentative hydrogen production from organic acids in outdoor conditions. *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* strains were used as microorganisms.

EU project "Hyvolution" targets to combine thermophilic fermentation with photofermentation for the conversion of biomass to hydrogen. In this study, the effluent obtained by dark fermentation of Miscanthus hydrolysate by *T. neapolitana* was fed to photobioreactor for photofermentation by *R. capsulatus*. Hydrogen yield was 1.4 L/Lculture showing that the integration of dark and photofermentation is possible.

Innovative elements were introduced to the photobioreactor design such as removal of argon flushing. An online gas monitoring system was developed which became a commercial product. It was found that the light intensity should be at least 270 W/m^2 on the bioreactor surface for the highest hydrogen productivity and the hydrogen production decreased by 43 % if infrared light was not provided to the bioreactor.

Scale-up of photofermentation process to 25L was achieved yielding 27L hydrogen in 11 days by *R. capsulatus* on acetate/lactate/glutamate (40/7.5/2 mM) medium.

The outdoor application of the system was made. Shading and water spraying were adapted as cooling methods for controlling the temperature of the outdoor bioreactor. It was found that uptake hydrogenase deleted mutant of *R. capsulatus* show better hydrogen productivity (0.52 mg/L.h) compared to the wild type parent (0.27 mg/L.h) in outdoor conditions. It was also shown that the hydrogen production depended on the sunlight intensity received.

Keywords: *R. capsulatus*, *R. sphaeroides*, Biological Hydrogen Production, Photobioreactor

ÖZ

GÜNEŞ BİYOREAKTÖRÜNDE MİKROORGANİZMALARLA
HİDROJEN ÜRETİMİ

Uyar, Başar

Doktora, Biyoteknoloji Bölümü
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Bu doktora tezinin ana amacı fotobiyolojik hidrojen üretimini etkileyen parametreleri araştırmak ve açık hava şartlarında mor kükürtsüz bakteriler ile organik asitlerin fotofermentasyonundan verimli hidrojen üretimi için anaerobik fotobiyoreaktör geliştirmektir. Mikroorganizma olarak *Rhodobacter capsulatus* ve *Rhodobacter sphaeroides* türleri kullanılmıştır.

AB Projesi Hyvolution'ın amacı biyokütleyi hidrojene çevirmek için termofilik fermentasyonu fotofermentasyonla birleştirmektir. Bu çalışmada Miscanthus hidrolizatının *T. neapolitana* ile karanlık fermentasyonuyla elde edilen çıktı *R. capsulatus* tarafından fotofermentasyonda kullanılması için fotobiyoreaktöre beslenmiştir. Hidrojen eldesi 1.4 L/Lkültür olmuş ve iki prosesin birleştirilmesinin mümkün olduğu gösterilmiştir.

Fotobiyoreaktör tasarımına argon gazının kaldırılması gibi yenilikçi yaklaşımlar getirilmiştir. Geliştirilen sürekli gaz ölçüm sistemi ticari ürün haline getirilmiştir. En iyi hidrojen üretimi için biyoreaktör yüzeyindeki minimum ışık

şiddetinin 270W/m^2 olması gerektiği ve biyoreaktöre kızılötesi ışık sağlanmadığı takdirde hidrojen üretiminin % 43 azaldığı bulunmuştur.

Fotofermentasyon prosesi 25L ölçeğe çıkarılmış, *R. capsulatus* ile asetat/laktat/glutamat (40/7.5/2 mM) besiyerinde 11 günde 27L hidrojen elde edilmiştir.

Sistemin açık hava uygulaması yapılmış, biyoreaktörün sıcaklığını kontrol etmek için gölgeleme ve su püskürtme soğutma yöntemleri olarak uygulanmıştır. Dış koşullarda *R. capsulatus*'un hidrojenazı silinmiş mutantının yaban tip'e göre daha hızlı hidrojen ürettiği (0.52 mg/L.h'ye karşılık 0.27 mg/L.h) bulunmuştur. Hidrojen üretiminin biyoreaktörün aldığı güneş ışığı şiddetine bağlı olduğu gösterilmiştir.

Anahtar Kelimeler: *R. capsulatus*, *R. sphaeroides*, Biyolojik Hidrojen Üretimi, Fotobiyoreaktör

To My Family

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A: Irradiated area (m^2)

Acetyl-CoA: Acetyl Coenzyme A

ADP: Adenosine di-Phosphate

ATP: Adenosine tri-Phosphate

GC: Gas Chromatography

gdw: Gram dry weight of bacteria

H_2: Hydrogen gas

hup^-: Uptake hydrogenase deficient (mutant)

HPLC: High Performance Liquid Chromatography

I: Light intensity (W/m^2)

k_c: Specific growth rate constant obtained by logistic model, (1/h)

PHB: Polyhydroxybutyrate

pK_a : minus the decimal logarithm of K_a (weak acid dissociation constant)

PNS: Purple Non-Sulphur

t: time (hours)

V: volume (ml or L)

$X_{0,e}$: Experimental initial bacterial concentration at the growth lag time, (g/L)

$X_{0,m}$: Initial bacterial concentration obtained by logistic model, (g/L)

$X_{max,e}$: Experimental maximum bacterial concentration, (g/L)

$X_{max,m}$: Maximum bacterial concentration obtained by logistic model, (g/L)

Greek Letters:

ρ_{H2}: Density of the hydrogen gas (g/L)

η: Light conversion efficiency (%)

μ_{max}: Specific growth rate constant obtained by exponential model, (1/h)

CHAPTER 1

INTRODUCTION

Much attention is being given to the development of clean and renewable energy systems with the potential to supplement and even substitute the fossil fuel based energy production. The main reasons, which make people consider and attempt in this direction, are the rapid depletion of limited fossil resources on one hand and the global environmental problems caused by their utilization on the other. Molecular hydrogen is one of the environmentally acceptable fuel alternatives, since a worldwide conversion from fossil fuels to hydrogen would eliminate many of these problems (Eroglu, 2006).

In many respects, molecular hydrogen is the ideal energy carrier and has been widely discussed as a possible carrier of energy on an economy-wide scale. It has the highest energy to mass ratio of any known fuel. 1 kg of hydrogen contains the same amount of energy as 2.7 kg of natural gas or 3.0 kg of gasoline. Consumption of hydrogen produces no carbon dioxide or toxic residues, but just water. The utilization of hydrogen in fuel cells is also gaining worldwide interest.

In order to meet the requirements for CO_2 reduction, to put the "Hydrogen Civilization" into practice and to make the future "Hydrogen Economy" fully sustainable, renewable resources instead of fossil fuels have to be employed for hydrogen production.

Molecular hydrogen is not available on Earth in convenient natural reservoirs. Hydrogen is presently most economically produced using fossil fuels. In practice this is usually methane, though hydrogen can also be produced via steam reforming or partial oxidation of coal. More expensively it can also be produced via electrolysis using electricity and water.

At present, most of the H_2 is produced from steam reforming of natural gas. In this process, natural gas and steam are passed over a usually nickel-based catalyst, at temperatures of 650 – 700 °C producing mixtures of hydrogen and carbon monoxide.

Another widely used method for the production of hydrogen is the splitting of water into hydrogen and oxygen by electrolysis. The application of the electrolytic process is restricted to the areas having cheap hydroelectric energy.

These industrial methods are energy and environment intensive, since they mainly consume fossil fuel as an energy source. Thus, alternative raw materials and processes for hydrogen production are being investigated or already at the research and development stage; such as thermochemical, photochemical, photoelectrochemical, and photobiological processes.

Some thermochemical processes, such as the sulfur-iodine cycle, can produce hydrogen and oxygen from water and heat without using electricity, however none of these processes have been demonstrated at production levels, although several have been demonstrated in laboratories.

The biological production of hydrogen offers an opportunity to utilize renewable resources; because they represent an ecological and a less energy intensive method as a result of operating at ambient process conditions and facilitating waste recycling.

The concept of biological production of hydrogen is based on the exploitation of bacteria, which freely and efficiently produce pure hydrogen as a by-product during growth on biomass. The reward of this approach will be enormous since it allows the greatest reduction in CO_2 emission and provides independence of fossil imports. Both topics are dominant in all global agreements on climate protection and because of the urgency in mitigating the greenhouse effect, it is of prime importance to start this research now.

In nature, some microorganisms such as algae, cyanobacteria, anaerobic and photosynthetic bacteria can produce hydrogen in accordance with their metabolisms. Both algae and cyanobacteria split water and make oxygenic photosynthesis by evolving O_2. However, these two microorganisms cannot

produce the hydrogen gas efficiently, compared to the remaining processes. Photosynthetic bacteria are favorable candidates for biological hydrogen production due to their high conversion efficiency and versatility in the substrates they can utilize.

An EU 6th framework integrated project "Non-thermal production of pure hydrogen from biomass" with the acronym "Hyvolution" is accepted to 2nd stage of evaluation by the EU commission and was started by the 1st of 2006. METU Biohydrogen Group leads the photofermentation researchs of this five year project.

The main scientific objective of this project is the development of a 2-stage bioprocess for the cost-effective production of pure hydrogen from multiple biomass feedstocks. The bioprocess starts with a thermophilic fermentation of feedstock to hydrogen, CO_2 and intermediates. In a consecutive photo-heterotrophic fermentation, all intermediates will be converted to more hydrogen and CO_2.

On the other hand, the main technological objective of this project is the construction of prototype modules of the plant which, when assembled, form the basis of a blue print for the whole chain for converting biomass to pure hydrogen.

Besides scientific and technological objectives, also socio-economic activities are included to increase public awareness and societal acceptance, and for identification of future opportunities, stakeholders and legal consequences of this specific bioprocess for decentral hydrogen production.

The new approach focuses on employing thermophilic bacteria which produce hydrogen together with acetic acid. The co-product acetic acid, is a prime substrate for H_2 production in a consecutive photofermentation for further increase of the final amount of H_2 produced per unit biomass. The combination of a thermophilic fermentation with a photofermentation enables the complete conversion of biomass to hydrogen with the highest efficiency theoretically possible.

The development of dedicated bioreactor prototypes for photofermentation both in terms of design and material usage with associated monitoring and control

strategies will comprise a basis for process optimization and facilitate final industrial production.

Rhodobacter species are known to be the metabolically most diverse species among the prokaryotes, thus it is easier to isolate such kind of bacteria from environment.

Hydrogen production by those bacteria occurs under illumination in the presence of an inert, anaerobic atmosphere (such as argon), from the breakdown of organic substrates such as malate and lactate. The culture medium should be under a nitrogen limitation (i.e. a high C/N ratio), which forces the bacteria to 'dump' the excess energy and reducing power through the production of hydrogen. Several individual components make up the overall production system and these may conveniently be grouped as: i) the enzyme systems, ii) the carbon flow – specifically the TCA cycle and iii) the photosynthetic membrane apparatus. These groups are interconnected within the hydrogen production scheme by means of the exchange of electrons, protons and ATP.

In addition to hydrogen, some valuable by-products such as biodegradable polymer "PHB" and a kind of pigment "carotenoid" are also produced by the photosynthetic bacteria.

The METU Biohydrogen Research Group works on photofermentative hydrogen production by PNS bacteria field since 1990's. Previous studies completed by the research group include:

Production of hydrogen by *Rhodobacter sphaeroides* O.U. 001 (Arik, 1995), Use of milk industry wastewater for biohydrogen production (Türkaslan, 1998), Use of sugar refinery wastewater for biohydrogen production (Yetiş, 1999), Identification of by-products in hydrogen producing bacteria (Yiğit, 1999), Hydrogen metabolism and factors affecting for biohydrogen production (Koku, 2001), Investigation of polyhydroxybutyrate (PHB) production by *Rhodobacter sphaeroides* O.U.001 (Suludere, 2001), Use of olive mill wastewater for biohydrogen production (Eroğlu, 2002, 2006), Biohydrogen production in a solar bioreactor (Tabanoglu, 2002), Effect of bacteriorhodopsin on hydrogen gas production (El-Kahlout, 2002), Biohydrogen production by bacteria with

genetically modified cytochrome systems (Ozturk, 2005), Development of helical tubular reactors (Sari, 2007).

Those researches showed that many types of wastewaters can be utilized for hydrogen production. Moreover, the metabolism of the bacteria was investigated, by-products were defined, hydrogen producing enzymes were genetically classified, the mutant bacterial strains were obtained and various photobioreactors were developed.

However, more studies were required to be carried out to advance the researches made so far. The physiological parameters that affect the bacteria required a more extensive research for optimization of the process conditions. Additionally the coupling of the dark fermentation and photofermentation had to be investigated and shown. On the other hand, there was a need for improved photobioreactor designs, further scale up of the process had to be made and more research were required in outdoor applicability of the process.

For the highly efficient hydrogen production in photobioreactors by photoheterotrophic bacteria the utilization of the effluent of the thermobioreactor for photofermentation is a key step. The maximum fermentative hydrogen production is achieved when acetate is the end product. Thus, the end-product effluent of thermophilic fermentations is expected to be composed mainly of acetate and some lactate. Therefore the photofermentation studies needs to be focused on the use of acetate as the sole or primary substrate to produce hydrogen.

It is also targeted to feed the real dark fermentation effluent into the photofermentation step to show whether the coupled hydrogen production process is possible or not. The pretreatments required (if any) to the dark fermentation effluent had to be determined and documented.

The construction and successful operation of the photobioreactors is a critical part of the study. The design parameters have to be set to ensure a simple design, low material and production costs and high utilization of sunlight within the optimum wavelength range. The optimal penetration of light in the photo-bioreactor should be established to give high photochemical efficiency.

Since the biological production of hydrogen at a competitive cost in industrial scales can only be possible in photobioreactors which are illuminated by sunlight, applications in outdoor conditions are also necessary.

With the accumulation of data and results of the test runs in these fields an efficient anaerobic photobioreactor design and setting the optimum process parameters for highest efficiency can be made, to set a solid basis for a stable and high performance photobiological hydrogen production process in industrial scale photobioreactors.

In the next chapter (Chapter 2), a general knowledge on the biological hydrogen production is given in details. Biological hydrogen production processes are explained on the core basis of photofermentative hydrogen production processes. Properties of photofermentative microorganisms as well as their metabolic pathways and the possible substrate sources for an efficient hydrogen production are described. Recent literature studies about the two-stage biological hydrogen production processes (dark fermentation followed by photofermentation) and the development of the photobioreactors were evaluated.

Experimental methods and procedures are given for the analyzing techniques in Chapter 3. In addition, the experimental setups for indoor and outdoor hydrogen production processes were also explained.

Experimental results are given and discussed in Chapter 4, 5 and 6. Chapter 4 gives the results of the physiological studies such as the photofermentative hydrogen production from different substrates and effect of different parameters on photofermentative hydrogen production; Chapter 5 focuses on photobioreactor design and improvement studies; Chapter 6 is where the outdoor applications of the process were given.

As a final point, achieved conclusions and further recommendations are explained throughout the last chapter (Chapter 7).

The thesis was completed by References section where the cited publications were listed and Appendices part in which additional data were provided.

CHAPTER 2

BIOLOGICAL HYDROGEN PRODUCTION

Renewable resources such as sunlight, water and biomass are utilized for biological hydrogen production. Therefore, it is an environmentally acceptable process. Biological hydrogen production is performed under mild operating conditions, and it does not require complex equipment. Biological hydrogen production processes are categorized as follows:

a. Biophotolysis of water using algae and cyanobacteria

b. Fermentative hydrogen production from organic compounds

c. Photodecomposition of organic compounds by photosynthetic bacteria

Biophotolysis process initially gives an attractive impression, since water is decomposed into hydrogen and oxygen without the requirement of any organic compounds. However, the production rates are low and the evolution of oxygen as an end product inhibits hydrogen production.

During fermentative hydrogen production, anaerobic bacteria produce hydrogen by decomposing organic substrates under dark conditions. As the decomposition is incomplete, lower molecular weight organic compounds are produced together with hydrogen and carbon dioxide. Thus, hybrid systems using photosynthetic and fermentative bacteria become an efficient way for the biological hydrogen production. The organic acids, produced as a result of fermentation, can further be utilized by photosynthetic bacteria for hydrogen generation (Wakayama and Miyake, 2001).

2.1 Microorganisms Producing Hydrogen

Different microorganisms participate in the biological hydrogen generation system such as green algae, cyanobacteria (or blue-green algae), photosynthetic bacteria and fermentative bacteria (Das and Veziroğlu, 2001). The overview of these processes are given in Table 2.1, the name of the microorganisms for each class is given in Table 2.2:

Table 2.1 Overview of biological hydrogen production processes

Microorganism Type	**Process**	**Reaction**
Algae	Biophotolysis	$H_2O \rightarrow H_2 + (½)\ O_2$
Cyanobacteria	Biophotolysis	$H_2O \rightarrow H_2 + (½)\ O_2$
Fermentative bacteria	Dark fermentation	$C_6H_{12}O_6 \rightarrow H_2 + CO_2$ + organic acids
Photosynthetic bacteria	Photofermentation	Organic acids + $6H_2O$ + light $\rightarrow 12H_2 + 6CO_2$

Table 2.2 Microorganisms used for hydrogen generation
(Modified from Das and Veziroğlu, 2001)

Broad Classification	**Name of the Microorganism**
Green algae	*Scenedesmus obliquus, Chlamydomonas reinhardii, Chlamydomonas moewusii*
Cyanobacteria	*Anabaena azollae, Anabaena variabilis, Anabaena cylindrical, Nostoc muscorum, Nostoc spongiaeforme, Westiellopsis prolifica, Plectonema boryanum, Oscillatoria limnetica, Synechococcus sp., Aphanothece halophytico, Mastidocladus laminosus, Phormidium valderianum*
Photosynthetic bacteria	*Rhodobacter sphaeroides, Rhodobacter capsulatus, Rhodobacter sulidophilus, Rhodopseudomonas palustris, Rhodospirillum rubrum, Chlorobium limicola, Chloroflexu aurantiacus, Thiocapsa roseopersicina, Halobacterium halobium,*
Fermentative bacteria	*Enterobacter aerogenes, Enterobacter cloacae, Clostridium butyricum, Clostridium pasteurianum, Desulfovibrio vulgaris, Magashaera elsdenii, Citrobacter intermedius, Escherichia coli*

2.1.1 Hydrogen Production by Biophotolysis of Water

Both algae and cyanobacteria split water and make oxygenic photosynthesis by evolving O_2. This method uses the same processes found in plants and algal photosynthesis, but adapts them for the generation of hydrogen gas instead of carbon containing biomass. Photosynthesis involves the absorption of light by two distinct photosynthetic systems operating in series: a water splitting and O_2 evolving system ("photosystem II" or PSII) and a second photosystem (PSI), which generates the reducetant used for CO_2 reduction. In this coupled process, two photons (one per photosystem) are used for each electron removed from water and used in CO_2 reduction or H_2 formation (Ramachandran et al, 1998).

This is an inherently attractive process since solar energy is used to convert a readily available substrate, water, to oxygen and hydrogen: $2H_2O \rightarrow 2H_2 + O_2$. However, direct biophotolytic processes, though inherently attractive, appear to suffer from the insurmountable barriers of oxygen sensitivity, intrinsic limitations in light conversion efficiencies, problems with gas capture and separation, and very onerous economics (Hallenback et al, 2002 and Melis, 2002).

2.1.2 Hydrogen Production by Dark Fermentation

During dark fermentative hydrogen production, anaerobic bacteria produce hydrogen by decomposing organic substrates under dark conditions. In case organic compounds are the sole carbon and energy source providing metabolic energy, the process is termed as 'dark' hydrogen fermentation. When light is required to provide additional energy, the process belongs to the category of photofermentative processes (Eroglu, 2006).

Fermentative processes can use either biomass obtained in a first stage light conversion process (e.g. higher plant or microalgae biomass high in starches or other fermentable substrates) or perhaps more attractively, various waste streams. The majority of microbial hydrogen production is driven by the anaerobic metabolism of pyruvate, formed during the catabolism of various substrates. Thus

in both these biological systems, the pyruvate generated by glycolysis is used, in the absence of oxygen, to produce acetyl CoA, from which ATP can be derived, and either formate or reduced ferredoxin, from which hydrogen can be derived. The enteric bacteria derive hydrogen from formate and strict anaerobes derive hydrogen from ferredoxin. The overall yields in these metabolisms are relatively low; one to two hydrogen produced per molecule of pyruvate. For one thing, this is a natural consequence of the fact that fermentations have been optimized by evolution to produce cell biomass and not hydrogen. Thus a portion of the substrate (pyruvate) is used in both cases to produce ATP giving a product (acetate) that is excreted. Also, in many organisms the actual yields of hydrogen are reduced by hydrogen recycling due to the presence of one or more uptake hydrogenases, which consume a portion of the hydrogen produced. The major issue is the feasibility of a dark fermentative reaction yielding close to the 12 mol of H_2 stored in each molecule of glucose metabolized. From a thermodynamic perspective, the most favorable products from the breakdown of 1 mol of glucose are 2 mol of acetate and 4 mol of H_2 (Hallenback et al, 2002).

Dark fermentative process can be obtained from anaerobic mixed cultures isolated from sewage sludges or some pure cultures that are known to produce hydrogen from carbohydrates include species of *Enterobacter, Bacillus* and *Clostridium*. The latter two are characterized by the formation of spores in response to unfavorable conditions (Hawkes et al, 2002).

In a digester, a consortium of anaerobic organisms work together to bind about the conversion of organic sludges and wastes. One group of organisms are responsible for hydrolyzing organic polymers and lipids to basic structural building blocks such as monosaccharides, amino acids, and related compounds. A second group of anaerobic bacteria ferments the breakdown products into simple organic acids, the most common of which is acetic acid. This group of microorganisms is described as non-methanogens, consisting of facultative and obligate anaerobic bacteria. They are often identified in the literature as “acidogenic” bacteria. Acidogenic bacteria commonly found are species of *Butyrivibrio, Propionic, Clostridium, Bacteroides* and *Ruminococcus, Acetivibrio*,

Eubacterium, Selenomonas, Lactobacillus, Streptococcus, and members of the *Enterobacteriaceae* (Zinder, 1984).

As a third group of bacteria, strict anaerobic methanogens converts the hydrogen and acetic acid into methane and carbon dioxide. Methanogens are present in sewage sludge at populations up to 108 per ml and contribute to 10% of the volatile solids. Most methanogenic bacteria utilize H_2 and CO_2, but species of only two genera, *Methanosarcia* and *Methanothrix*, can produce methane from acetic acid (Speece, 1996).

It might be feasible to harvest hydrogen at the acidification stage of anaerobic treatment, leaving the remaining acidification products for further processes such as photofermentation. Both from an economic and environmental standpoint, hydrogen is more attractive than methane as an energy source for replacing conventional fossil fuels (Ueno et al., 1996) and efforts have been directed toward production of hydrogen rather than methane (Ueno et al., 1995; Lay et al.,1999).

It is necessary to avoid the presence of organisms utilizing H_2, particularly methanogens, and this has been achieved in laboratory studies by operating at low pH and short retention times since methanogens are more affected by lower pH (usually pH<5) and are growing much slower than fermentative organisms (Kim et al., 2004).

Ueno et al. (1995) studied the hydrogen production potential of natural anaerobic mixed microflora with artificial wastewater containing cellulose. The microflora in sludge compost was found to produce a significant amount of hydrogen and carbondioxide, in addition to the generation of lower fatty acids (mainly acetate and butyrate) that constitute more than 90% of the total soluble metabolites.

Chemical compositions of the inoculated media have very significant effect on H_2 yield, as they influence the fermentation end products. Fermentations of hexose to acetate or butyrate produce H_2 and CO_2. Reduced fermentation end products such as ethanol and other alcohols contain additional H atoms not present in the corresponding acids, so alcohol production gives correspondingly lower H2

yields. It is important therefore to establish bacterial metabolism resulting in acetate and butyrate as end products. Therefore, if we know the actual metabolic pattern, it would be possible to drive the pathway towards a higher acetate/butyrate ratio so as to enhance hydrogen production by controlling environmental conditions such as pH, mixing intensity, hydraulic retention time (HRT), organic loading rate or the temperature (Khanal et al., 2004).

2.1.3 Hydrogen Production by Photofermentation

Photosynthetic bacteria are favorable candidates for biological hydrogen production due to their high conversion efficiency and versatility in the substrates they can utilize. For large-scale hydrogen production, an integrated view of the overall metabolism is necessary in order to interpret results properly and facilitate experimental design.

Photosynthetic bacteria are designated as the most promising microbial system among the biological hydrogen production processes (Fascetti and Todini, 1995; Miyake and Kawamura, 1987). Major benefits of using photosynthetic bacteria can be listed as follows:

a. They have higher substrate conversion efficiency into hydrogen

b. There is diversity in sources of substrates either for growth or hydrogen production. This facilitates their potential to be used in association with waste treatment.

c. They can remain functional under many different environmental conditions such as aerobic, anaerobic, with light or without light, and salty waters.

d. They can trap energy at a wide range of the light spectrum and can withstand high light intensities.

e. Large database is available for both future genetic improvement attempts and the photosynthetic hydrogen production mechanism.

2.1.3.1 General Characteristics of Purple Non-sulphur Bacteria

Purple non-sulphur (PNS) bacteria are prokaryotic and unique photosynthetic organisms since they have a single photosystem (lack photosystem II). Thus, they carry out anoxygenic photosynthesis. PNS bacteria have requirements for one or more water-soluble vitamins for phototropic growth, can grow at a pH of 6-9 that primarily depends on substrate source, and have an optimum temperature between 25 and 35 ºC (Sasikala et al., 1993). Additionally, they can live in both dark and light conditions; and all species are microaerophilic (Biebl and Pfennig, 1981). The cells are motile by polar flagella. They divide by binary fission and produce capsules and slime. When culture is matured, they become viscous due to the production of slime. Aged anaerobic cultures have a brown color, ranging from light-dirty greenish brown to dark brown. However, the brown color of an anaerobic culture can turn into red when exposed to air (Holt et al., 1984). The color of the bacteria is due to the pigments of bacteriochlorophyll and carotenoid, in which *R. sphaeroides* includes the photosynthetic pigments of bacteriochlorophyll a (with characteristic absorption maxima values; 372-375, 586-588, 800-805, 850-852 and 870-875 nm for living cells), and carotenoids of spheroidene series (with absorption maxima; 414-416, 446-450, 474-481 and 507-508 nm for living cells) (Pellerin and Gest, 1983).

Table 2.3 The classification of *Rhodobacter* species (Tabanoglu, 2002)

Domain	Bacteria
Phylum	Proteobacteria
Class	Alphaproteobacteria
Order	Rhodobacterales
Family	Rhodobacteraceae
Genus	Rhodobacter
Species	Capsulatus - Sphaeroides

2.1.3.2 Overview of Metabolism

Hydrogen production by *R. sphaeroides* and other purple non-sulphur (PNS) bacteria occurs under illumination in the presence of an inert, anaerobic atmosphere (such as argon), from the breakdown of organic substrates such as malate and lactate. The culture medium should be under a nitrogen limitation (i.e. a high C/N ratio), which forces the bacteria to 'dump' the excess energy and reducing power through the production of hydrogen. Several individual components make up the overall production system (Figure 2.1 and 2.2) and these may conveniently be grouped as: i) the enzyme systems, ii) the carbon flow – specifically the TCA cycle and iii) the photosynthetic membrane apparatus. These groups are interconnected within the hydrogen production scheme by means of the exchange of electrons, protons and ATP (Koku *et al.*, 2002).

Photosynthetic membrane apparatus converts light energy into ATP, which is directed into the nitrogenase together with protons and electrons. Protons are supplied in part by the TCA cycle, and the remaining are supplied by the action of ATP-synthase; working as a part of photosynthetic apparatus. Finally; nitrogenase reduces the protons to molecular hydrogen (Sasikala *et al.*, 1990). In the presence of hydrogenase; it functions primarily in the direction of H_2 consumption by producing ATP, protons and electrons. Therefore, the net collected H_2 amount is the amount produced by nitrogenase minus consumed by hydrogenase (Vignais et al. 1985).

Though a wide variety of substrates can be used for growth, only a portion of these is suitable for hydrogen production. The efficiency of a certain substrate depends on factors such as the activity of the TCA cycle, the carbon-to-nitrogen ratio, the reduction-state of that material and the conversion potential of the substrate into alternative metabolites such as PHB (Yiğit et al, 1999).

Hydrogen production and consumption in photosynthetic bacteria are mediated by nitrogenases and hydrogenases. While nitrogenase produces hydrogen, membrane-bound uptake hydrogenase consumes it (Vignais et al. 1985).

i. Nitrogenase:

H_2 evolution by photosynthetic bacteria attributed mainly to this enzyme, it is repressed by O_2. Reaction catalyzed:

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i \quad (2.1)$$

One mol of hydrogen and 2 mol of ammonia are formed by Mo-nitrogenase catalyzed reaction at the expense of 16 moles of ATP (Kars et al, 2006).

ii. Hydrogenase:

Responsible mainly for H_2 uptake (consumption) in photosynthetic bacteria. The enzyme catalyzes the simplest chemical reaction:

$$H_2 \leftrightarrow 2H^+ + 2e^- \quad (2.2)$$

The reaction is reversible, and its direction depends on the redox potential of the components able to interact with the enzyme. In the presence of H_2 and an electron acceptor, it will act as a H_2 uptake enzyme; in the presence of an electron donor of low potential, it may use the protons from water as electron acceptors and release H_2. Most of the known hydrogenases are iron-sulfur proteins with two metal atoms at their active site, either a Ni and an Fe atom (in [NiFe]-hydrogenases) or two Fe atoms (in [FeFe]-hydrogenases) (Vignais et al, 2007).

All these individual components of the hydrogen production interact and are subject to strict regulatory controls. An overall scheme for the hydrogen production metabolism and carbon flow is given in Figures 2.1 and 2.2, respectively.

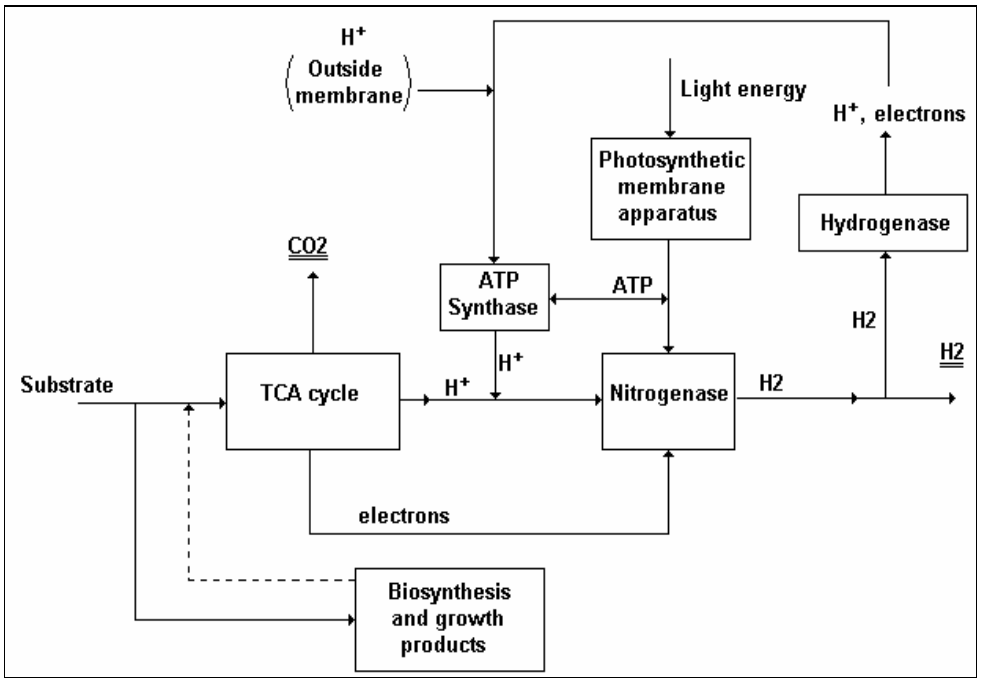


Figure 2.1 The overall scheme of hydrogen production by PNS bacteria (Koku *et al.*, 2002)

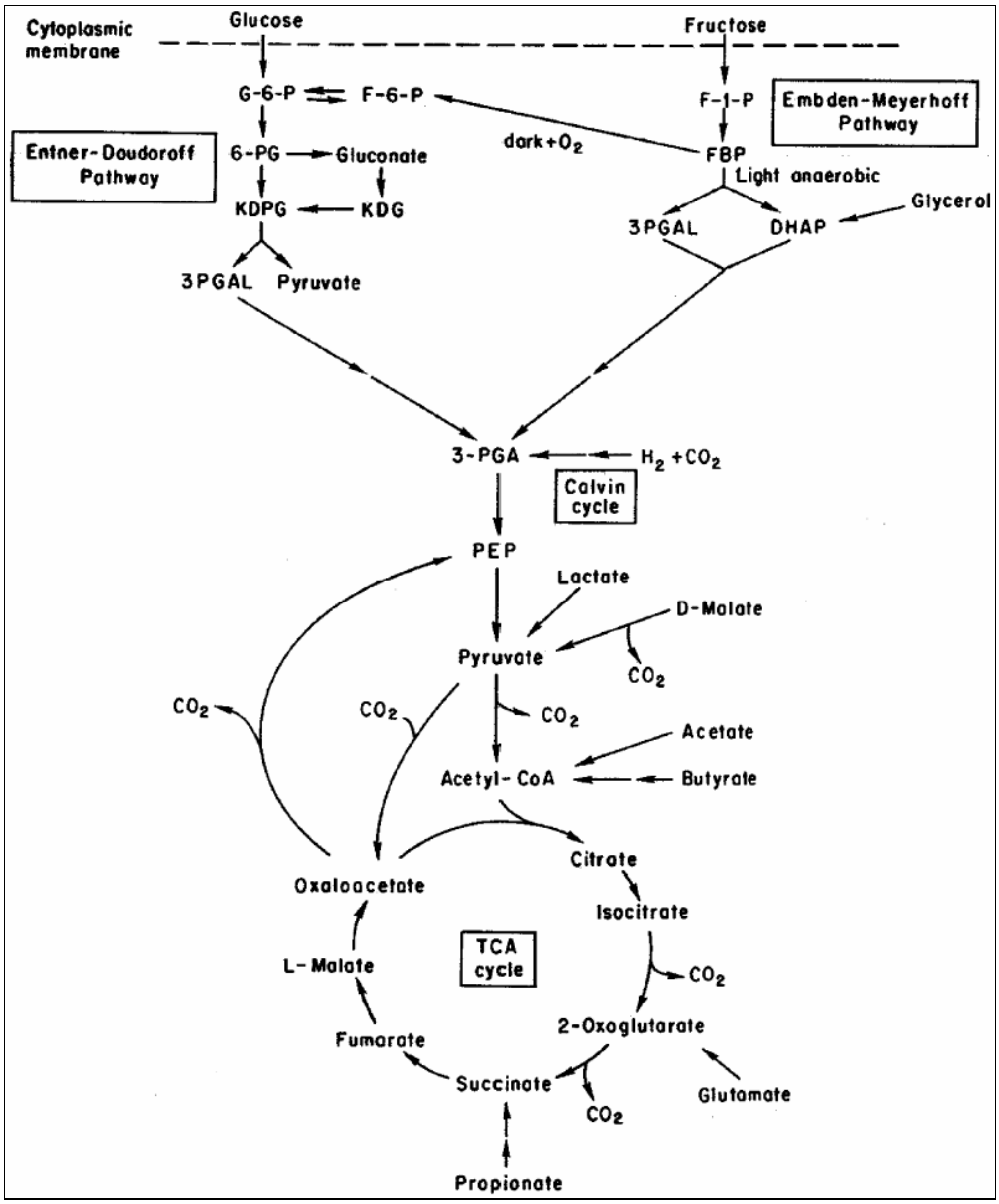


Figure 2.2 Simplified overall scheme of the carbon metabolism in PNS bacteria. (Koku et al., 2002)

2.1.3.3 By-products of Photofermentation Processes

One tool to overcome the economic restrictions of biological hydrogen production by photosynthetic bacteria is the simultaneous production of by-products which increase the added value of overall process. One of these by-products is obviously, the biomass itself. Cells from photosynthetic bacteria are rich in high quality protein and also contain biological co-factors and B group vitamins (Rocha et al., 2001).

Another important by-product of photofermentative hydrogen production process is polyhydroxybutyrate (PHB), which is an expedient source for various biotechnological processes. The thermoplastic properties of this polymer and its biodegradability set its importance as a substitute for petrochemical plastics. It has important industrial applications, particularly to construct biodegradable carriers for long-term dosages, either in the agriculture for herbicides and insecticides, or in the medical field for drugs and also for surgical sutures (Khatipov et al., 1998; Yiğit et al., 2000; Suludere, 2001).

PHB is mostly synthesized during unfavorable growth conditions, particularly under stress conditions through the stationary phase of growth, as an intracellular carbon and energy storage material for the bacteria and is accumulated as granules at different sites of cytoplasm. PHB accumulation inside the cells of photosynthetic bacteria, when grown under anaerobic conditions, depends on carbon and nitrogen availability, as well as the pH of the medium. The highest levels of PHB produced by *R. sphaeroides* were obtained with acetate (Krahn et al., 1996) under both ammonium and nitrogen poor conditions.

Carotenoid pigments are stated as another valuable by-product, which are essential for photosynthesis, since they transfer nearly half of the absorbed light energy to bacteriochlorophyll, and are to such an extent functional as light harvesting pigments. Moreover, its fundamental importance is due to the protection of photosynthetic bacteria from the photooxidative effects of light.

They are a class of hydrocarbons (carotenes) and their oxygenated derivatives (xanthophylls). During H_2 production process if any air is leaked into

the system, carotenoids of spheroidene series are oxidized with O_2 and then converted into their keto groups, which result the shifting of color from yellowish-brown to a deep rose red. Several carotenoid-lacking mutant strains of photosynthetic bacteria are known to be extremely sensitive to such photooxidations (Sistrom et al., 1956). Carotenoids has been used commercially during cancer chemoprevention; and also as a food colorant, natural antioxidant, or provitamin A source. Sasaki (1999) obtained the extracellular production of 5-aminolevulinic acid (ALA) from acetic and propionic acid containing medium prepared from the effluent of the anaerobic digestion of swine waste, using the cells of *Rhodobacter sphaeroides*. ALA can be applied to agricultural fields as herbicide, insecticide, and growth-promoting factor, or enhancer of salt tolerance for plants. In addition, ALA has applications in the medical field as a cancer treatment of diagnosis of heavy-metal poisoning and as medication.

2.1.4 Coupled Hydrogen Production

The combination of photosynthetic bacteria with anaerobic bacteria can provide a system for hydrogen photoproduction from residual carbohydrates such as organic wastes. In such a system, anaerobic fermentation of organic wastes produces low-molecular-weights organic acids in a first step, which are then converted to hydrogen by photosynthetic bacteria at the expense of light energy, in a second step (Barbosa et al, 2001).

The composition of the fermentor liquor obtained after first step fermentation depends on the substrate, microorganism type used and pH of the process. Table 2.4 lists the compositions reported in literature.

Table 2.4 The composition of the dark fermentation outlet liquor

m/o type	substrate	pH	Ace. (mM)	Prop. (mM)	Buty. (mM)	Ethanol (mM)	Ref.
mesophilic	sewage sludge	5-7	10-21	0-7	29-36	1-6	Horiuchi et al, 2002
mesophilic	sewage sludge	8	41-49	19-24	3-6	3-11	Horiuchi et al, 2002
mesophilic	refined sucrose	5.2	17-27		33-39	2	Hussy et al, 2005
mesophilic	municipal solid waste	5	17	8	32	22	Lay et al, 1999
mesophilic	sewage sludge	6.8	41	23	86	34	Lin et al, 2005
thermophilic	foodwaste	4.5-6.5	1-4		7-11		Shin et al, 2004
mesophilic	foodwaste	4.5-6.5	3-9	2-6	2-7		Shin et al, 2004
mesophilic	rice-waste	4-7	16-29	0-4	18-25		Fang et al, 2006
mesophilic	fruit-vegetable waste	5	83	37	250		Fascetii etl al,1998
mesophilic	molasses	5-6.5	9-22	0-3	1-2	32-60	Ren et al, 2006
mesophilic	steam exploded corn straw	4.3-5.4	61	20	25	12	Li et al, 2007
mesophilic	cornstalk wastes	4.5	15	9	13	5	Zhang et al, 2007
mesophilic	cattle dung and sludge	6.6	13	1	1		Tao et al, 2007

The types and concentrations of organic compounds obtained after the fermentation covers a wide range, however the main fermentation products can be identified as acetate and butyrate. Propionate was also present in considerable amounts and ethanol was reported in some cases.

The conversion of these acids to hydrogen would be advantageous in order to couple energy production with organic-waste treatment.

On the other hand, for hydrogen photoproduction, malate and lactate were reported as the most favorable substrates giving the highest hydrogen production rates (Hillmer et al 1977, Kim et al 1980, Miyake et al 1984). The drawback of the malate is its availability and high cost for large scale hydrogen production compared to other cheaper substrates such as acetate. However, little is known

about the conversion of acetic and butyric acids to hydrogen by photosynthetic bacteria (Sasaki et al. 1998, Segers et al. 1983), acetate and butyrate are reported to be favourable for production of PHB instead of hydrogen (Koku et al 2002). Moreover, PHB production competes with light dependent hydrogen production; the reason for this competition is that PHB production and hydrogen production use the same reducing power that results from the metabolism of organic acids (Yigit et al 1999).

2.2 Bioreactor Types and Construction Materials

A bioreactor is a device or system that supports a biologically active environment. Photobioreactors can be defined as culture systems in which light has to pass through the transparent reactor's wall to reach the cultivated cells that carry out a light dependent biological process (Tredici, 2004).

Many photobioreactor types constructed from different materials researched so far for biological hydrogen production processes; these include channel plate bioreactors made of acrylic sheet (Modigell et al, 1998), spiral tubular bioreactors made of transparent PVC tubing for H_2 production by cyanobacteria (Markov et al, 1997), bubble column bioreactors (650 ml in liquid volume) made of glass for the photoproduction of hydrogen by the green alga (Kojima et al, 2004) pneumatically agitated flat-panel photobioreactor made of a stainless-steel frame and polycarbonate panels for H_2 production by *Rhodopseudomonas* sp (Bijmans et al, 2002) and glass bottles with different sizes used for the hydrogen production by *Rhodobacter sphaeroides* (Sasikala et al 1995, Eroğlu et al 2004, Miyake et al, 1999), by *Rhodobacter capsulatus* strains (He et al 2006) and by *Rhodopseudomonas palustris* (Chen et al, 2006). Apart from those custom-made photobioreactors, commercially available fermenters were also used after modification: Younesi et al (2007) used 2 L such a fermenter for biohydrogen production by *R. rubrum*.

Utilization of different photobioreactor materials was also reported for different processes than hydrogen production: Sierra et al (2007) used U-shaped

disposable plastic bag located between two iron frames as a 250 L photobioreactor to produce microalgae.

2.3 Outdoor Bioreactors

Kim et al. (1987) examined continuous outdoor hydrogen production for 45 days in Sendai, Japan, using *Rhodobacter sphaeroides* B5.

Miyake et al. (1987) studied efficiency of light energy conversion to hydrogen by *Rhodobacter sphaeroides* and termed conversion efficiency (%) to be combustion enthalpy of H_2 / absorbed light energy x 100.

In Japan, Miyake and Asada (1993) studied photosynthetic bacterial hydrogen production by solar energy. They used agar immobilized *Rhodobacter sphaeroides* 8703 cells. The efficiency of solar energy conversion was as high (7%) at low intensities of light (below 100 W/m^2), and it dropped down to 2% at high intensities (1000 W/m^2).

Ogbonna et al. (1999) state that, day–night cycles and diurnal variation in light intensity is a major problem with the use of solar light energy. Depending on the location and season, the number of hours per day when the light intensity is high enough to support photosynthetic cell growth can be very short. In the absence of light energy or some other metabolizable organic carbon source in the medium, cells metabolize the cell components to obtain maintenance energy, thus leading to a decrease in cell weight. In order to overcome the problems of diurnal variation in solar light intensity and prolonged periods of bad weather, an illumination system with integrated solar and artificial light sources was developed. Solar light is used for illumination during the day but when the solar light intensity decreases below a set value (during cloudy days and at night) the system switches automatically to artificial light source, thus ensuring continuous light supply to the reactor.

Rechenberg (1998) screened purple bacteria on world wide and directed evolution experiment in the laboratory. This research resulted in the isolation of the strain *Rhodobacter sphaeroides* DSM 9483 which had the maximum total

hydrogen evolution (4.55 ml hydrogen/ml culture) at 40 °C. Later, the experiments were carried out at Sahara. The column bioreactor of 1.4 L of capacity was made of two glass flasks with transparent jackets. Instead, they were filled with laser dye filter. The optimum laser dye has an absorption range from 420-520 nm, which corresponds with the absorption range of the carotenoids. Hydrogen production was thought to be enhanced because the laser dye transforms the absorbed wavelengths into longer ones, which are more effective in photosynthesis. They were able to produce 10 L of hydrogen in 7 days.

Kitajima et al. (1998) examined hydrogen production for 12 days in an outdoor experiment using plane type PBR with different depths and reported a conversion efficiency of 1.1 %.

Modigell and Holle (1998) investigated hydrogen production with an outdoor reactor in the form of hollow channel plates made of acrylic glass that are connected at the top and at the bottom to form a loop and erected vertically and placed at east-west position. The total area was $8m^2$ and they used *R. rubrum* cells and used lactate for outdoor hydrogen production experiments. Constant hydrogen production was observed with exchange of the half of the medium every fifth day for almost two months, indicating that there was no significant contamination of the reactor although no sterile methods or substrates were applied. They were able to obtain 40 $L_{hydrogen}/m^2_{ground\ area}$·day.

Arai et al. (1998) investigated hydrogen production by *Rhodobacter sphaeroides* RV using lactate and propionate as carbon sources. They used outdoor flat bioreactors. The experiments were held in winter season in Japan, from October 1994 to March 1995. The bioreactors were made of acrylic resin and had the irradiated area of 20 x 44 cm^2, with inner thickness of 5 cm and capacity of 4.4 L. Hydrogen production rate was 0.0125 $L_{hydrogen}/L_{culture}$·h.

Ogbonna et al. (1998) constructed an internally illuminated PBR by solar light with optical fibers was studied for carbon dioxide fixation by *Chlorella pyrenoidosa* and α-tocopherol production by *Euglena gracilis* in a 20L PBR. However, still this type of reactor has to use artificial light during cloudy days and the optical fibers that are used are very expensive.

Miyake et al. (1999) studied the simulation of a daily sunlight illumination pattern for photohydrogen production. As a reference they also carried out outdoor experiments with reactors facing south. For outdoor operations hydrogen production rate was 14-28 $L_{hydrogen}/m^2 \cdot day$ and for indoor operations hydrogen production rate was 79.2 $L_{hydrogen}/m^2 \cdot day$. They found conversion efficiencies as 1.1, 0.9, and 1.0%, respectively. Also they stated that, 12 hours light and 12 dark light cycle operation simulated outdoor conditions very well.

Carlozzi et al. (2001) studied biomass production by *Rhodopseudomonas palustris* grown in an outdoor temperature controlled underwater tubular photobioreactor. The PBR consisted of a loop: the loop was made up of ten 2 m long parallel glass tubes with ID of 4.85 cm and OD of 5.0 cm. These tubes were connected by polyvinylchloride (PVC) U-bends with watertight flanges. The reactor pipes were 0.05 m spaced, determining the smaller side of the reactor 1.0 m while the larger side was 2.0 m. The total length of the illuminated portion of the tubes exposed to the sun was 20 m. The occupied area of the PBR was 2.0 m^2.

Hu et al. (1996) stated that, when the front surface of the flat panel reactor was inclined towards to the south, it receives the major thrust of solar irradiance and the back side of the reactor surface was also illuminated by diffuse and reflected light which may be very effective for photosynthesis. In this research, the individual reactors were facing south with inclinations of 30° and 60° for summer and winter, respectively.

Zhang et al. (1999) stated that when the illuminated surface was placed in an east-west facing orientation, the productivity was higher that that in a north-south facing orientation, because more solar energy was received in the former case than in the latter.

Richmond et al. (2001) states that, flat reactors made of glass represent very promising photobioreactor type due to well-illuminated entire surface area, which is an essential advantage for high phototrophic activity. When tilted, the front surface exposed directly to the sun receives the major thrust of solar irradiance, but the back panel as well as the side walls are also illuminated by

diffuse and reflected light rather low photon flux densities, but very effective for photosynthesis.

Eroglu et al (2007) reported the performance of an eight liter flat plate solar bioreactor operating in outdoor conditions by using different organic acids (malate, lactate, and acetate) and olive mill waste water as carbon sources and determined the accumulation of byproducts, such as PHB and carotenoid.

2.4 Scope of the Thesis

The main objective of this PhD thesis is exploring the parameters affecting photobiological hydrogen production and to develop anaerobic photobioreactors for efficient hydrogen production from photofermentation of organic acids by purple non-sulphur bacteria.

In order to achieve this goal, some of the key parameters of the bioreactor and process design were defined and studied extensively:

Physiological Studies

The studies in this area are carried out to screen the hydrogen yield and productivity of the wild type and mutant *Rhodobacter* strains (*R. Sphaeroides* and *R. capsulatus*) in organic acids. For this purpose, well-defined laboratory scale photobioreactors are designed, constructed and operated, in which continuous hydrogen evolution data can be recorded. Effect of the physiological conditions such as the temperature, pH, substrate concentrations on the growth rate and the hydrogen production rate are investigated.

Rhodobacter species are photoheterotrophic bacteria that can easily switch to fermentative mode if the illumination is below a threshold value. Hydrogen can not be produced during fermentation but other organic acids that are the products of fermentation can be used for hydrogen production if it is illuminated again. Therefore the effect of the properties of the incident light (such as intensity and wavelength distribution of photons) and illumination protocols (such as light-dark cycles) on the growth and hydrogen production of the bacteria are investigated.

The data are analyzed to find kinetic models of substrate utilization, growth, hydrogen production. Hydrogen productivity and yield are studied. The light distribution and absorption in the photobioreactor are documented. These studies provide data and set a basis for photobioreactor design, operation conditions and scale up evaluation.

Flat Panel Photobioreactor Development

The studies in this area are carried out to design, construct and operate anaerobic flat panel photobioreactors at different scales for biological hydrogen production. Test runs are carried out and the obtained results are analyzed and evaluated in terms of light and substrate conversion efficiencies, rate of growth and hydrogen production, stability and reproducibility of the operation. The light and temperature distribution inside and on the surface of the bioreactors are also determined to better understand and document the process conditions. The last step of this stage is to scale-up, that is, test the applicability of biological hydrogen production at large scales.

Process Technology for Outdoor Applications

The biological production of hydrogen at a competitive cost in industrial scales can only be possible in photobioreactors which are illuminated by sunlight. The process is adapted to outdoor conditions by examining the uncontrolled parameters such as light intensity, diurnal cycle and temperature changes. The response of the system to the changes in these parameters is investigated. The bacteria generates heat by absorbing sunlight increasing the temperature in outdoor bioreactor to lethal levels thus cooling methods (such as shading and water spraying) are adapted to the system. The test runs in outdoor are carried out under clear sky and cloud conditions by both wild type and mutant bacteria and the results are evaluated.

CHAPTER 3

MATERIALS AND METHODS

3.1. The Microorganisms

Several photosynthetic bacteria strains were used throughout this research. Wild type strains used were *Rhodobacter sphaeroides* O.U.001 (DSM 5864), *Rhodobacter capsulatus* (DSM 1710), *Rhodobacter capsulatus* (DSM 155), *Rhodosprillum centenum (synonym: Rhodocista centenaria) (DSM 9894), Rhodosprillum rubrum* (DSM 107). Those strains were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH - German Collection of Microorganisms and Cell Cultures, Germany). A mutant strain lacking uptake hydrogenase enzyme (hup^-) that was used in outdoor studies part, *Rhodobacter capsulatus* YO3, was genetically modified by Dr. Yavuz Öztürk (GMBE, TÜBİTAK-MAM, Gebze) from *Rhodobacter capsulatus* MT1131. The microscopic views of *R. rubrum* and *R. capsulatus* (DSM 155) were given in Appendix A.

3.2. Culture Media

3.2.1. Liquid Media

3.2.1.1 Growth Media

The bacteria were grown on Biebl and Pfennig (1981) medium with some modifications. The Biebl and Pfennig medium was prepared with malate (7.5 mM) as the carbon source and glutamate (10 mM) as the nitrogen source. Additional

components of the medium were vitamins, trace elements and iron-citrate. The preparation of the solutions were given in Appendix B. After mixing the substrates, the pH of the medium was adjusted to 6.4-6.8 by the addition of NaOH. An experiment attempting to find out the minimal nutrient requirements for the bacteria were given in Appendix C.

3.2.1.2 Hydrogen Production Media

Hydrogen production media was the same of the growth medium except the carbon and nitrogen sources. Types and concentrations of the C and N sources were different for many of the experiments conducted but typically malate (15 mM) as the carbon source and glutamate (2 mM) as the nitrogen source were used for *R. sphaeroides* and acetate (40 mM) + lactate (7.5mM) as the carbon source and glutamate (2 mM) as the nitrogen source were used for *R. capsulatus* unless otherwise stated. The primary difference from the growth media is the nitrogen limitation to promote hydrogen production.

3.2.2 Solid Media

Solid media were used for plate counts and contamination detection. The solid media is the same of the liquid growth media except that it contains agar (0.9-1.5 % w/w). In order to prepare agar-solidified medium, agar was dissolved in the growth medium and boiled for 10 minutes then dispensed into plates while still molten. After the media cooled down and solidified, liquid culture spread on the agar plates. The plates were incubated at 36 °C under light.

3.3 Experimental Setup for Hydrogen Production and Operating Conditions

For many of the indoor hydrogen production experiments, rubber-tapered glass bottles with 55-500 ml of volume were used. The temperature of the photobioreactor was maintained at 30-32ºC in the incubator. The illumination was

provided by 75-100 W tungsten lamp, adjusted to provide a uniform light intensity of 3500 lux (200 W/m^2) at the surface of the bioreactor. A schematic diagram and photograph of the experimental setup is shown below in Figure 3.1 and 3.2.

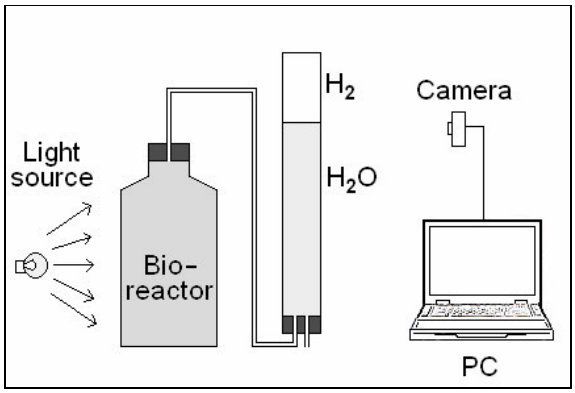


Figure 3.1 Schematic presentation of the experimental setup

Figure 3.2 Picture of the experimental setup

The vessels were either fully filled with medium to create anaerobic conditions (dissolved oxygen in the medium is neglected) or a 10% headspace was left and argon gas was flushed through the media to strip off the oxygen and provide anaerobic atmosphere in the bioreactor.

The sterilization of the photobioreactors and media were accomplished by autoclaving. Sterilized medium was added into the reactor near flame or in a sterile chamber. The amount of inoculation to the bioreactors was 10% by volume of the fresh medium (25% for outdoor experiments). During the experiments, the evolved gas was collected and measured volumetrically by graduated-glass burettes by the reversible replacement of water. The produced gas amounts were monitored by a digital camera continuously.

The operational conditions were:

Microorganism used: Purple non-sulphur photosynthetic bacteria

Temperature: 30-32 ºC

Starting pH: 6.4-6.8

Light Intensity: 200 W/m^2

Atmosphere: Anaerobic

Inoculation amount: 10%

Bioreactor operating mode: Batch

3.4 Experimental Procedure

3.4.1 Preparation of the Inoculum

Growth medium was prepared in glass bottles with rubber caps. These medium containing bottles were sterilized in an autoclave for 15 minutes at 121 ºC. After cooling to room temperature, filter-sterilized (20µm pore size) vitamin solutions were added and the bottles were sparged by argon gas (>99 % purity) with a flow rate of 100-150 ml /min for about 5 minutes, in order to obtain an anaerobic atmosphere inside.

Then, 10% inoculum of the bacteria from active culture was injected into the prepared anaerobic liquid media by using sterile syringe needles. In the end, the incubation was carried out at 30-33 ºC in an incubator under the illumination of a 100 W tungsten lamp, which was placed at a distance of 15-20 cm from the front surface of the bottle.

The inoculum is ready once it is in mid-logarithmic phase (at around OD 1.0), which usually takes 1-2 days of incubation.

For storage purpose of bacteria, after 48 hours of growth, sterile glycerol (10% v/v) of was injected into the bottle with a sterile syringe, in order to protect the cells from damage during freezing. Then, the bottle was stored at -80 ºC. When inoculating a new culture from that stock, the bacteria were transferred at least two times into fresh media to get rid of the glycerol.

3.4.2 Cleaning and Sterilizing the Photobioreactors

The photobioreactors were sterilized in autoclave if possible (i.e. glass bottles with rubber caps). Larger photobioreactors made of plexiglas were sterilized chemically; by using H_2O_2 (3% solution), benzalkonium chloride or ethanol (70% solution) then cleaned with sterile distilled water.

3.4.3 Inoculation of the Photobioreactor

The prepared anaerobic liquid media was put into the photobioreactor, then 10% inoculum of the bacteria from active culture was added. Argon gas flushed to remove the air from the bioreactor and the incubation was carried out at 30-33 ºC under the illumination of a 100 W tungsten lamp (for indoor studies).

3.4.4 Sampling and Shut-down of the Photobioreactor

The sampling was mostly made by using a sterile syringe, 0.5-10 ml of samples were collected depending on the analysis to be performed on the sample.

For small photobioreactors (<500ml) where the volume difference due to sampling is important, basal medium (medium has same composition with the growth medium but without N and C sources) of equal amount was injected into the photobioreactor while taking the sample. In the bigger photobioreactors where culture loss due to sampling was negligible, basal medium was not added. In the biggest photobioreactors (5-25L), samples were collected by opening a valve mounted on the photobioreactor, without using a syringe.

Once the process ended, the photobioreactor was emptied, the effluent was sterilized either by autoclaving or chemically and then discarded. The photobioreactor was washed to remove cell debris.

3.5 Analyses

3.5.1 Gas Amount and Composition

Produced gas amount were tracked by a device and software developed during the present study which allowed monitoring of the produced gas amount in real-time with high precision (0.1ml/h). This system has been commercialized under tradename ObiGasMaster by a biotechnology company (OBİTEK LTD ŞTİ) in 2006. The details of the commercial product were given in Appendix D.

Gas composition was analysed by gas chromatography. Two gas chromatogprahy devices were used for the analysis throughout the study. One was Hewlett-Packard 5890 Series II with thermal conductivity detector. The oven, injector and detector temperatures were 30, 40 and 50 ºC, respectively. Nitrogen was used as a carrier gas at a flow rate of 11 mL/min for gas determination with Propak Q column. The other device was Agilent Technologies 6890N with thermal conductivity detector. Argon was used as a carrier gas at a flow rate of 1.4 mL/min for gas determination with HP19091F-413 column. The oven, injector and detector temperatures were 150, 50 and 250 ºC, respectively. A typical gas analysis chromatogram is given in Appendix E.

3.5.2 Organic Acid Analysis

Liquid samples were initially filtered through a 20µm Nylon filters (R0-000381-55, Varian) for organic acid analysis by HPLC system (VarianProStar HPLC) at Middle East Technical University Central Laboratory. Liquid samples (20 µL) were analyzed by a MetaCarb 87H (300x 7.8 mm, 5 µm) HPLC column. 0.008 M H_2SO_4 was used as the mobile phase. The standard analysis of photobioreactor effluents was performed at 35 °C with a mobile phase flow rate of 0.6 mL/min. The flow rate and temperature were adjusted and maintained by an HPLC pump (ProStar 240 Quaternary Gradient Solvent Delivery Module).

ProStar 330 PDA (210 nm) was used to detect the column separation. Recording and integration of the chromatogram data was carried out through an electronic data acquisition unit. The relation between peak areas and component concentrations were determined by the construction of calibration curves for different concentrations of pure organic acid standards.

3.5.3 Cell Concentration

The cell concentration was obtained by measuring the optical density of culture or performing the dry cell weight analysis.

Measuring the optical density of the culture is one of the simplest and direct ways of the bacterial cell concentration determination. For this purpose, absorbance of the culture at 660 nm was detected by a visible spectrophotometer (Shimadzu UV-1201). Fresh medium was used as a blank solution. Absorbance values were converted to dry cell weights by the help of the calibration curve which was constructed by dry cell weight analysis.

For the determination of the bacterial dry cell weight, first 10 mL samples were taken from the reactor and centrifuged (Sigma) at 10000 g for 20 minutes. Then, the supernatant was removed and the pellet residue was transferred to small aluminum caps, which were previously weighed. The pellets were dried overnight at 40 ºC in an oven (Thelco, Model-18, Precision Scientific). Then, the caps

containing dried bacteria were weighed again. The bacterial dry cell weight was determined by subtracting the weight of empty cap from the total weight of cap and dried pellet.

Then, dry cell weight versus OD_{660} calibration curve was obtained from the samples corresponding to the various points of the growth. The calibration curves of dry cell weight (gdw) versus OD_{660} were given in Appendix F. Calibration gives that, OD_{660} value of 1.0 corresponds to 0.56 gdw/$L_{culture}$. That calibration factor was found to be almost same for *R. capsulatus* and *R. sphaeroides*.

3.5.4 pH Analysis

The pH of the culture was measured with a standard combination of pH electrode (Mettler Toledo 3311) and an electronic transmitter (Nel pHR-1000 Transmitter).

3.5.5 Temperature Analysis

The temperature inside the bioreactors were measured by using a digital thermometer probe. The surface temperature of the photobioreactors were measured by using an infrared thermometer (Testo 830-T1).

3.5.6 Light Intensity and Wavelength Analysis

Light intensity and spectrum measurements were made by a luxmeter (Lutron) and a spectroradiometer (StellarNet EPP2000-VIS-50), respectively.

3.5.7 Bacteriochlorophyll *a* Analysis

For the determination of the Bacteriochlorophyll a content of the bacteria, sample taken (usually 1 ml) from the photobioreactor was centrifuged at 10000 rpm for 10 minutes. Then the supernatant was discarded and acetone-methanol

mixture (7:2 v/v) were added for the extraction of the bacteriochlorophyll *a*. The mixture was vortexed one minute for homogenization. The homogenate was centrifuged again at 10000 rpm for 10 minutes to remove almost all proteins under ambient conditions (Hirabayashi *et al.*, 2006). The supernatant was separated and the concentration of bacteriochlorophyll *a* was determined from the absorbance at 770nm (extinction coefficient=76 $mM^{-1}cm^{-1}$), acetone-methanol mixture was used as blank (Clayton, 1966). Cellular bacteriochlorophyll *a* content was calculated by considering the cell dry weight. Greater than 92% of the bacteriochlorophyll was extracted by this method (Biel, 1986).

3.5.8 Software Used

Gas Chromatography: Agilent Chemstation ver.B.01.01 (Agilent Technologies)
Gas Amount: ObiGasMaster ver3.0 (Obitek Ltd Şti)
Light wavelength analysis: Spectrawiz Spectrometer ver.4.0g (StellarNet Inc.)
Online temperature: Elimo Data Logger Manager ver5.1
Online light intensity: Lutron data acquisition software ver.V9812TW
Cell growth modelling: Curve Expert ver1.3

CHAPTER 4

PHYSIOLOGICAL STUDIES ON PURPLE NON-SULPHUR BACTERIA

Physiological parameters such as pH, temperature, medium composition and light intensity affect the growth and hydrogen production of PNS bacteria. The bacteria can grow in a limited range of these parameters (i.e. non-acidic pH, mesophilic temperatures) and produce hydrogen under even more limited conditions (i.e. high C/N ratio, neutral pH, under illumination). It is of primary importance to determine the ranges and optimum values of physiological parameters to obtain high hydrogen production rate and yield, in a stable and reproducible manner.

In this study the culture media constituents (type and concentration of C and N sources, minor nutrients such as Fe, Mo, Ni and buffer amount) were optimized. Other parameters that affect the process such as pH and temperature were also studied. Lastly, optimization of light and illumination patterns was carried out. The experiments were conducted with two PNS bacteria species: *R. sphaeroides* and *R. capsulatus*.

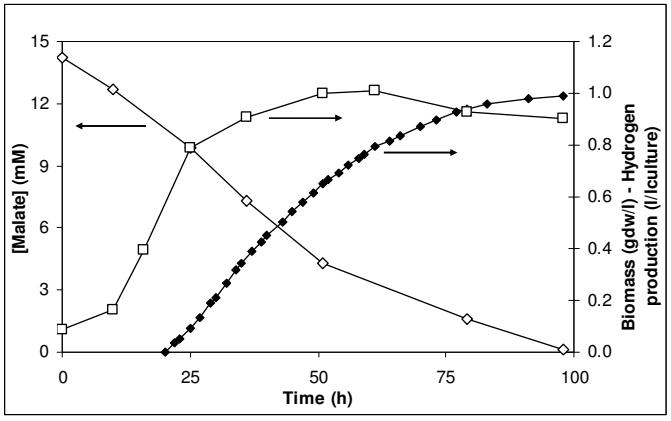
4.1 Hydrogen Production in Organic Acid by *R. sphaeroides*

This part of the thesis study had two objectives: one is the analysis of the biomass growth and hydrogen production in a photobioreactor with media containing different organic acids as carbon sources (malate, acetate, propionate, lactate, pyruvate and butyrate) in order to determine alternative carbon sources for biohydrogen production and to document the efficiencies and production rates for each of the substrates. Second objective was to focus further on the utilization of acetate, butyrate, propionate and the mixtures of those carbon sources in order to

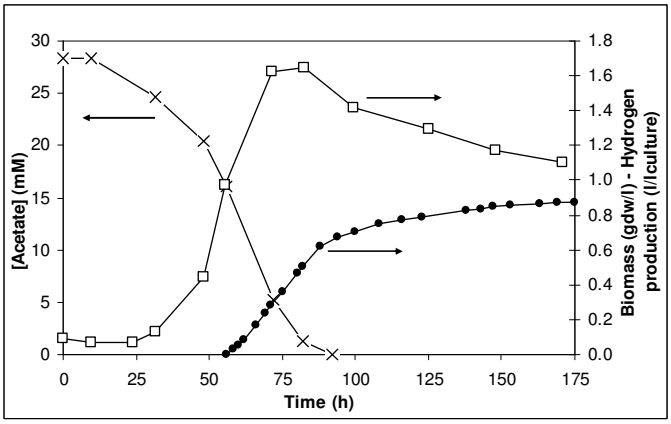
investigate the appropriate conditions of coupling fermentation with photofermentation for biological hydrogen production. *Rhodobacter sphaeroides* O.U. 001 was used as bacteria. The experiments were carried out in 55 ml vessels.

Hydrogen production experiments were conducted in media containing different carbon sources; malate (15mM), acetate (30mM), propionate (20mM), lactate (20mM), pyruvate (20mM) and butyrate (15mM). All other parameters were kept same but the carbon sources. The C/N ratio in the media is a critical parameter for hydrogen production process and previously optimized for *R. sphaeroides* for malate and glutamate as 60/2 (Eroglu et al,1999). The same ratio was used in this experiment and kept fixed for all bioreactors. Thus the concentrations of the organic acids were proportional to their C content. The nitrogen source, Na-Glutamate, was 2mM.

Figure 4.1 and 4.2 show the bacterial growth, hydrogen production and substrate concentration (available for malate, acetate and butyrate only) data obtained with respect to time. Table 4.1 lists the overall results obtained.



(a)



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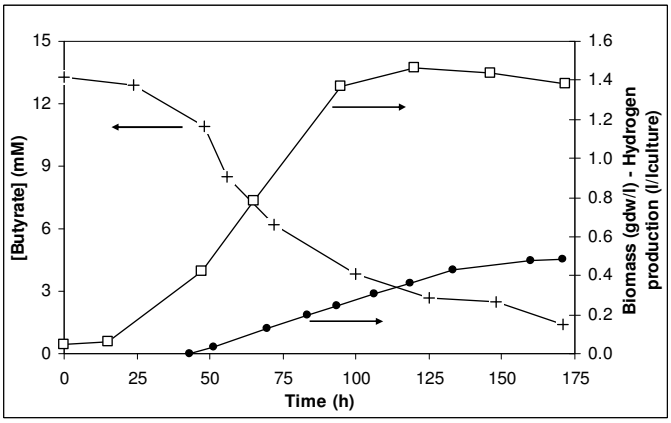
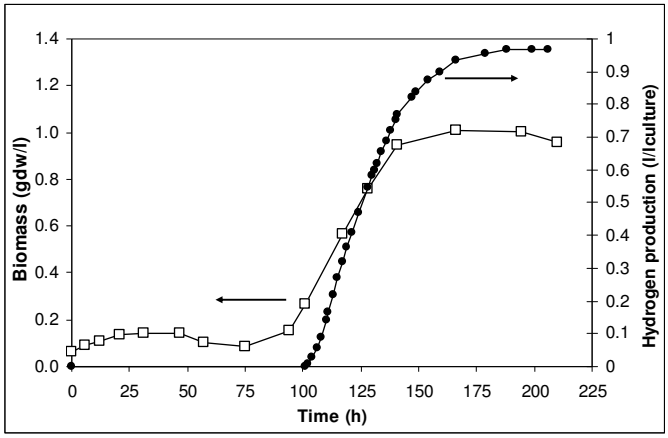
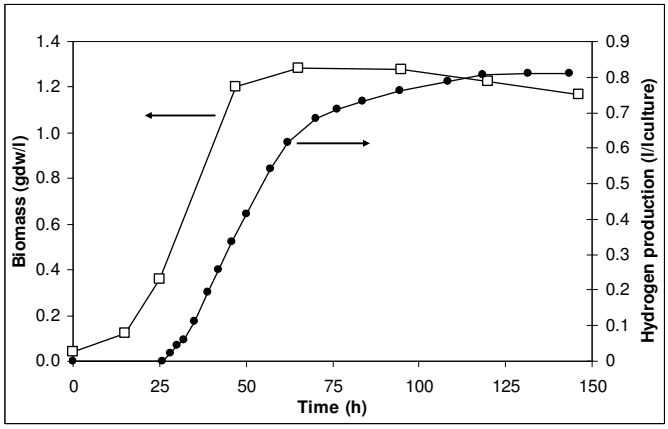


(c)

Figure 4.1 Bacterial growth of *R. sphaeroides* ( ), hydrogen production (●) and substrate consumption (malate (◊), acetate (X) and butyrate (+)) obtained in 55ml bioreactors containing a) 15 mM of malate, b) 30 mM of acetate, c) 15 mM of butyrate as the carbon source with 2 mM of glutamate as the N source.



(a)



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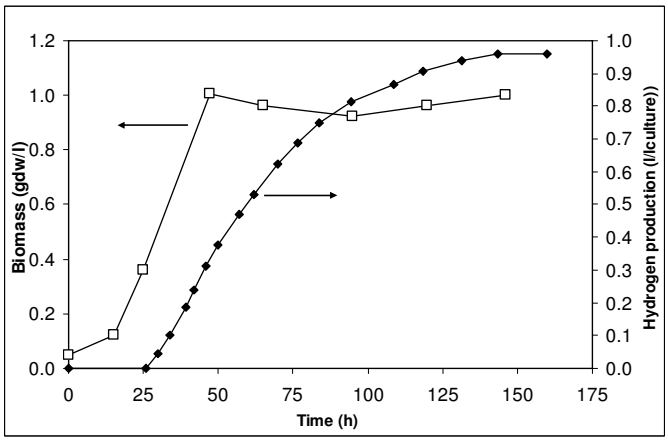


(c)

Figure 4.2 Bacterial growth of *R. sphaeroides* ( ) and hydrogen production (●) in 55ml bioreactors containing a) 20 mM of propionate, b) 20 mM of lactate, c) 20 mM of pyruvate as the carbon source with 2 mM of glutamate as the N source.

Table 4.1 Hydrogen production by *R. sphaeroides* in organic acid as substrate

	Malate	Acetate	Propionate	Lactate	Butyrate	Pyruvate
Initial C source concentration (mM)	15	30	20	20	15	20
C/N ratio (mol/mol)	60/2	60/2	60/2	60/2	60/2	60/2
Max. Cell concentration (g/L)	1.01	1.65	1.01	1.28	1.46	1.01
Final hydrogen produced ($l/l_{culture}$)	1.00	0.89	0.96	0.82	0.47	0.96
Hydrogen prod. lag time (h)	20	56	102	26	42	25
Hydrogen prod. duration (h)	63	78	65	69	96	92
Average gas prod. rate ($ml/l_{culture}.h$)	15.9	11.4	14.8	11.9	4.9	10.4
H_2 productivity (mg $H_2/l_{culture}.h$)	1.31	0.94	1.22	0.98	0.40	0.86
Theoretical H_2 yield (mol H_2/mol org. acid)	6	4	7	6	10	5
Molar H_2 yield (mol H_2/mol organic acid)	3.14	1.40	2.14	1.83	1.76	2.14
H_2 yield (g H_2/g substrate)	0.041	0.046	0.054	0.037	0.030	0.045
Substrate conversion efficiency (%)*	52	35	31	31	18	43
Light conversion efficiency (%)	0.66	0.47	0.61	0.49	0.20	0.43

*HPLC data were used if available, otherwise it was assumed that all the substrates were used by bacteria.

A particularly useful parameter for characterizing microbial hydrogen production is the substrate conversion efficiency, which is a measure of how much of the substrate has been utilized for hydrogen production rather than growth or alternative biosynthesis.

It was determined as the ratio of moles of hydrogen that have actually been produced per moles of hydrogen expected through stoichiometric conversion of the substrate according to the following hypothetical reaction:

$$CxHyOz + (2x - z)H_2O \rightarrow (y/2 + 2x - 2)H_2 + xCO_2 \quad (4.1)$$

or more precisely, for the substrates that were used in this study:

$$\text{Malate:} \quad C_4H_6O_5 + 3\,H_2O \rightarrow 6\,H_2 + 4\,CO_2 \quad (4.2)$$

$$\text{Lactate:} \quad C_3H_6O_3 + 3\,H_2O \rightarrow 6\,H_2 + 3\,CO_2 \quad (4.3)$$

Acetate: $C_2H_4O_2 + 2\,H_2O \rightarrow 4\,H_2 + 2\,CO_2$ (4.4)

Propionate: $C_3H_6O_2 + 4\,H_2O \rightarrow 7\,H_2 + 3\,CO_2$ (4.5)

Butyrate: $C_4H_8O_2 + 6\,H_2O \rightarrow 10\,H_2 + 4\,CO_2$ (4.6)

Pyruvate: $C_3H_4O_3 + 3\,H_2O \rightarrow 5\,H_2 + 3\,CO_2$ (4.7)

Here, only organic acid was considered as the substrate for hydrogen production, Na-glutamate (N source in the media) was not taken into account since it is mainly used for biomass formation. Another C source present in the media is Fe-citrate, it is neglected due to the very low concentration (Appendix B).

It should also be noted that the CO_2 produced remains in the media as bicarbonate (HCO_3^-) and the bacteria used in this study are able to utilize it back as a C source, thus those stoichiometric equations do not reflect the actual gas output compositions. The gas collected typically had more than 98% hydrogen for all of the runs.

In the literature, the substrate conversion efficiencies for photofermentative hydrogen production were reported to cover a wide range depending on the microorganism type and the substrates used: 6% to 72% for *R. capsulatus* and 24% to 80% for *R. sphaeroides* (Koku et al, 2003).

Sample calculation of the substrate conversion efficiency was given in Appendix G.

Average gas production rate and hydrogen productivity are useful tools to determine the efficiency of the process based on the bioreactor culture volume. Hydrogen yield shows the efficiency of the process based on substrate used. Sample calculations of the hydrogen production rate, hydrogen productivity and hydrogen yield were provided in Appendix H.

The obtained results showed that the bacteria were capable to metabolize all the different organic acids tested. The growth and hydrogen production varied however. It was also observed that the bacteria enters into death phase once the carbon source was depleted however hydrogen production continues for a while, possibly by using the endogeneous reserves of bacteria such as PHB.

Malate was the best carbon source for hydrogen production as expected: The highest substrate and light conversion efficiencies, highest hydrogen production rate, shortest lag time and highest amount of hydrogen and yield, were obtained in malate containing media. Malate is utilised by two different pathways, depending on the optical isomer. L-malate is metabolised by the TCA cycle while D-malate enters the carbon flow by first being converted to pyruvate, as shown in Figure 2.2.

Butyrate is expected to produce 10 mol H_2/mol butyrate theoretically but only 1.4 mol H_2 /mol butyrate was produced, it has the lowest substrate conversion efficiency. The rest is possibly converted to other metabolites and the reserve material PHB (Hillmer et al., 1977).

Pyruvate is utilised by practically all strains of PNS bacteria, though the manner of utilisation might be quite different (Tabita, 1995). In *R. sphaeroides* or *R. capsulatus* pyruvate assimilation under phototrophic conditions starts by conversion of pyruvate into acetyl-CoA or oxaloacetate (Willison, 1988). When nitrogenase is active, pyruvate is also a good substrate for hydrogen production.

Since the inoculum was grown in malate containing media, long lag times for hydrogen production were observed when carbon source was changed. It might be possible to reduce these by growing the inoculum in the same carbon source as photobioreactor media.

Highest biomass accumulation was observed in the acetate containing media. However, high growth is not desired in the hydrogen production process, available substrates are used for growth instead of hydrogen production and high biomass decreases light intensity in deeper parts of the photobioreactors due to shading effect.

4.1.1 Stabilizing Hydrogen Production in Acetate medium

During dark fermentation by thermophilic bacteria, the highest hydrogen production is achieved when acetate is the end product. Thus, the dark fermenter

effluent mainly contains acetate and some amount of lactate. Therefore special emphasis was given on H_2 production in acetate by *R. sphaeroides*.

It is observed that pH in acetate media tends to increase rapidly during the batch process. The best pH range for hydrogen production is 6.7-7.5, higher pH values decreased hydrogen production and above pH 9-9.5 hydrogen production completely stopped (Sasikala et al. 1995).

Such an effect was also observed in malate media but was not that significant and the buffer in the media was able to hold pH within the optimum range desired. This increase was observed in both *R. capsulatus* and *R. sphaeroides* studies and that problem in acetate media caused many of the early studies to fail due to extremes of pH. The reason for this significant pH increase remained unknown however the problem was solved by adapting several solutions:

The possible solutions to keep pH stable at neutral values includes; increasing buffer content of media, decreasing starting pH, addition of acid during process. Last option is not preferred due to anaerobic conditions and contamination risk. The optimum buffer concentration was determined by trial and error method, the results of the trials were given in Table 4.2.

Table 4.2 Effect of different buffer concentrations on final pH in acetate media

Buffer*:	Maximum pH	Hydrogen production:
1 x	9.9	no
1 x	10.5	no
1 x	10.4	no
1 x	10.3	no
2 x	10.8	no
2 x	10.3	no
4 x	10.3	no
6 x	8.0	49 ml (/55 ml reactor)

*1 x buffer = 3.68 mM KH_2PO_4 (original amount in the Biebl&Pfennig medium)

According to those results, 6 times more buffer (22mM) was needed for acetate media studies to keep pH under control, at neutral level. The increased buffer capacity of the media between pH 6-7 was shown in Figure 4.3.

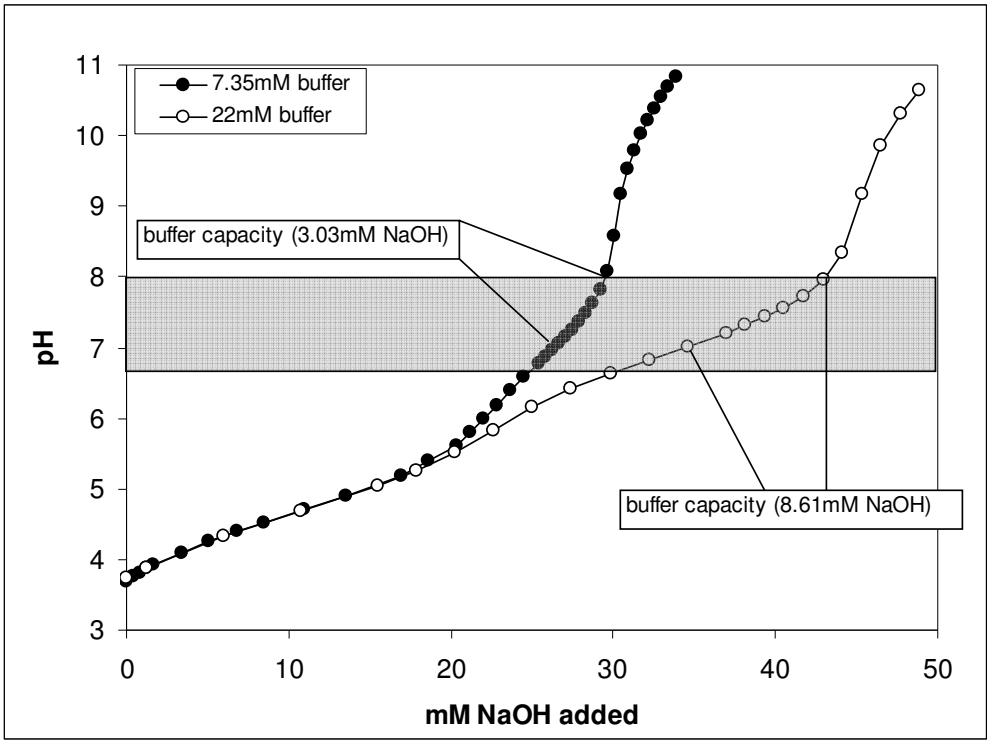


Figure 4.3 The titration of culture media containing 2x and 6x buffer content (C source is 20 mM of acetate and N source is 2 mM of glutamate)

In addition to increase in buffer content, starting pH of media was decreased to 6.5 from 6.8 resulting in more available buffer capacity.

However, six times increase in buffer amount increases ionic strength of the culture media. Ionic strength may cause unwanted stress on the bacteria, thus the share of buffer in total ionic strength of solution was determined. The contribution of individual compounds to the ionic strength of the media was calculated and given in Appendix I.

Six times increased buffer amount increases ionic strength from 0.071 to 0.087 and buffer is responsible from 34% of the total ionic strength in the media. In order to compensate that increase, NaCl amount in the media was halved and thus, ionic strength was reduced to 0.084, later on, NaCl was completely removed

(in *R. capsulatus* studies) since there are plenty of Na^+ and Cl^- ions in the media from other compounds.

After those modifications, pH was remained between 6.7 - 7.5 and its effect on the hydrogen production was eliminated.

The major drawback of the acetate was the unstability of the hydrogen production by *R. sphaeroides*. That is, although the bacteria well adapted to acetate and grew fast, hydrogen production was not always observed. The data given in single substrate comparison section belongs to one of the successful runs. The hydrogen production in acetate had to be stabilized. Substantial effort was made to overcome the stability problem.

Presently, there is no plausible explanation for this phenomenon, a detailed evaluation was made in Section 4.6.1 (Selection of bacterial strain). However substantial effort was made to stabilize hydrogen production in acetate media. Addition of Na_2CO_3, changing type and concentration of nitrogen source (urea, ammonium chloride, ammonium sulphate), changing acetate concentration, changing inoculum ratio were tried but all failed to stabilize hydrogen production.

Many runs were carried out between years 2003-2006 to study this case in detail. The summary of the studies made with *R. sphaeroides* in acetate media was given in Table 4.3.

Table 4.3 Growth and hydrogen production of *R. sphaeroides* in acetate media

		Experimental conditions							Results	
Run	**Date of study**	**[acetate] mM**	**N type**	**[N] mM**	**Buffer amount**	**pH**	**Inoculum amount**	**inoc. [C] type**	**Growth**	**H_2**
1	2003	30	glutamate	2	x1	too high	10%	malate	+	-
2	2003	30	glutamate	2	x1	too high	25%	malate	+	-
3	2003	30	glutamate	2	x1	too high	10%	malate	+	-
4	2003	30	glutamate	2	x1	too high	25%	malate	+	-
5	2003	30	glutamate	2	x2	too high	10%	malate	+	-
6	2003	30	glutamate	2	x2	too high	10%	acetate	+	-
7	2003	30	glutamate	2	x6	moderate	10%	acetate	+	+
8	2003	30	glutamate	2	x10	too high	10%	malate	+	-
9	2004	15	glutamate	2	x4	too high	10%	malate	+	-
10	2004	15	glutamate	0.5	x6	moderate	10%	malate	+	+
11	2004	15	glutamate	1	x6	moderate	10%	malate	+	-
12	2004	15	glutamate	2	x6	moderate	10%	malate	+	+
13	2004	30	glutamate	0.5	x6	moderate	10%	malate	+	+
14	2004	30	glutamate	1	x6	moderate	10%	malate	+	+
15	2004	30	glutamate	2	x6	too high	10%	malate	+	-
16	2004	60	glutamate	0.5	x6	moderate	10%	malate	-	-
17	2004	60	glutamate	1	x6	moderate	10%	malate	-	-
18	2004	60	glutamate	2	x6	moderate	10%	malate	-	-
19	2004	15	glutamate	0.25	x6	moderate	10%	acetate	+	-
20	2004	15	glutamate	0.5	x6	moderate	10%	acetate	+	-
21	2004	15	glutamate	1	x6	moderate	10%	acetate	+	-
22	2004	15	glutamate	1.5	x6	moderate	10%	acetate	+	-
23	2004	15	glutamate	2	x6	moderate	10%	acetate	+	-
24	2004	15	glutamate	2	x6	moderate	10%	malate	+	-
25	2004	15	glutamate	2	x6	moderate	10%	acetate	+	-
26	2006	30	glutamate	2	x6	moderate	10%	malate	+	+
27[1]	2006	40	glutamate	2	x6	moderate	10%	malate	+	+
28	2006	40	urea	0	x6	(no growth)	10%	malate	-	-
29	2006	40	urea	0.1	x6	(no growth)	10%	malate	-	-
30	2006	40	urea	0.25	x6	(no growth)	10%	malate	-	-
31	2006	40	urea	0.5	x6	(no growth)	10%	malate	-	-
32	2006	40	urea	0.75	x6	(no growth)	10%	malate	-	-
33	2006	40	urea	1	x6	(no growth)	10%	malate	-	-
34[2]	2006	15	glutamate	2	x6	moderate	10%	malate	+	+
35[3]	2006	15	mixed	2	x6	moderate	10%	malate	+	-
36	2006	40	glutamate	10	x6	moderate	10%	malate	+	-
37[4]	2006	40	urea	0.25	x6	moderate	10%	acetate	+	+
38	2006	40	urea	0.5	x6	moderate	10%	acetate	+	-
39	2006	40	urea	1	x6	moderate	10%	acetate	+	-
40	2006	40	urea	2	x6	moderate	10%	acetate	+	-
41	2006	40	glutamate	0.5	x6	too high	10%	acetate	+	-
42	2006	40	glutamate	1	x6	too high	10%	acetate	+	-
43	2006	40	glutamate	2	x6	too high	10%	acetate	+	-
44	2006	40	glutamate	4	x6	too high	10%	acetate	+	-
45	2006	40	glutamate	8	x6	too high	10%	acetate	+	-
46	2006	40	glutamate	0.5	x6	(no growth)	10%	acetate	-	-
47	2006	40	glutamate	1	x6	(no growth)	10%	acetate	-	-

Table 4.3 Growth and hydrogen production of *R. sphaeroides* in acetate media (continued)

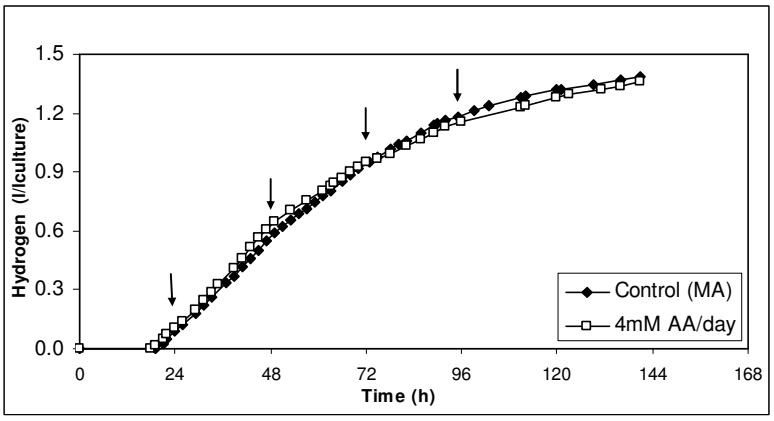
48	2006	40	glutamate	2	x6	(no growth)	10%	acetate	-	-
49	2006	40	glutamate	4	x6	(no growth)	10%	acetate	-	-
50	2006	15	glutamate	2	x6	too high	10%	acetate	+	-
51[5]	2006	15	glutamate	2	x6	too high	10%	acetate	+	-
52[6]	2006	15	glutamate	2	x6	too high	10%	acetate	+	-

[1]5mM propionate and 8mM butyrate was added in Run 27,
[2]15 mM malate also present in Run 34,
[3]N type: glut.-NH_4Cl-urea each one was 0.66mM in Run 35,
[4]H_2 very small amount in Run 37,
[5]2mM Na_2CO_3 added in Run 51,
[6]5mM Na_2CO_3 added in Run 52.

As can be seen from the Table 4.3, only 8 out of 52 runs yielded hydrogen.

Moreover, the following set of experiment clearly demonstrated that acetate was sometimes utilized for biomass increase only (possibly in the form of reserve polymer PHB) instead of hydrogen production.

Two 55 ml bioreactors were set and started with malate media, 4mM of acetate (adjusted pH) was added into one of them everyday and the change in growth, hydrogen production and pH was compared to the control bioreactor. The results were given below; arrows on the graphs indicate the acetate additions.



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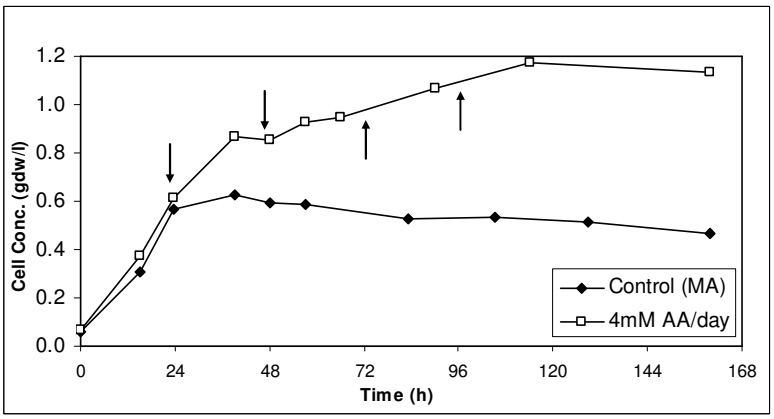
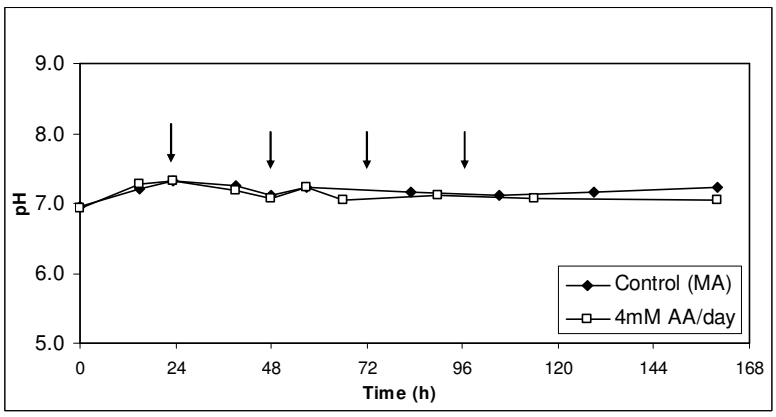


(b)



(c)

Figure 4.4 Acetate addition into malate (15 mM) media, effect on: a) bacterial growth, b) hydrogen production, c) pH of *R. sphaeroides* in 55ml bioreactors. Arrows show acetate additions. (●) control bioreactor, ( ) acetate added bioreactor.

As a result, growth, hydrogen production and pH in both parallel bioreactors were almost identical until the first acetate addition, then two bioreactors began to differ from each other: addition of acetate did not affect hydrogen production but cell growth was significantly increased. pH was not affected since the pH of the added acetate was set to 7.0 by NaOH titration before addition. This result suggests that *R. sphaeroides* may utilize acetate for growth and not produce any hydrogen.

Tao et al. (2007) reported similar cases with *R. sphaeroides* SH2C, they did not observe hydrogen production when acetate concentration was between 10 - 15 mM but reported hydrogen production when butyrate accompanied acetate as substate or acetate concentration was increased up to 50 mM. They concluded that quantity of acetate less than 15mM might mainly contribute to cell growth rather than hydrogen production and hypothesized that the phototrophic hydrogen production came mainly from butyrate in the fermentation effluents.

4.1.2 H_2 Production in Acetate Mixed with Other Organic Acids

The hydrogen production of *Rhodobacter sphaeroides* O.U. 001 was investigated in media containing mixtures of carbon sources: acetate and other organic acids. The experiments were carried out in 55 ml vessels.

4.1.2.1 Malate – Acetate Mixtures

In order to stabilize the hydrogen production in acetate medium, malate was introduced into the acetate media. Malate concentration was initially 7.5 mM in these mixtures containing different acetate concentrations (7.5 – 30 mM). N source was Na-glutamate and its amount was 2mM in all of the bioreactors.

In order to ensure the stability of the process, the runs were repeated up to 5 times, growth and hydrogen production were observed in all of the runs. Average values and ranges obtained were given in Figure 4.5 below:

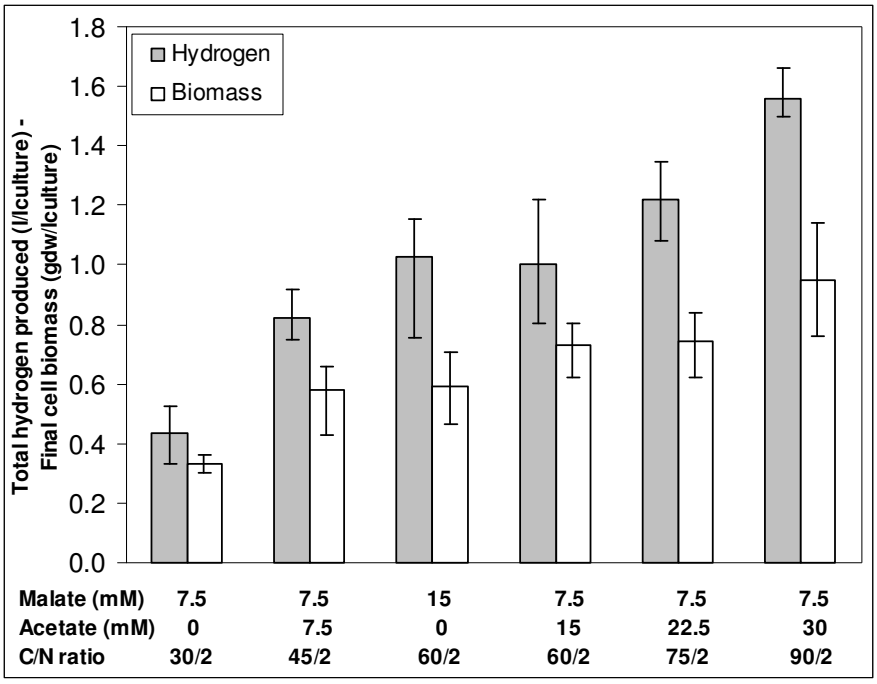
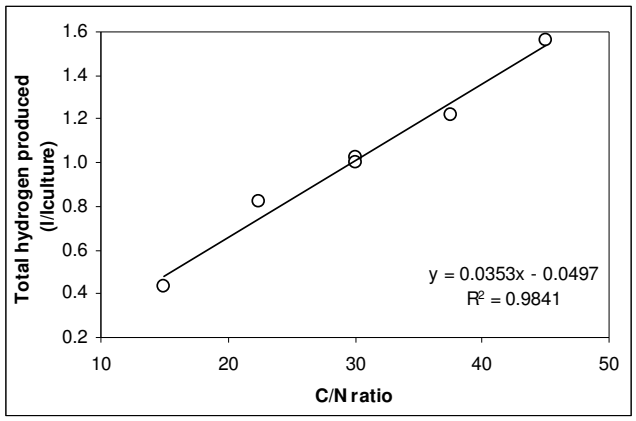


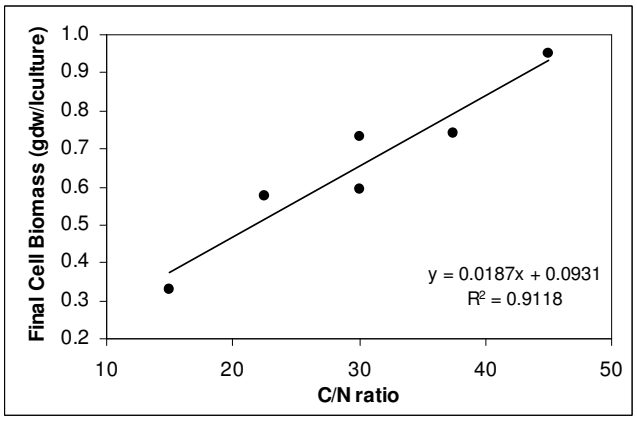
Figure 4.5 Total amount of hydrogen produced and final biomass concentration of *R. sphaeroides* in 55ml bioreactors containing different ratio of acetate-malate mixtures as the carbon source and 2 mM of glutamate as the nitrogen source (average of five runs).

It is clearly observed from Figure 4.5 that malate triggered hydrogen production when acetate was the main carbon source in the media and resulted in a stable hydrogen production process. As acetate concentration increased both the biomass concentration and total H_2 produced increased.

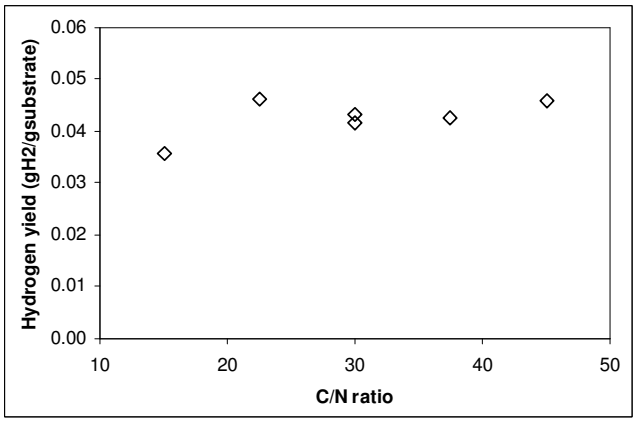
The effect of C/N ratio on hydrogen production and biomass was shown in Figure 4.6.



(a)



(b)



(c)

Figure 4.6. Correlation between C/N ratio and a) Total hydrogen produced, b) Final cell biomass, c) Hydrogen yield obtained, in acetate-malate mixtures by *R. sphaeroides.*

It can be seen that there is a linear correlation between the C/N ratio and the final hydrogen and biomass amounts as shown in Figure 4.6 a and b. On the other hand the H_2 yields were almost the same for all of the C/N values analyzed and ranged between 0.042-0.046 $gH_2/g_{substrate}$ except the first data which was slightly lower (0.036 $gH_2/g_{substrate}$) compared to the others.

4.1.2.2 Acetate – Lactate Mixtures

In this part of the study, lactate was introduced into the acetate media instead of malate, to provide a stable hydrogen production process. N source was 2 mM of glutamate in all of the runs. Different mixtures of acetate and lactate were experimented. C/N ratio was kept fixed at 30/2 (except last bioreactor). The obtained results were given in Figure 4.7 below:

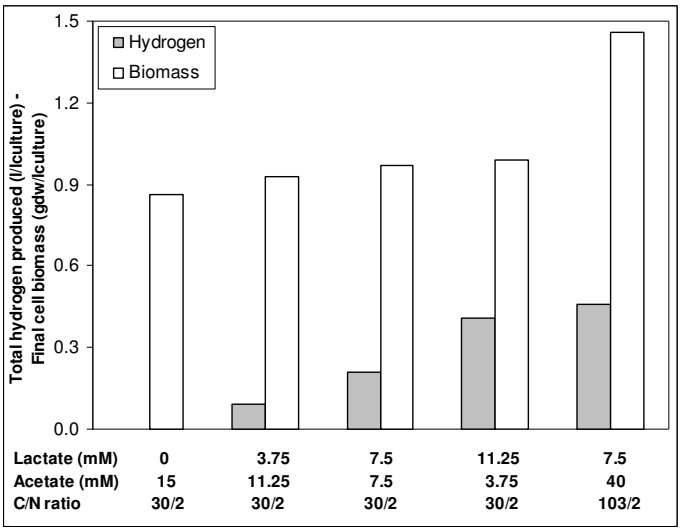


Figure 4.7. Total amount of H_2 produced and final biomass concentration of *R. sphaeroides* in 55ml bioreactors containing different ratio of acetate-lactate mixtures as the carbon source and 2 mM of glutamate as the nitrogen source.

Figure 4.7 shows that cell growth was present in all of the bioreactors. Hydrogen was not produced in acetate-only media. In the bioreactors that contained acetate-lactate mixtures, hydrogen production was observed. Final cell biomass was almost the same in all bioreactors that had 30/2 C/N ratio. Hydrogen production increased as the lactate ratio in the mixture increased.

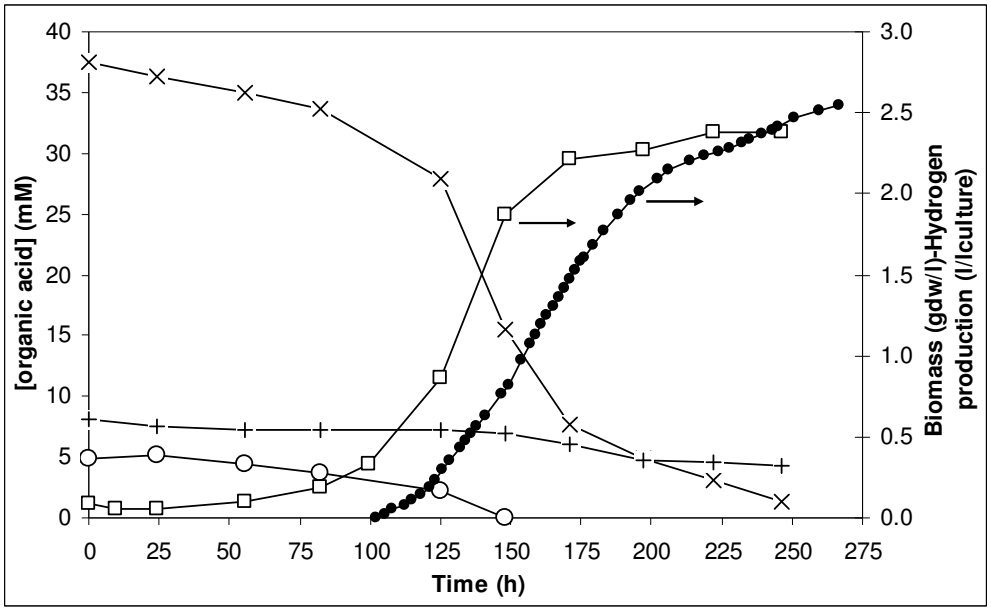
Also, out of the two bioreactors that contained 7.5 mM of lactate, it can be concluded that the one that contained 40 mM of acetate produced 2.2 times more hydrogen than the bioreactor that contained 7.5 mM of acetate.

4.1.2.3 Acetate – Propionate – Butyrate Mixtures

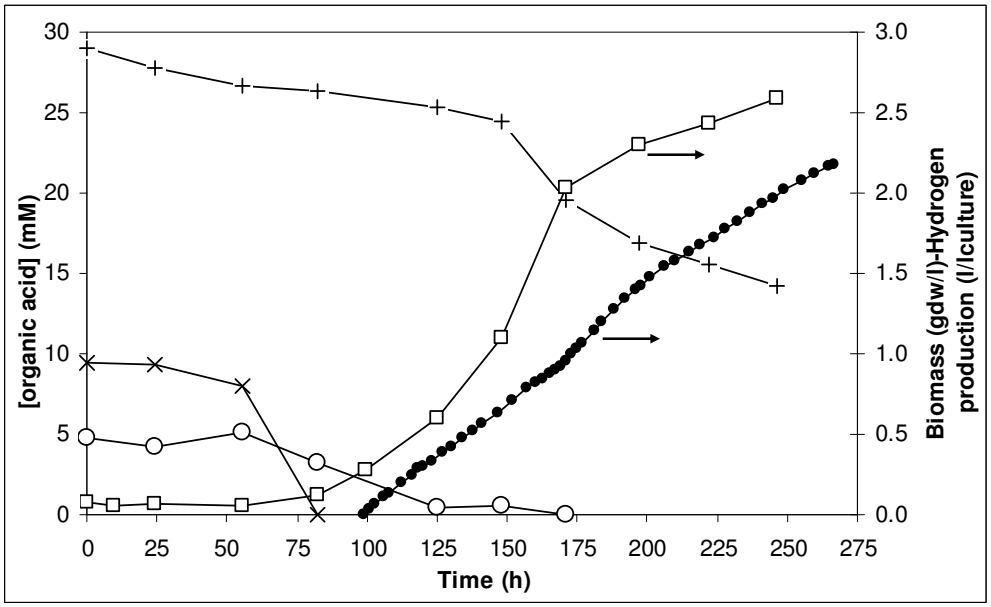
Triple mixtures of propionate, butyrate and acetate that reflect the composition of dark fermentation effluent as tabulated in Table 2.4 were tested. N source was Na-glutamate (2mM). Different mixtures of acetate and lactate were experimented and the obtained results were given in Figure 4.8.

It is shown that those carbon sources also triggered hydrogen production and provided a stable process. In the first run main substrate was acetate. Bacteria consumed acetate first, then propionate and lastly butyrate (Figure 4.8a). In the next run, where butyrate was the main substrate, bacteria consumed acetate and propionate first (acetate faster) then used butyrate (Figure 4.8b). Compiling both figures together, one concludes that bacteria prefer to use substrates in the order of acetate, propionate and butyrate. It can be suggested that small substrates are metabolized faster by the bacteria.

Further studies are required on using mixtures of acids as carbon source in order to support these findings and for in detail evaluation of bacterial metabolism and hydrogen production.



(a)



(b)

Figure 4.8. Bacterial growth ( ), hydrogen production (●), acetate concentration (X), butyrate concentration (+) and propionate concentration (○) data of *R. sphaeroides* obtained in 55ml bioreactors containing a) mixture of acetate (40mM), butyrate (10mM) and propionate (5mM), b) mixture of butyrate (30mM), propionate (5mM) and acetate (10mM).

4.2 Effect of Nitrogen Sources on Biological H_2 Production

4.2.1 Glutamate, Ammonium Chloride, Urea, Ammonium Phosphate

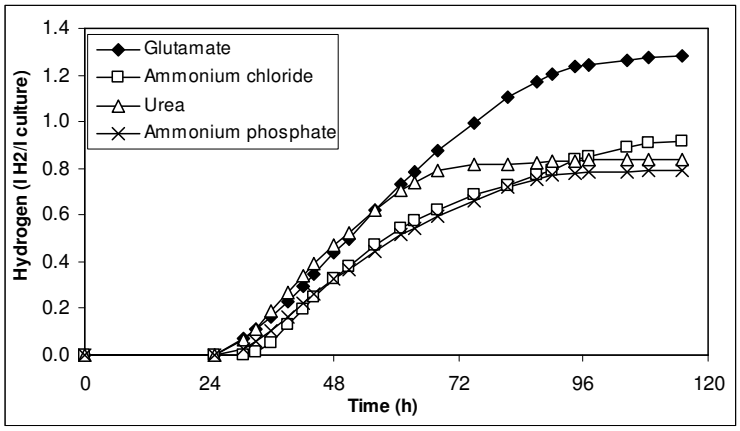
In the previous section, it is shown that different organic acids can be used as substrates for the hydrogen production process. The nitrogen source was glutamate in those studies. Glutamate has been found to be a suitable nitrogen source for the process and the optimum concentration was given as 2mM in previous works (Eroglu et al, 1999 and Koku et al, 2002).

However glutamate is not suitable as an additive for large scale applications of the process due to high cost. It contains five carbon atoms per nitrogen atom and is a significant additional carbon source for the bacteria at high concentrations.

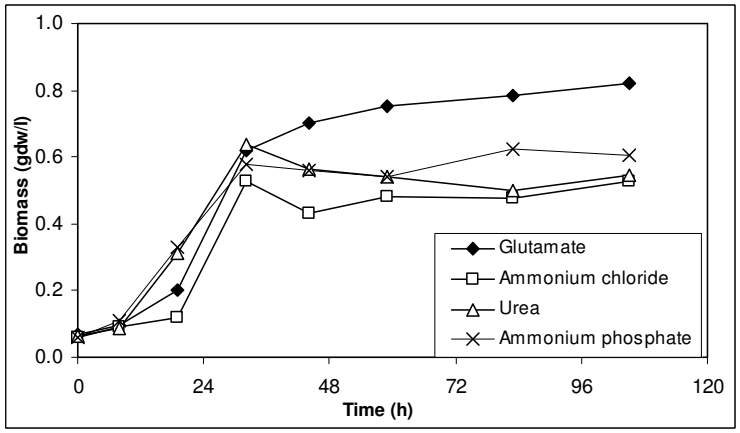
In this study, the growth and hydrogen production by *R. sphaeroides* was studied in media containing different types of nitrogen sources, in order to find a cheaper and more available substitute(s) for glutamate that can be used in large scale applications.

Four 55ml bioreactors were run in parallel. *R. sphaeroides* was used as the bacteria. The media contained 15 mM malate as C source. Different N sources were added into each of the bioreactors but C/N ratio was kept fixed at 60/2. First bioreactor had 2 mM of sodium glutamate ($Na\text{-}C_5H_8NO_4$), second had 2mM of ammonium chloride (NH_4Cl), third had 1 mM of urea ($(NH_2)_2CO$), and last bioreactor had 2mM of ammonium dihydrogen phosphate ($NH_4H_2PO_4$).

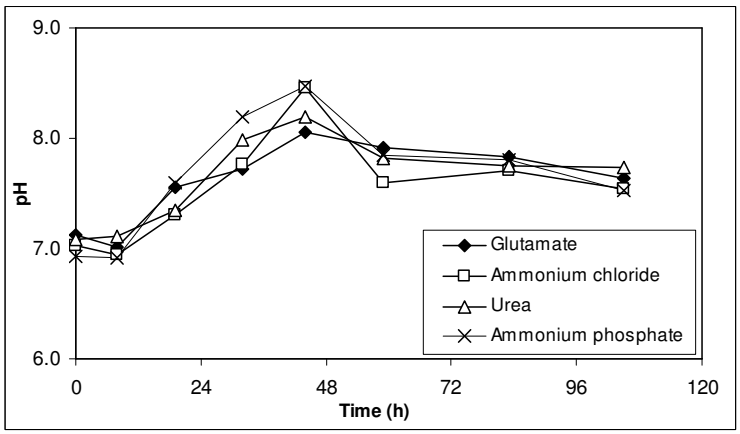
Figure 4.9 and Table 4.4 illustrate the effect of different N sources on hydrogen yield, biomass and pH change.



(a)



(b)



(c)

Figure 4.9 Hydrogen production (a), biomass concentration (b) and pH changes (c) of *R. sphaeroides* in 55ml bioreactors containing different nitrogen sources (C source was 15 mM malate).

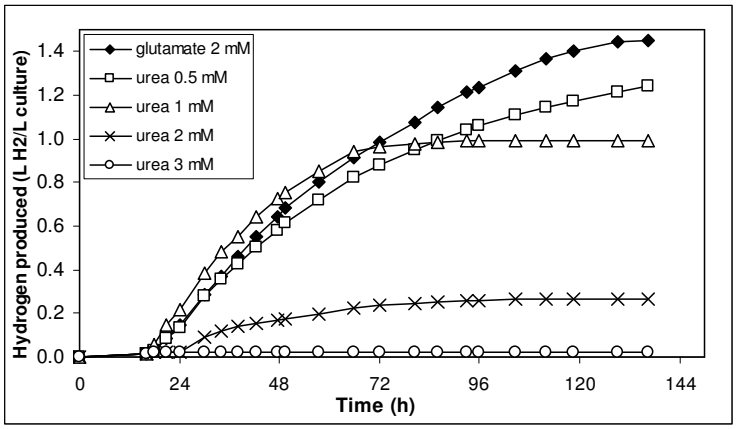
Table 4.4 Hydrogen production by *R. sphaeroides* in bioreactors containing different nitrogen sources. C source was 15 mM of malate

	$C_5H_8NO_4$	NH_4Cl	$(NH_2)_2CO$	$NH_4H_2PO_4$
Initial N source conc. (mM)	2	2	1	2
C/N ratio (mol/mol)	60/2	60/2	60/2	60/2
Max. Cell concentration (g/L)	0.82	0.53	0.64	0.63
Final H_2 produced ($l/l_{culture}$)	1.28	0.92	0.84	0.79
Aver. H_2 prod. rate ($ml/l_{culture}.h$)	23	20	23	17
H_2 productivity (mg $H_2/l_{culture}.h$)	1.9	1.7	1.9	1.4
H_2 yield (g H_2/g substrate)	0.052	0.038	0.034	0.032

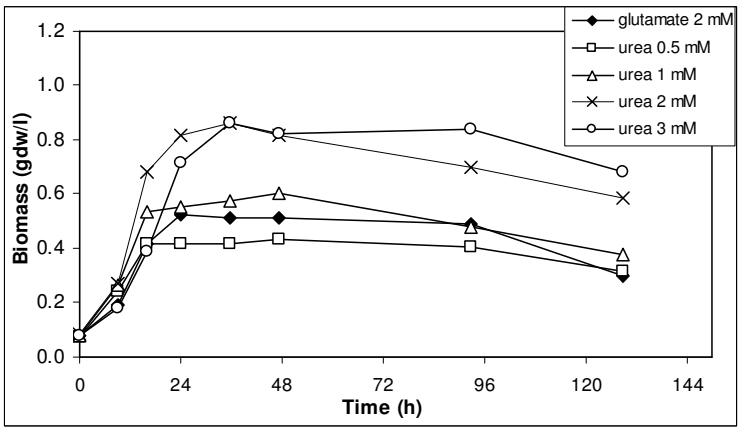
The results show that the bacteria adapted well to all four types of N sources: Growth and hydrogen productions were observed in all cases. Highest hydrogen productivity and yield was obtained with glutamate, this may be attributed to the fact that glutamate is also an amino acid and has the most carbon atoms/molecule compared to others therefore it is probably the most useful substrate to the bacteria among the tested compounds. Hydrogen production in urea containing medium is also quite promising (Figure 4.9a) however it stopped after three days. This "low" hydrogen production capacity may be resulted due to low concentration of urea. This has been tested in the next section.

4.2.2 Optimization of Urea Concentration for Hydrogen Production

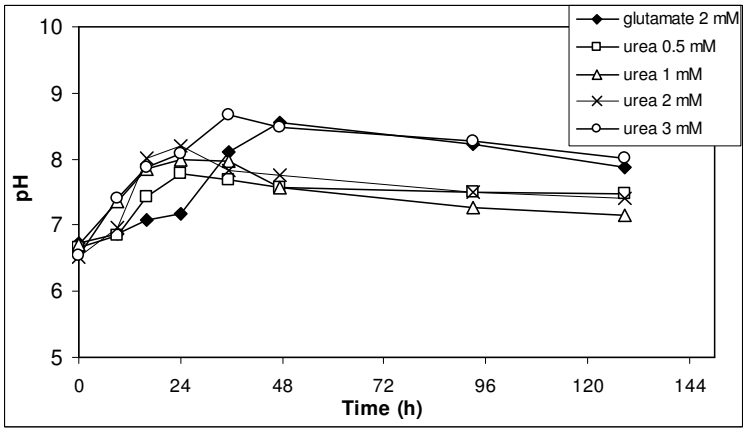
In the second part of the study, the most promising substitute of the previous set, that is urea, was chosen for further investigation and optimization. The experimental set was designed to include different concentrations of urea as N source (0.5, 1, 2 and 3 mM) as well as a control bioreactor containing 2 mM glutamate for comparison. Five 55ml bioreactors were run in parallel. *R. sphaeroides* was used as the bacteria. The media contained 15 mM malate as C source. The results obtained are given in Figure 4.10 and Table 4.5.



(a)



(b)



(c)

Figure 4.10 Hydrogen production (a), biomass concentration (b) and pH changes (c) of *R. sphaeroides* with respect to time in 55ml bioreactors containing different urea concentrations and glutamate (C source was 15 mM malate)..

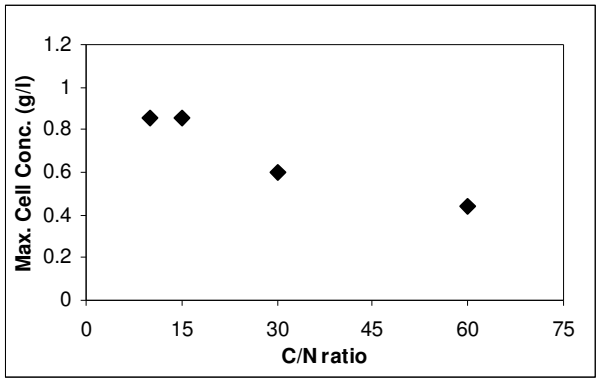
Table 4.5 Hydrogen production by *R. sphaeroides* in bioreactors containing different urea concentrations and glutamate. C source was 15 mM of malate

N source type	Glutamate	Urea	Urea	Urea	Urea
Initial N source conc. (mM)	2	0.5	1	2	3
C/N ratio (mol/mol)	60/2	60/1	60/2	60/4	60/6
Max. Cell concentration (g/L)	0.53	0.44	0.60	0.86	0.86
Final H_2 produced ($l/l_{culture}$)	1.45	1.24	0.99	0.27	0.02
Aver. H_2 prod. rate ($ml/l_{culture}.h$)	14.2	11.5	18.8	4.1	0.0
H_2 productivity (mg $H_2/l_{culture}.h$)	1.2	1.0	1.6	0.3	0.0
H_2 yield (g H_2/g substrate)	0.059	0.051	0.040	0.011	0.001

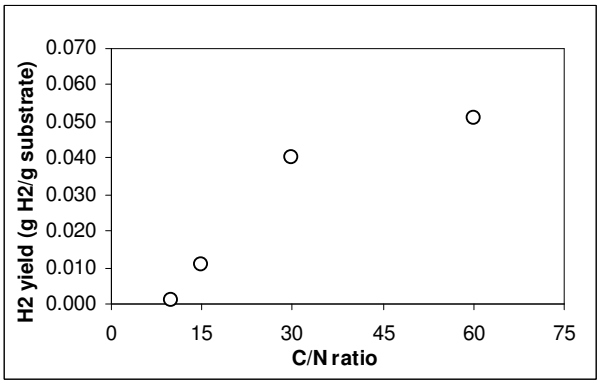
The results show that high urea concentration results in higher growth but inhibits hydrogen production. Almost no hydrogen was produced in 3 mM urea containing media although cell concentration was highest compared to other bioreactors. Highest hydrogen yield was obtained with 2mM of glutamate media (control), although comparable results were obtained in 0.5 mM urea containing media as well. On the other hand, highest productivity was obtained in 1 mM urea containing media, followed by glutamate media.

As a result, it may be concluded that urea can be used as substitute for glutamate when malate is the C source in the media; the large-scale availability and low price of urea are also advantages over glutamate.

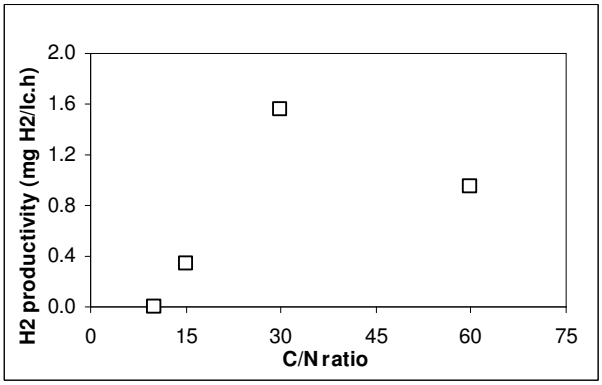
For urea containing bioreactors, C/N ratio versus maximum cell concentration, hydrogen productivity and hydrogen yield were plotted in Figure 4.11.



(a)



(b)



(c)

Figure 4.11 Maximum cell concentration (a), H_2 yield (b) and H_2 productivity (c) for different C/N ratios in bioreactors containing urea as N source (C source was 15 mM malate).

From Figure 4.11, it is clearly seen that as C/N ratio increases, that is, the N source becomes more limited compared to the C source, maximum cell concentration decreases (Figure 4.11a) and hydrogen yield increases (Figure 4.11b). Hydrogen productivity increases up to C/N=30 but then decreases again (Figure 4.11c). It can be suggested that C/N ratio should be at least 30 for optimum hydrogen production in media containing urea as N source and malate as C source.

In a comparable study, Eroglu et al (1999) suggested the same optimum C/N ratio (60/2) for same bacteria (*R. sphaeroides*) in media containing glutamate as N source and malate as C source.

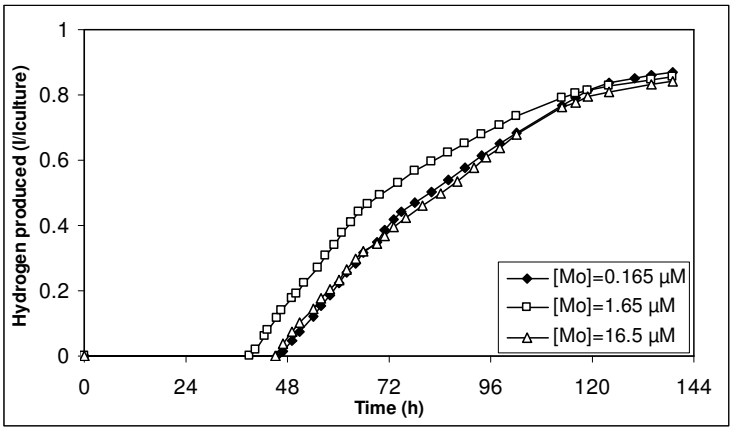
4.3 The Significance of Minor Nutrients

The effect of the minor nutrients on the growth and hydrogen production was investigated. It was targeted to:

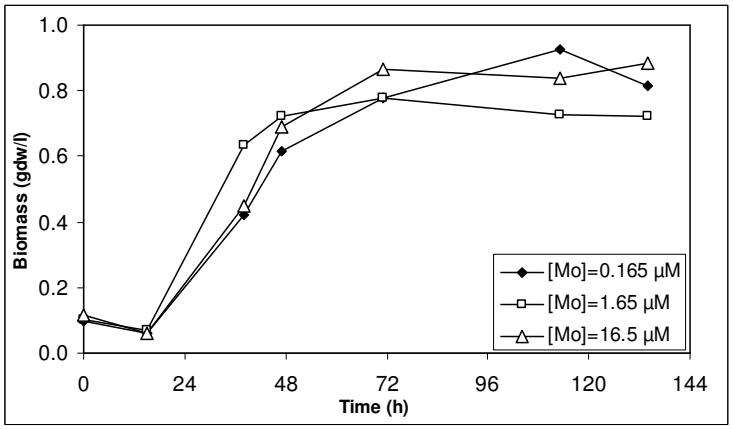
- find the optimum concentrations of these nutrients,
- obtain data for dark-photofermentation integration process (requirement of the addition of essential minor nutrients to the effluent),
- decrease the cost by eliminating or reducing the amounts of minor nutrients used.

4.3.1 Effect of Molybdenum Concentration

R. sphaeroides was used as photosynthetic bacteria in 55 ml vessels. Molybdenum is a chemical element with the symbol Mo and atomic number 42. It is the cofactor of the nitrogenase enyzme. Therefore, increase in [Mo] in the media may enhance nitrogenase activity and consequently the hydrogen production. By default, molybdenum concentration is 0.165μM in the defined media. In this study, three bioreactors were run in parallel, all the parameters were kept constant except the molybdenum concentration in the media, which is increased 10 and 100 times compared to the default. The results are given in Figures 4.12.



(a)



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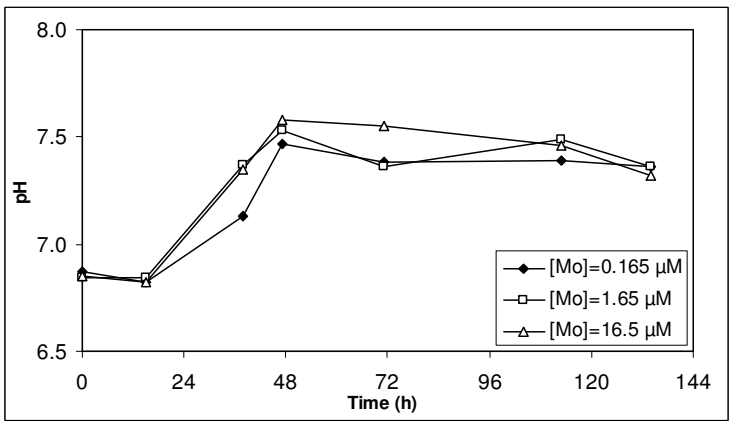


(c)

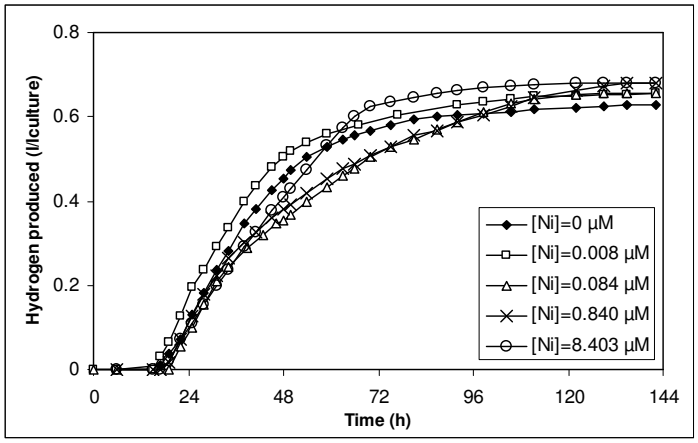
Figure 4.12 Hydrogen production (a), biomass concentration (b) and pH changes (c) of *R. sphaeroides* with respect to time in 55ml bioreactors containing different concentration of molybdenum (C source was 15 mM malate).

As can be seen in the Figures, there is no significant difference in biogrowth, hydrogen production and pH curves obtained. This leads to the conclusion that the [Mo] in the media is already in excess amount and it is not a limiting substrate for the process, increasing the concentration did not improve the hydrogen production. Another conclusion that can be made is that the increased concentration of Mo up to 100 times than default did not have any negative effect on the biogrowth and hydrogen production.

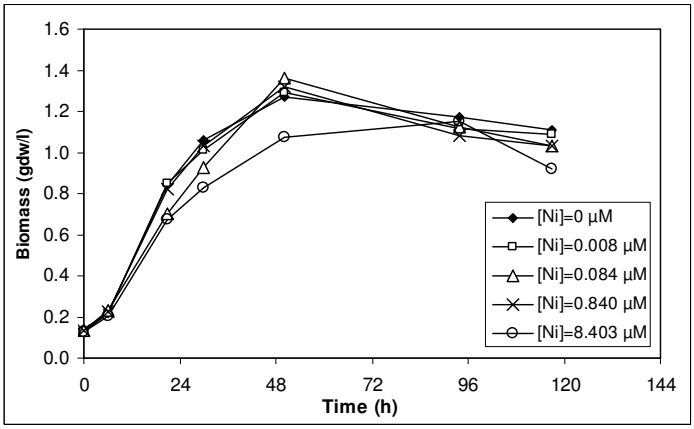
Kars et al (2006) carried out a similar study and reported that there was almost no hydrogen production in medium which is not containing molybdenum and little hydrogen production in 0.0165µM Mo medium; however, it was observed that there was an increase in total hydrogen production up to 30% accompanied with elevated molybdenum concentrations (1.65µM and 16.5µM).

4.3.2 Effect of Nickel Concentration

Rhodobacter sphaeroides O.U. 001 was used as photosynthetic bacteria in 55 ml vessels. Nickel is a metallic chemical element in the periodic table that has the symbol Ni and atomic number 28. It is the cofactor of the Hydrogenase enyzme in *R. sphaeroides*. Therefore, change in [Ni] in the media may affect hydrogenase activity and consequently the hydrogen production. By default, nickel concentration is 0.084µM in the media. In this study, five bioreactors were run in parallel, all the parameters were kept constant except the nickel concentration in the media, which is changed as 0, 0.1, 10 and 100 fold compared to default. The results are given in Figure 4.13.



(a)



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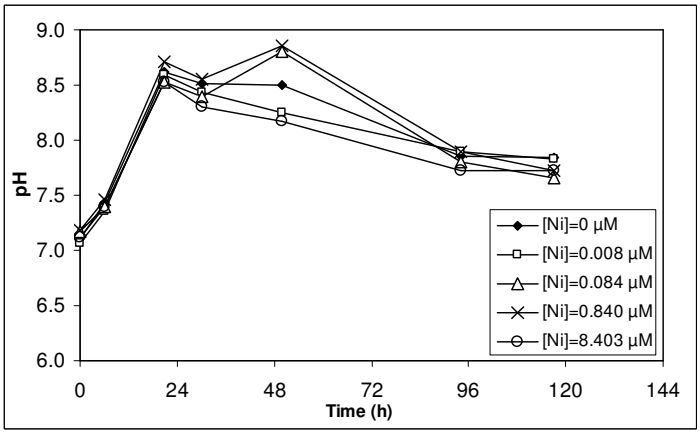


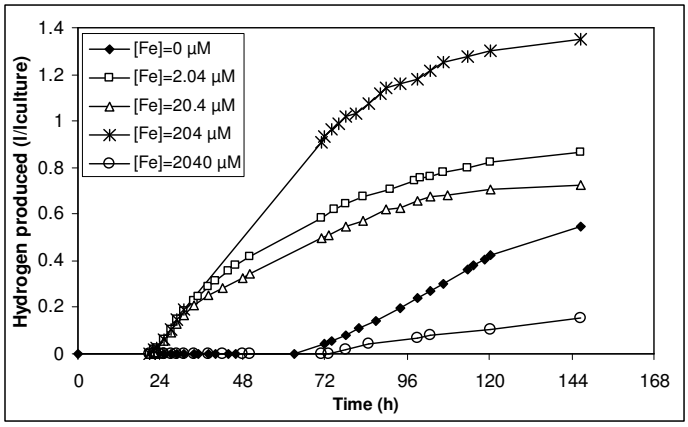
(c)

Figure 4.13 Hydrogen production (a), biomass concentration (b) and pH changes (c) of *R. sphaeroides* with respect to time in 55ml bioreactors containing different concentration of nickel (C source was 15 mM malate).

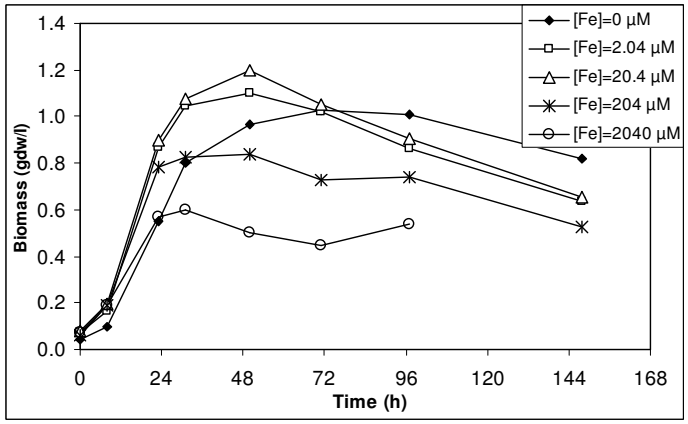
As can be seen in the figures, there is no significant difference in biogrowth, hydrogen production and pH curves obtained. This result may suggest that the activity of the hydrogenase in the bacteria is negligible compared to the nitrogenase activity and the change in hydrogenase activity is not affecting the overall process significantly. Another conclusion may be that the [Ni] in the media is already in much excess amount and it is not a limiting substrate for the hydrogenase activity, even the very trace amount of nickel in "no-nickel containing" media which is brought by the inoculum was enough. The enzyme expression levels should be determined for a more accurate conclusion. In any case, it was also shown that the increased concentration of Ni up to 100 times than default concentration did not have any negative effect on the biogrowth and hydrogen production.

4.3.3 Effect of Iron Concentration

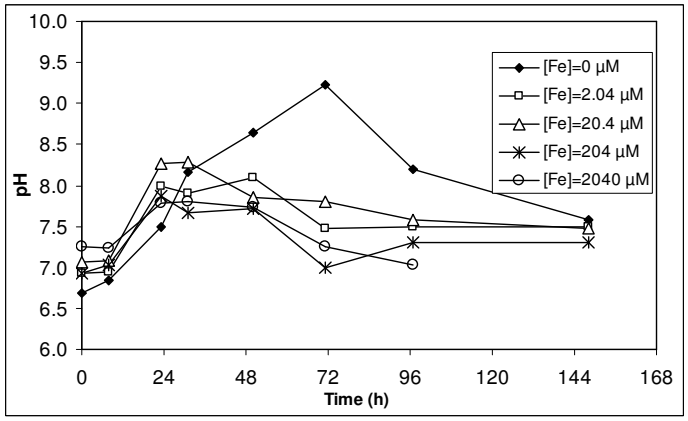
Rhodobacter sphaeroides O.U. 001 was used as photosynthetic bacteria in 55 ml vessels. Iron is a metallic chemical element in the periodic table that has the symbol Fe and atomic number 26. It is the cofactor of the hydrogenase enyzme in *R. sphaeroides*, also is present in ferrodoxin. Therefore, change in [Fe] in the media may affect hydrogenase activity and electron transport chain of the bacteria. By default, iron concentration is 20.4µM in the media in the form of Fe(III)citrate. In this study, five bioreactors were run in parallel, all the parameters were kept constant except the iron concentration in the media, which is changed as 0, 0.1, 10 and 100 fold compared to default. The results are given in Figure 4.14.



(a)



(b)



(c)

Figure 4.14 Hydrogen production (a), biomass concentration (b) and pH changes (c) of *R. sphaeroides* with respect to time in 55ml bioreactors containing different concentration of iron (C source was 15 mM malate).

As can be seen in the Figures, unlike the [Mo] and [Ni], [Fe] affected the results significantly: The maximum biomass amount in the bioreactors were measured as 1.03, 1.10, 1.20, 0.84, 0.60 gdw/l and the total hydrogen produced were 0.54, 0.86, 0.73, 1.35, 0.15 $l/l_{culture}$ for 0, 2.04, 20.4, 204 and 2040 µM iron containing bioreactors, respectively. The maximum hydrogen was produced in 10x (204µM) iron containing bioreactor. The hydrogen production was severely inhibited in non-iron and 100x iron containing bioreactors; not only the collected gas amounts were much less, but also the gas productions started two days later compared to other three bioreactors, which is a significant lag. In 100x Fe containing (2040µM) bioreactor, a dark green-black colored biomass coagulation at the bottom of the bioreactor has been formed during the run, possibly indicating the toxic effect of that concentration of iron on the bacteria, very small amount of hydrogen was produced in this case (0.15 $l/l_{culture}$). In non-iron containing bioreactor there is smaller bacterial growth and considerably less hydrogen production compared to iron-containing bioreactors, however as iron is an essential element for the bacterial metabolism, the growth should not happen at all.

This unexpected result is probably due to the trace amount of iron present in the media, which might be brought in by the inoculum media.

This study showed the importance of iron element for the growth and the hydrogen production of the bacteria, these findings were verified once more in dark-fermentation effluent studies (Section 4.5).

Kars et al (2006) carried out a similar study and reported that there was a growth delay in No Fe and 1/10X Fe media indicating the vitality of iron for cellular functions. It was also recorded that there was almost no hydrogen production in the medium which had no Fe, showing that iron is vital for the hydrogen evolution, the highest iron concentration (1mM) caused a decrease in the hydrogen production which might be due to a toxic effect and the optimum iron salt concentration was suggested to be is 0.1mM.

4.4 Light Effects

The investigations of the effect of light intensity, wavelength of the light and illumination protocols are important to set a basis in outdoor applications. The design and scale up of more efficient photobioreactors, require knowledge on the relation between the hydrogen production and the light intensity, wavelength, and the illumination patterns. Therefore the effects of these parameters, different light-dark cycle and initial dark period protocols on growth and hydrogen production of *Rhodobacter sphaeroides* O.U. 001 was investigated.

4.4.1 Light Absorption Spectra of Bacteria

The light absorbance of *R. sphaeroides* O.U.001 is obtained for a large spectrum range by two different devices for comparison; a spectrophotometer and a spectroradiometer. The accuracy and operation ranges of these devices are different. The absorbance graph is given in Figure 4.15.

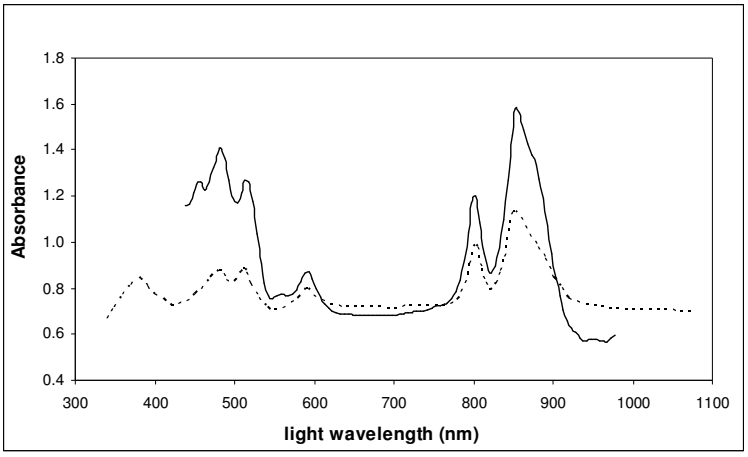


Figure 4.15 Absorption spectra of *R. sphaeroides* O.U. 001, (---) obtained by spectrophotometer (range: 340-1080nm, normalized), (—) obtained by spectroradiometer (range: 430-980nm)

It is clearly seen that the bacteria absorb light at some specific wavelengths. This is due to the bacteriochlorophyll and carotenoid pigments of bacteria. Bacteriochlorophyll *a* absorbs light at 375, 590, 805 and 830-910 nm wavelength where spheroidene (carotenoid of *R. sphaeroides*) absorbs light at 450, 482 and 514 nm wavelength. From these data, it can be concluded that the photobioreactor should receive light rich at these specific wavelengths and the bioreactor construction material should not absorb/reflect these wavelengths.

4.4.2 Light Emission Spectra of Different Light Sources

As suggested in previous part (4.4.1), the photobioreactor should receive light rich at the wavelengths where absorbance peaks of bacteria are present. Actually this may be considered as a knock-out criterion for the light source selection. In order to find which light sources meet this requirement, the spectral analyses of various light sources (natural and artificial) were made. The normalized plot is given in Figure 4.16.

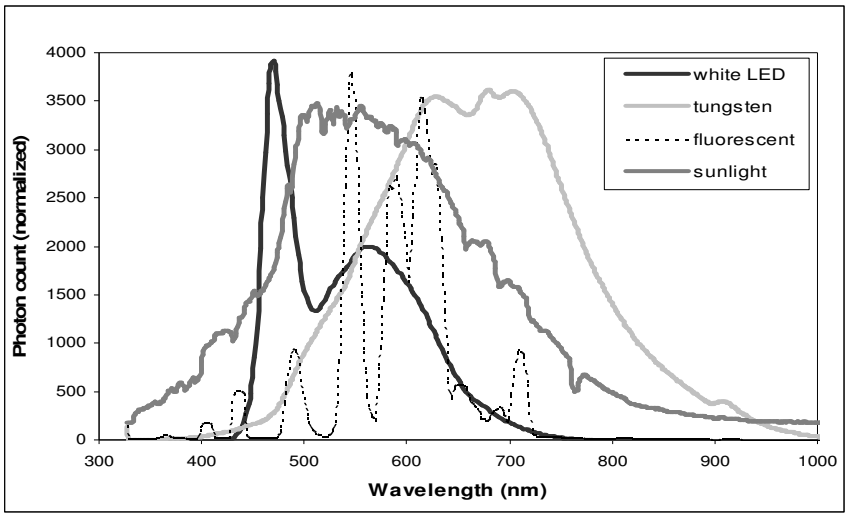


Figure 4.16. Emission spectra of different light sources

It can be seen that sunlight and tungsten lamp provides enough photons at the desired wavelengths, whereas LED (light emitting diode) and fluorescent lamps do not emit light at near-infrared part of the light spectrum. The cost of the illumination should also be considered while choosing the appropriate light source. Sunlight is free, LED and fluorescent lamps consume up to 80% less electricity to produce same illumination than incandescent lamps (tungsten, carbon filament, halogen etc).

From these data, it can be concluded that sun is the best light source, and in case of indoor operation tungsten lamp should be employed. LED and fluorescent lamps can not be used, since they do not emit red-infrared light which would severely drop the efficiency of the process.

4.4.3 Effect of the Wavelength of Light

In this study, the bacteria (*R. sphaeroides*) were exposed to light at specific wavelengths in order to observe the effect of the light wavelength on growth and hydrogen production. The light source was tungsten lamp. Rhodamin B solution (6.5mM) and $CuSO_4$ solution (135mM) were used as optical filters in order to block specific ranges of light wavelength. These filters were prepared by trial and error, through a scanning of many colored compounds including indicators, pigments etc. During the run, the control photobioreactor received the normal bell-shaped light spectrum from the lamp (370-1030nm) whereas optical filters were placed in front of the other two photobioreactors to obtain the light spectra given in Figure 4.17 at the surface of the photobioreactors.

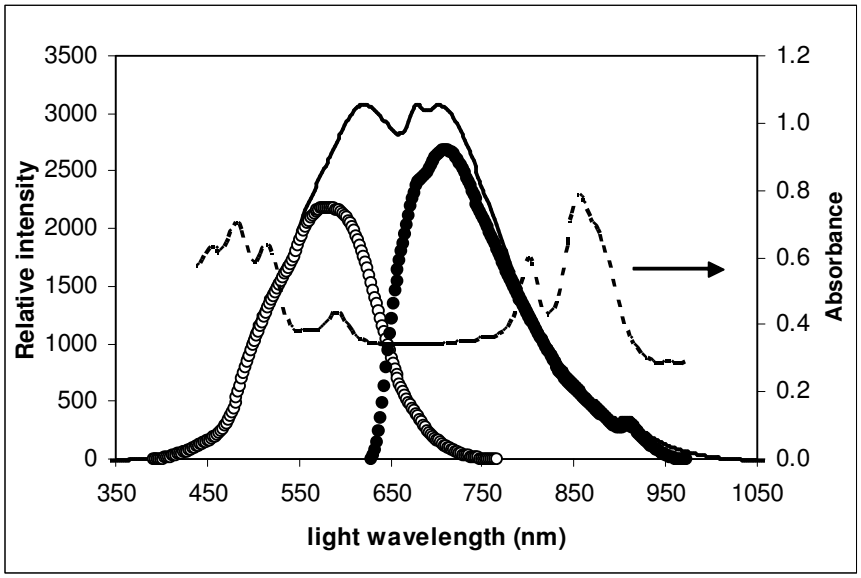
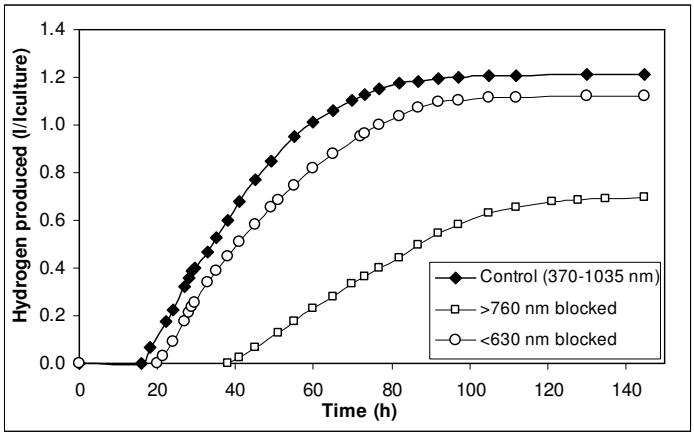
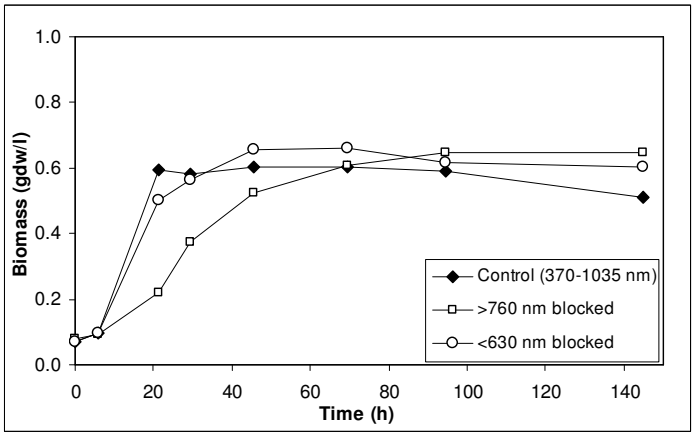


Figure 4.17 The light spectrum at the surface of the (—): Control photobioreactor, no filters used, 370-1035nm range, (○): Rhodamin B solution filter (>760nm blocked), (●) $CuSO_4$ solution filter (<630nm blocked), (---) Absorbance spectrum of *R. sphaeroides*

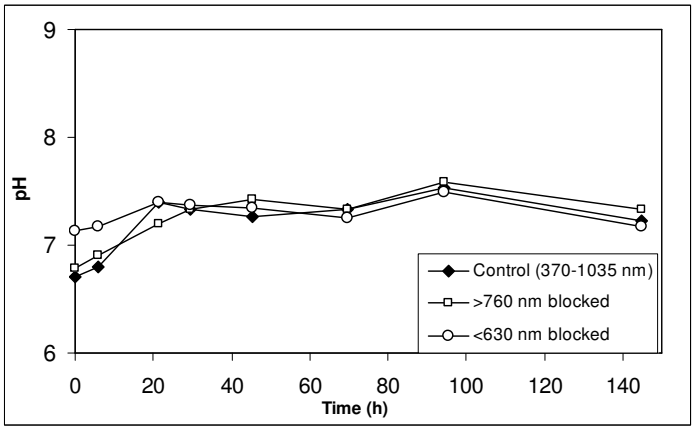
It can be seen that Rhodamin B solution acted as an optical filter that transmitted all of the light with wavelength less than 560nm, while blocking the light with wavelength greater than 760nm completely. On the other hand, $CuSO_4$ solution acted as an optical filter that transmitted almost all of the light with wavelength greater than 720nm while blocking the light with wavelength less than 630nm completely. The other sides of photobioreactors were wrapped by aluminum folio to prevent light scattering. Photobioreactors used had 55ml working volume. The results are presented in Figure 4.18 and 4.19.



(a)



(b)



(c)

Figure 4.18 Effect of incident light wavelength on hydrogen production (a), biomass concentration (b) and pH changes (c) of *R. sphaeroides* in malate (15 mM) media. 55ml bioreactors were used.

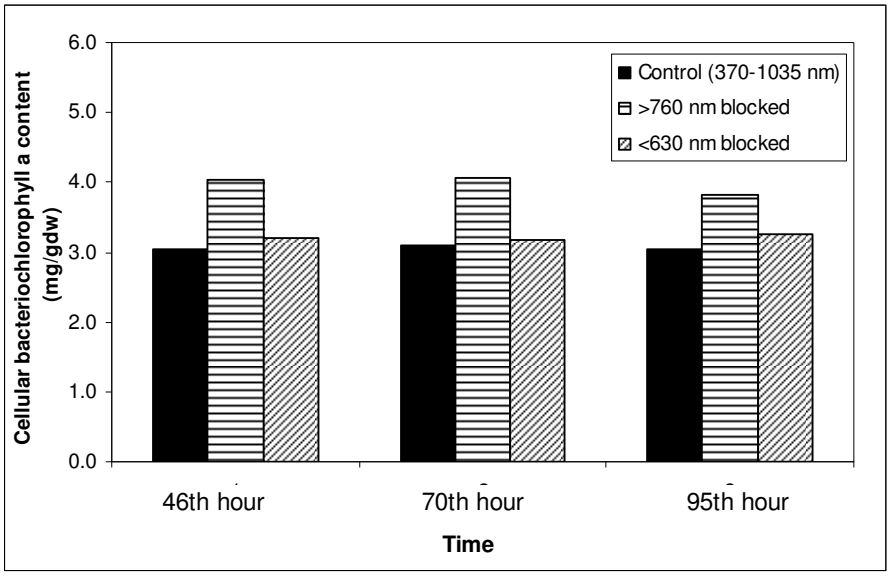


Figure 4.19 Effect of incident light wavelength on cellular bacteriochlorophyll *a* levels of *R. sphaeroides* in malate media (15 mM). 55ml bioreactors were used.

It is clearly seen that in case of infrared light blocked photobioreactor, growth and hydrogen production are affected negatively: Hydrogen production lag time was 40 hours compared to 17 hours for the control photobioreactor, produced hydrogen gas was 57% of the control and growth was slower. Moreover, cellular bacteriochlorophyll *a* content in this photobioreactor was 30% higher than in the control photobioreactor (Figure 4.19), which means that there is a great energy stress on bacteria and they produce more bacteriochlorophyll a to overcome that shortage. In case of the blue light blocked photobioreactor, hydrogen production was slightly affected compared to the control: Hydrogen production lag time was 4 hours longer, 7% less hydrogen gas was obtained. Cellular bacteriochlorophyll *a* content in this photobioreactor was quite close to that in the control photobioreactor. Growth was also not significantly affected by that light-filter. These results showed that the infrared region of light spectrum where the

bacteriochlorophyll *a* absorption maxima exist is very important for hydrogen production, whereas the left part of spectrum where the carotenoid absorption maxima exist is not significantly effective. Thus, it is recommended that the outdoor photobioreactors should be located and oriented such that they receive the sunlight during sunset and sunrise to ensure that they receive plenty of red and infrared light. Moreover, in case of indoor operatons, the artificial light source which does not emit light in the red-infrared region (750-950nm) are not suitable for illumination of the photobioreactor for hydrogen production.

4.4.4 Effect of the Light Intensity

This study was carried out to show the effect of incident light intensity on hydrogen production. Sasikala et al. (1991) indicated the saturation of hydrogen production at 5000 lux. In the present study, a more precise experiment around this optimum was performed to obtain a better insight: photobioreactors with very short light path (0.5 cm light path, 4.1 ml total volume) were used for high precision and photobioreactors are exposed to 7 different light intensities between 88 - 405 W/m^2 (measured at the surface of the photobioreactors).

The results are listed in Table 4.6. From the total hydrogen gas produced versus time data, the maximum hydrogen production rate (calculated from the linear hydrogen production phase during exponential bacterial growth), light conversion efficiency and the substrate conversion efficiency were estimated.

Substrate conversion efficiency was determined as the ratio of moles of hydrogen that have actually been produced per moles of hydrogen that would have been produced if all of the substrate had been converted to hydrogen through the stoichiometric equation.

A performance evaluation parameter that has gained widespread acceptance is the solar (or light) conversion efficiency. This parameter is the ratio of the total energy (heat of combustion) value of the hydrogen that has been obtained to the total energy input to the photobioreactor by light irradiation.

It is calculated by

$$\eta = [(33.61 \cdot \rho_{H2} \cdot V_{H2})/(I \cdot A \cdot t)] \cdot 100 \quad (4.8)$$

where V_{H2} is the volume of produced H_2 in l, ρ_{H2} is the density of the produced hydrogen gas in g/l, I is the light intensity in W/m^2, A is the irradiated area in m^2 and t is the duration of hydrogen production in hours.

Incident light intensity was used in the calculations instead of the absorbed light intensity since the runs were carried out in batch mode, where the cell concentrations and thus the absorbed light intensities vary throughout the process.

In the literature, light conversion efficiencies were reported to be as low as 0.1% up to 10% for photoheterotrophic bacteria depending on the microorganism type (including mutants) and the incident light intensity. High efficiencies were obtained mostly at the lower light intensities (less than 50 W/m^2) with generally associated hydrogen production rates that were too low to be interesting from a practical point of view. For *R. sphaeroides* O.U. 001, the reported values were mostly in the range of 0.21-0.76% on malate (Koku et al, 2003).

A sample calculation of the light conversion efficiency was given in Appendix J.

Table 4.6 The effect of light intensity on hydrogen production by *R. sphaeroides* in 4.1 ml photobioreactors

Light Intensity (W/m^2)	Maximum H_2 production rate ($ml/l_{culture}.h$)	Total H_2 produced ($l/l_{culture}$)	Light conversion efficiency (%)	Substrate conversion efficiency (%)
88	18	0.68	1.11	34
118	22	0.58	0.82	27
169	28	0.64	0.76	31
209	31	0.65	0.65	30
277	33	0.80	0.50	36
338	34	0.75	0.45	37
405	34	0.60	0.25	30

According to the results an increase in light intensity up to 270 W/m^2 increased the maximum hydrogen production rate up to 33 ml/$l_{culture}$.h. Further increase in light intensity did not change the rate and no photoinhibition was observed. On the other hand, the substrate conversion efficiency was not significantly influenced by the light intensity within the given range.

Light conversion efficiency decreased from 1.11% to 0.25% as light intensity increased from 88 W/m^2 to 405 W/m^2. The decrease in light conversion efficiency was not actually a drawback since produced hydrogen (V_{H2}) remained the same. The decrease resulted from the high value of (I) that is the incident light intensity in Equation (4.8). In solar bioreactors the light intensity is not the limiting factor since in a sunny summer day, light intensities up to 850-950 W/m^2 are common for most of the Europe (40º - 55ºN).

The light intensity that should be attained is at least 270 W/m^2 at the darkest point of the photobioreactor for obtaining high hydrogen production rate. That could be one of the limitations in the design of photobioreactors. The light intensity at low intensities is the rate determining parameter of hydrogen production thus it is one of the most important parameters that should be monitored and controlled closely; if the sunlight intensity in an outdoor photobioreactor is below this threshold value, additional artificial illumination may be provided to keep the hydrogen production rate high.

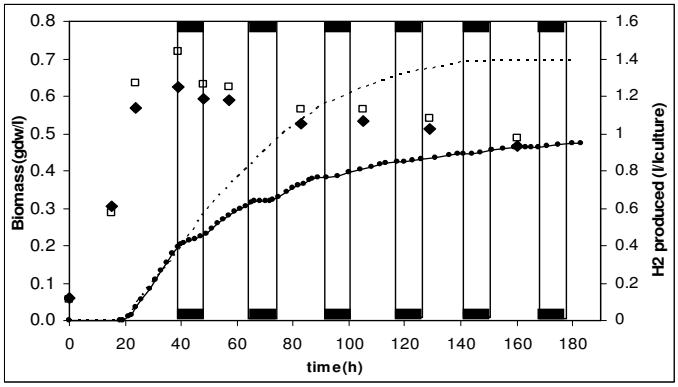
Kitajima et al. (1998) estimated the effect of the hydrogen uptake on the hydrogen production rate from lactate by *R. sphaeroides* RV in reactors with agitation and various depths. They showed that the hydrogen production rate decreased as the bioreactor depth increased (due to the insufficient light penetration into the bioreactors), to a compensation point where the rates of the hydrogen production and uptake were balanced in the reactor, so that no apparent production of hydrogen would be observed. They estimated that point to occur if the photobioreactor was 27 cm deep, under the highest outdoor sunlight illumination. They anticipated that the values of light compensation points or rates of hydrogen production and uptake will differ depending on the strain of bacteria,

substrate composition, and the coloration of the medium. These results confirm with their findings.

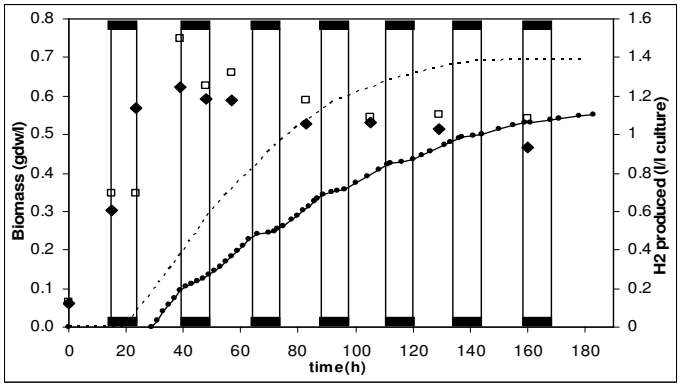
Nakada et al (1998) have reported that alteration of light intensity and light spectrum upon passage of the light through the reactor affected light energy conversion efficiencies to hydrogen. They also found that light energy decreased exponentialy with depth of the photobioreactor. According to their results, light energy conversion was low in the first compartment, that was the closest one to the light source, but light energy conversion was high in the last photobioreactor compartment which received the lowest light energy. In the present study, all photobioreactors received the same light spectrum at different intensities.

4.4.5 Effect of Light-Dark Cycles

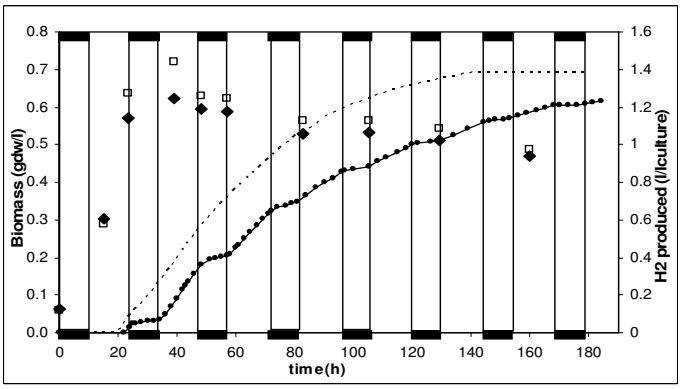
To get information about the tolerance of the growing cells to dark periods, four photobioreactors (55 ml in volume) were run in parallel; each one was subjected to different illumination protocols. The first photobioreactor was illuminated continuously (control); the second photobioreactor was illuminated after inoculation until hydrogen production started, then 14 h light - 10 h dark cycles were applied; the third photobioreactor was illuminated by 14 h light - 10 h dark cycles after inoculation; the fourth photobioreactor was illuminated by 10 h dark - 14 h light cycles, that is, it started with a dark period after inoculation and received the first light after 10 hours. Figure 4.20(a-b-c) illustrates the comparison of the variation of total hydrogen gas produced and cell concentration with respect to time for each illumination protocol with the results of the continuously illuminated photobioreactor.



(a)



(b)



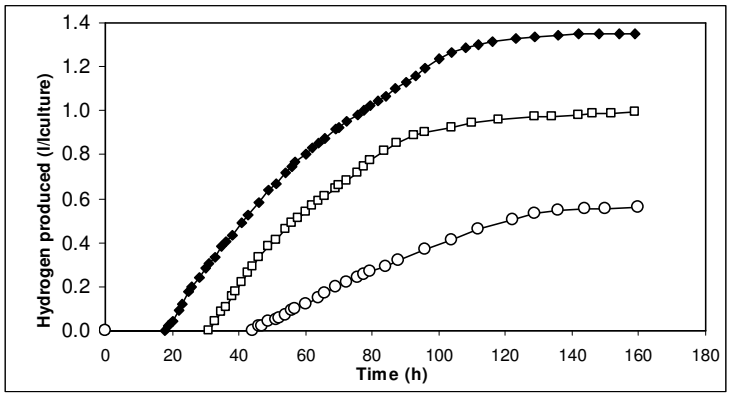
(c)

Figure 4.20. Effect of light-dark cycles on growth and H_2 production of *R. sphaeroides* in malate media (15 mM). 55ml bioreactors were used. Black bands indicate dark periods a) illuminated until H_2 production starts, then 14 h light - 10 h dark cycles were applied, b) illuminated with 14 h light - 10 h dark cycles from the start, c) illuminated with 10 h dark - 14 h light cycles from the start.
(▫): growth, (•): growth under continuous illumination (control); (—):H_2 production; (----):H_2 production under continuous illumination (control).

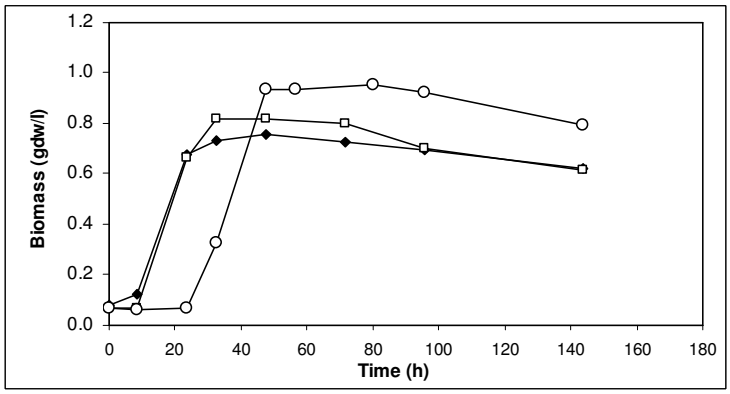
Figure 4.20 indicates that in the photobioreactors that are exposed to light-dark cycles, hydrogen production stopped during dark periods, and was restored when illumination started again. However, as summarized in Table 4.7, the average hydrogen production rate and the total hydrogen produced decreased compared to the continuously illuminated photobioreactor; from 15 ml/lculture.h to 8 ml/$l_{culture}$.h and from 1.36 l/$l_{culture}$ to 0.95-1.25 l/$l_{culture}$, respectively. This decrease is possibly due to the consumption of available substrates during dark periods by the bacteria. These results confirmed the findings of Miyake et al (1999), who reported that hydrogen production stopped during dark periods, and was restored when illumination started again. Similarly, cells did not grow during dark periods but survived and growth was restored when illumination started again (Figure 4.20.b). It is also observed that the dark period extends the lag time of hydrogen production from 18 hours to 22-28 hours. These results suggest that the overall hydrogen production rate and the total amount of hydrogen produced in an outdoor solar bioreactor which will be exposed to day-night cycle will be lower compared to the contiuously illuminated photobioreactor. Artificial illumination during night might be considered based on the gain reported and the cost of such an installment.

4.4.6 Effect of Initial Illumination

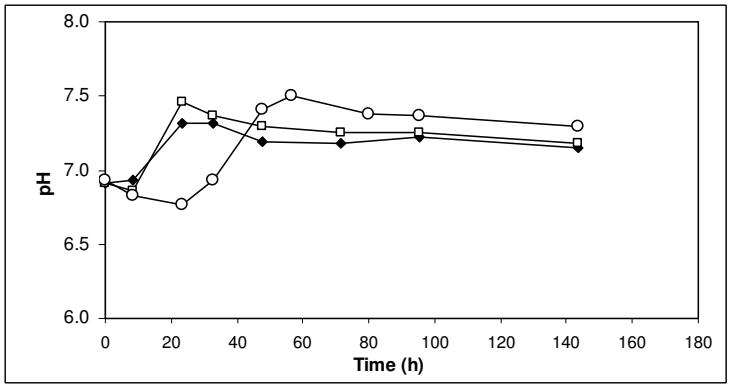
Figure 4.21 (a-b-c) illustrates the triggering effect of illumination after inoculation to biological hydrogen production by giving the total hydrogen production, growth and pH change. The photobioreactors were kept either 10h or 24 h at dark after inoculation, and then they were illuminated continuously. In order to see stimulation of light on hydrogen production, the results were compared with the results from a continuously illuminated photobioreactor. The results are compared with effect of the light/dark cycle runs and summarized in Table 4.7.



(a)



(b)



(c)

Figure 4.21 Effect of initial illumination a) on hydrogen production, b) on growth, c) on pH change of *R. sphaeroides* in malate media (15 mM). 55ml bioreactors were used. (•): Continuous illumination from the start (control); (▫): 10 hours of dark period at the start, then continuous illumination was applied; (○): 24 hours of dark period at the start, then continuous illumination was applied.

Table 4.7 The effect of different illumination protocols on hydrogen production by *R. sphaeroides* in 55ml photobioreactors

Illumination protocol	Average H_2 prod. rate ($ml/l_{culture}.h$)	Total hydrogen ($l/l_{culture}$)	Light conversion efficiency (%)	Substrate conversion efficiency (%)
Illuminated continuously	15	1.36	0.54	68
Illuminated until hydrogen production, then 14 h light - 10 h dark cycles	8	0.95	0.53	47
14 h light - 10 h dark cycles after inoculation	8	1.11	0.81	55
10 h dark - 14 h light cycle after inoculation	8	1.25	0.66	62
10 h at dark after inoculation, then continuous illumination	11	1.00	0.42	50
24 h at dark after inoculation, then continuous illumination	6	0.56	0.27	28

If the reactor was kept at dark after inoculation, the lag time of both growth and hydrogen production increased, and the total amount of produced hydrogen and the rate of hydrogen production decreased. It is deduced from Figure 4.21 that if the photobioreactor was kept at dark after inoculation, the cell did not grow until illumination started. Rhodobacter sphaeroides could not grow under dark anaerobic conditions; however it survived in fermentation mode by consuming malate. The decrease of pH during the dark period also confirmed that the bacteria survived in fermentation mode. Hydrogen production could not be achieved until a threshold cell concentration was obtained. These results confirm the literature: Gurgun et al. (1976) reported slow growth under dark anaerobic conditions. Uffen et al (1970) reported growth to a limited extent only when heavy inoculations were made under dark anaerobic conditions.

The inoculation should be made either in the morning to allow the solar bioreactor to receive daylight during first phase of the process or artificial illumination should be provided after inoculation if there is not enough light.

4.5 Integration of Dark and Photofermentation

4.5.1 Properties and the Composition of the Dark Fermentation Effluent

In order to combine dark and photo fermentation for an integrated biological hydrogen production process, it is of critical importance to test the dark fermentation effluent as a substrate in photofermentation. The composition of the dark fermentation effluent depends on the substrates used, the bacteria and the process parameters. For the testing purposes, samples of dark fermentation effluent were obtained from Agrotechnology & Food Sciences Group, Wageningen University, Netherlands. The samples were analyzed by HPLC and the composition of the samples was given in Table 4.8.

Table 4.8 Composition of dark fermentation effluent obtained from A&F, Wageningen University

Microorganism	Substrate used in dark fermentation	composition of effluent (mM)		
		acetate	lactate	formate
C.saccharolyticus	glucose/xylose 7/3 g/l	88	0.8	<0.2
C.saccharolyticus	miscanthus hydrolysate 14 g/l monosachharides	145	3.6	<0.2
C.saccharolyticus	miscanthus hydrolysate mix of 10&28g/l monosachharides	65	3.3	<0.2
T. neapolitana	miscanthus hydrolysate 14 g/l monosachharides	139	9.5	0.4
T. neapolitana	miscanthus hydrolysate 10 g/l monosachharides	94	8.9	<0.2
C.saccharolyticus	carrot press cake mix of 10&28g/l monosachharides	96	46.0	4.8
C.saccharolyticus	fructose 10 g/l	31	8.5	<0.2
C.saccharolyticus	fructose 20 g/l	108	14.3	0.3
C.saccharolyticus	glucose/fructose 7/3 g/l	59	0.4	<0.2
C.saccharolyticus	glucose/fructose 14/6 g/l	104	14.4	<0.2
T. neapolitana	glucose 10 g/l	77	0.8	<0.2

(Analysed in RWTH Aachen University, Germany)

The effluent sample which has closest organic acid composition to the optimized defined media (Section 4.6.1 - Selection of Bacterial Strain) for *R. capsulatus* was chosen for the study. This sample effluent was obtained by feeding miscanthus hydrolysate containing 10 g/l monosachharides to *T. neapolitana.* The resulting dark fermentation effluent obtained was analyzed by HPLC and the carbon sources present were identified as 94 mM of acetate, 8.9 mM of lactate and 2 mM of fructose, the pH of the effluent media was 7.53. After 1:1 dilution, the composition of this effluent is quite similar to the optimized defined medium, which is previously given as 40 mM of acetate and 7.5 mM of lactate.

4.5.2 Screening of PNS Bacteria in Defined (simulated) Media

Three different purple non-sulfur bacteria species (R. *capsulatus, R. rubrum, R. centenum*) were tested for growth in defined media, which had simulated organic acid content of the dark fermentation effluent.

The defined media contained 40mM of acetate and 7.5mM of lactate as C sources, and 2mM of glutamate as N source. The obtained results are given in Table 4.9 below. Data previously obtained by *R. sphaeroides* were also added to the table for comparison.

Table 4.9 Comparison of PNSB on acetate - lactate media in terms of growth and hydrogen production

Bacteria	Growth	Hydrogen production
R. capsulatus	+	+
R. rubrum	+	+
R. centenum	-	-
R. sphaeroides	+	+

Although three of the four species grew on the test media, significant differences were observed: *R. capsulatus* had 3 days of lag time for growth whereas *R. rubrum* had 7-8 days lag time. *R. centenum* did not grow within the tested time period. It is known from physiological studies part that *R. sphaeroides* also has a comparable lag time to *R. capsulatus* however its hydrogen production is not as stable and reproducible as *R. capsulatus* on acetate containing media.

Hydrogen production of these strains was not quantitatively measured and compared in this test.

Based on these findings and the results of the other studies performed within the Hyvolution project (Deliverable Report 3.2, 2006), *R. capsulatus* was chosen as the microorganism to be employed in the fermenter effluent studies.

4.5.3 Pretreatment of the Dark Fermentation Effluent

In order to feed the darkfermentation effluent into the photofermentation, the requirement of pretreatments was investigated.

Filtering

The fermenter effluent is a colored liquid and filtering helps reducing the color of the media. Since photofermentation depends on the light penetration into the media, reducing the color is important for dark colored effluents. In the current case, the fermenter effluent was centrifuged in A&F, Wageningen University after the dark fermentation process to remove the solid particles (i.e. bacteria) from the media. The resulting liquid was visually clear and further filtering was not applied.

Dilution

It is known from physiological studies part that acetate concentration above 40mM inhibits the growth of photofermentative bacteria and growth is virtually absent above 60mM of acetate. Since the fermenter effluent contained 94mM of acetate, it was diluted in 1:1 ratio with distilled water and the acetate concentration was halved to 47mM.

Buffer Addition

The filtered and diluted media was titrated with NaOH to see its resistance to pH increase, as photofermentation promotes a considerable pH increase in the media. The titration curves were given in Figure 4.22 below:

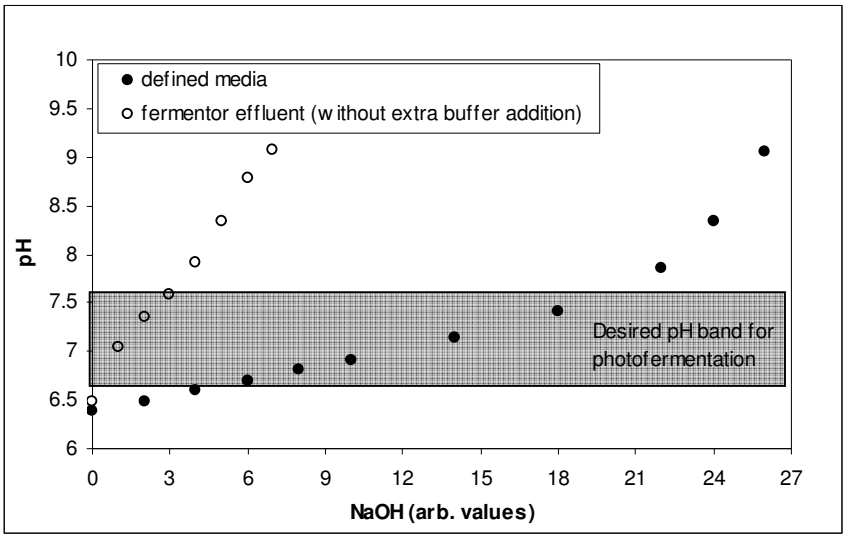


Figure 4.22. The comparison of defined media to fermenter effluent in terms of resistance to pH increase (buffer capacity). The shaded band shows the optimum pH levels for photofermentative hydrogen production process.

It is observed that the fermenter effluent does not have enough buffer capacity to keep pH stable at the desired band (6.7-7.5) and pH will immediately increase during photofermentation above 9 resulting in no hydrogen production. Therefore 20mM of KH_2PO_4 was added into the media as buffer to keep pH under control.

Sterilization

Final solution was sterilized by heat at 121°C for 15 minutes to prevent contamination. The sterilization may not be necessary in the future studies since thermophilic bacteria are not able to grow at photofermentation temperatures (30-35°C).

4.5.4 Hydrogen Production by *R. capsulatus* in Dark Fermentation Effluent

Studies were carried out to investigate the growth and hydrogen production of photofermentative bacteria on the effluent media (pretreated dark fermentation liquor). *R. capsulatus* was used, 10% inoculation was made. Experiments were carried out in 11 and 105 ml gas tight bottles.

In the first step, the growth of bacteria was tested in 105 ml sealed bottles, half filled under argon atmosphere. Bacteria grew without problem, lag time was shorter than one day. This run was repeated once more and the growth was confirmed.

In the second step, hydrogen productivity was investigated as well as the effect of the addition of other minor nutrients such as vitamins, iron and trace elements.

A picture of bioreactors showing the growth of bacteria was given in Figure 4.23.

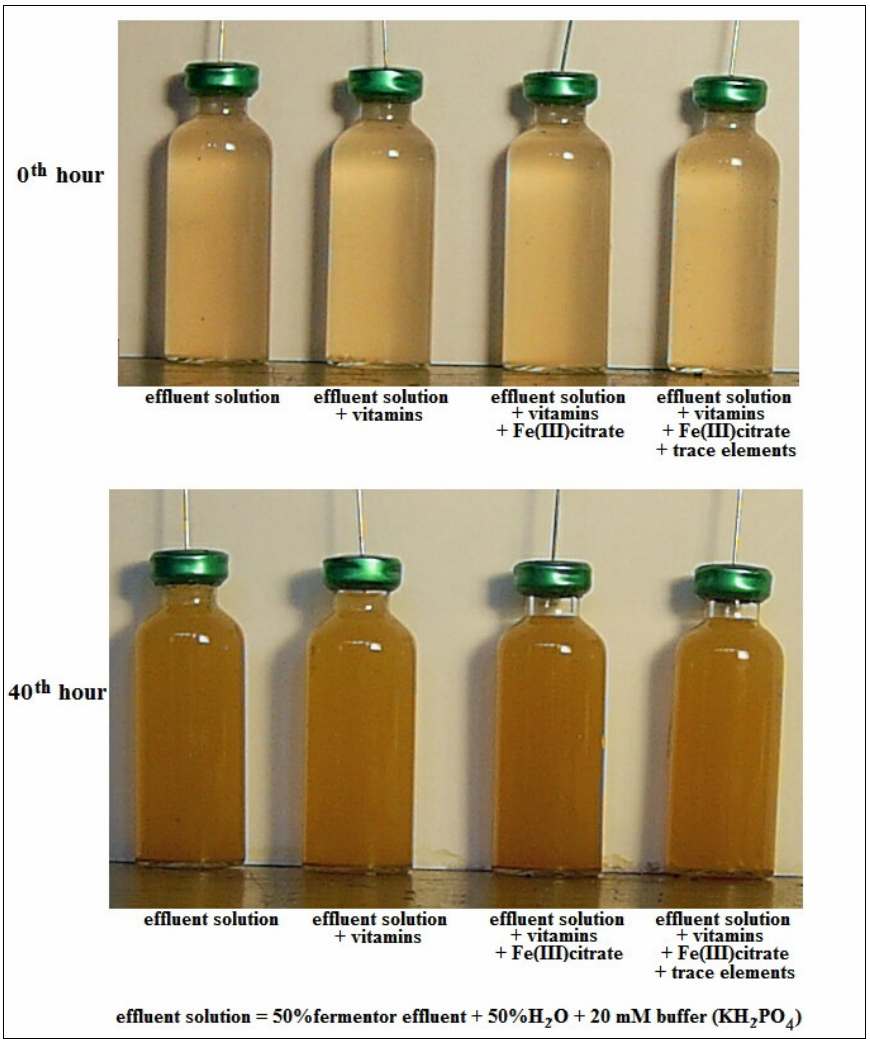


Figure 4.23. Growth of *R.capsulatus* in dark fermentation effluent media

In Figure 4.24, hydrogen production with respect to time for a run containing effluent media + iron + vitamins is given.

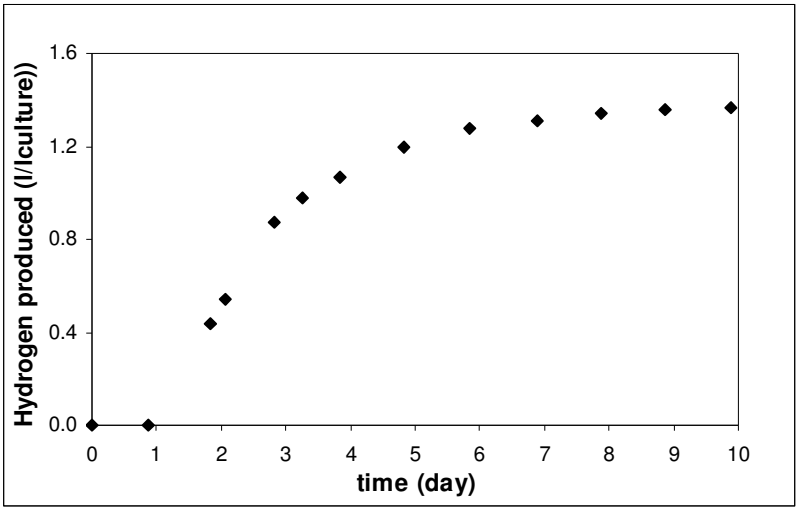


Figure 4.24. Hydrogen production by *R. capsulatus* in 105 ml bioreactor containing 1:1 diluted fermenter effluent, Fe-citrate, biotin, niacin and thiamine.

The composition of the gas obtained was 85.2% H_2 and 14.8%CO_2. The bacterial biomass was measured to be 0.49 gdw/l and the pH was 6.51 at the end of this run.

The results of hydrogen production experiments showing the effect of additives were given below in Table 4.10.

Table 4.10 Hydrogen production by *R. capsulatus* in bioreactors containing effluent media + iron + vitamins

	averaged runs:	average gas produced (ml/ml culture):
media[1]	3	0.33
media + vitamins[2]	3	0.33
media + iron[3]	1	1.18
media + iron + vitamins	4	1.40
media + iron + vitamins + trace elements	1	1.30

[1]media = pretreated dark fermentation effluent (Miscanthus hydrolysate)
[2]vitamins = biotin, niacin and thiamine
[3]iron = iron(III)citrate
(concentrations of the added nutrients were the same of defined media, which is given in Appendix B)

The final pH for all of the runs was in the range of 6.55-7.57. The final biomass obtained for media + iron + vitamins runs was 0.48 gdw/l in average and the composition of the gas obtained was 86% H_2 and 14%CO_2 in average. It should be noted that the hydrogen content of the produced gas is lower than the hydrogen content of the gas obtained by defined media (which had >95% H_2).

It is clearly observed that iron increased gas productivity drastically, therefore it is concluded that the iron addition is necessary into the media during pretreatment. This result is confirming the findings in physiological studies (Section 4.3.3 - Effect of iron concentration). Vitamins and trace elements slightly enhanced hydrogen production. When iron was added, produced hydrogen amount was comparable to defined media with similar C content.

4.6 Evaluation of Physiological Studies

4.6.1 Selection of Bacterial Strain

The PNS bacteria should be able to produce hydrogen from acetate and acetate/lactate mixtures, since the dark fermenter effluent contains mainly acetate and some lactate.

Although *Rhodobacter sphaeroides* O.U. 001 grew well on all of the substrates tested, it was found that acetate alone could not be a reliable carbon source for stable hydrogen production by *R. sphaeroides* in long term.

On the other hand, METU Biohydrogen Group showed that *R. capsulatus* (DSM1710) have better stability and performance in media where acetate or lactate/acetate mixtures were used as the carbon source(s) compared to *R. sphaeroides* and suggested the optimum acetate/lactate ratio as 40 mM / 7.5 mM. On the contrary, *R. rubrum* did not grow on acetate with any of the nitrogen sources (Hyvolution Project Deliverable Report 3.2, 2006).

Actually, how exactly the growth of PNS bacteria occurs on acetate may vary in different strains. During growth on acetate, new intermediates must be supplied to the TCA-cycle by special reactions. Such a special mechanism for the synthesis of four-carbon acids is glyoxylate cycle, in which acetate was converted to glyoxylate which then condenses with acetyl-CoA, yielding malate.

It is reported that the enzymes of glyoxylate cycle were present in *R. capsulatus* if the bacteria are cultivated in acetate containing media under both phototrophic or chemotrophic growth conditions (Fuller et al 1961, Kornberg et al 1960, Losada et al 1960).

In *R. rubrum* and *R. sphaeroides*, insignificant amount of isocitrate lyase (which is a key enzyme in glyoxylate cycle) has been found and the glyoxylate cycle cannot account for the synthesis of cell constituents from acetate, yet both grew well with acetate. In *R. sphaeroides* however glyoxylate has been detected, this suggests a rather direct conversion of acetate to glyoxylate by an unknown mechanism (Fuller 1959, Kornberg et al 1960, Kikuchi et al 1963).

Another study state that acetate-grown aerobic cultures of *R. sphaeroides* failed to incorporate (2-^{14}C) acetate into glyoxylate suggesting this organism uses a novel pathway to metabolize acetate (Payne et al 1969).

Subsequent studies verified that three different *R. sphaeroides* strains lacked isocitrate lyase, while four *R. capsulatus* strains were shown to have isocitrate lyase (Willison 1988).

Consequently, since *R. capsulatus* was found to produce hydrogen gas in a more stable manner in acetate media than *R. sphaeroides*, it has been concluded that at large scales *R. capsulatus* should be used instead of *R. sphaeroides* as the photosynthetic bacterium.

4.6.2 Effect of Temperature

The bacteria used in the studies are of mesophilic type and can not grow at elevated temperatures. A study has been made with *R. sphaeroides* to determine the maximum temperature for bacterial growth and hydrogen production. The bioreactors were placed inside the incubator and the temperature was set to a fixed value, however, the temperatures inside the bioreactors were also recorded by thermometer probes since bioreactor temperature is different than incubator temperature due to the absorbed light by the bacteria. Table 4.11 was constructed by the data obtained from different runs

Table 4.11 Effect of temperature on the growth and hydrogen production by *R. sphaeroides*

Bioreactor temperature (ºC)	growth	H_2 production
48	-	-
42	-	-
37	+	+
34	+	+
31	+	+

Since the data are not obtained in parallel runs, comparison graphs are not plotted, however it can be clearly concluded that the temperature in the bioreactor should not exceed 40°C during the operation.

Similar results were obtained with *R. capsulatus* in outdoor operations and bacteria did not grow when bioreactor temperature exceeded 40°C (Section 6).

Favinger et al (1989) similarly reported that most PNS bacteria are unable to grow at temperatures above 39°C. In a comparable work, Mahakhan et al (2005) reported that from 226 isolates of anoxygenic phototrophic purple non-sulfur bacteria, only 13 strains could grow under light-anaerobic conditions at 40°C. One of these strains (strain SB24) showed photoproduction of molecular hydrogen using raw cassava starch as an electron donor, when incubated with illumination under anaerobic conditions at 40°C at a maximum rate of 39 ml $H_2/l_{culture}$.h.

The best candidate for high-temperature operation seems to be *R. centenum*, which is reported to grow optimally at 40-42°C and up to 45°C (Favinger et al, 1989), however it can not grow at high substrate concentrations which limits its usability for hydrogen production operations.

4.6.3 Substrate Consumption Kinetics

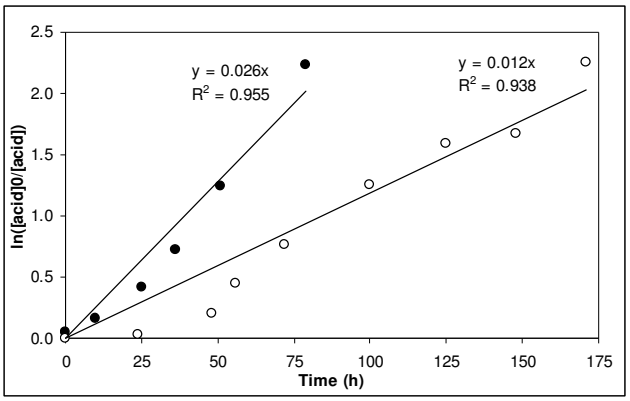
Recently, Eroglu et al, (1999) and Koku et al, (2003) reported that the consumption rate of malate is first order with respect to malate concentration with a consumption rate constant of 0.015 - 0.037 h^{-1}. In this study, the consumption rate analyses were made for the runs that contained malate, butyrate and acetate as sole substrates, and for the runs that contained mixtures of those. The first order consumption rate equation:

$$-r_S = -dS/dt = -kS \qquad (4.9)$$

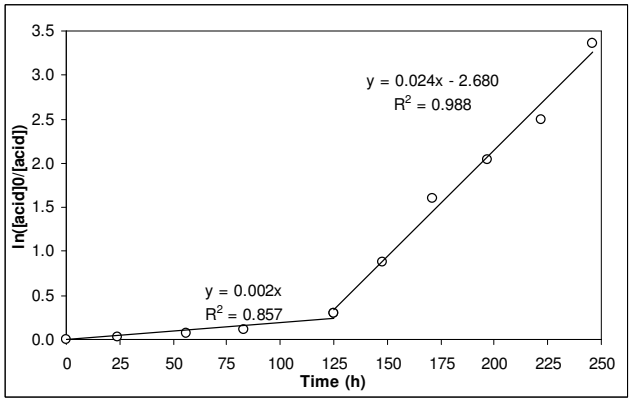
can be integrated to obtain the expression:

$$S = S_0 \exp(-k_s t) \qquad (4.10)$$

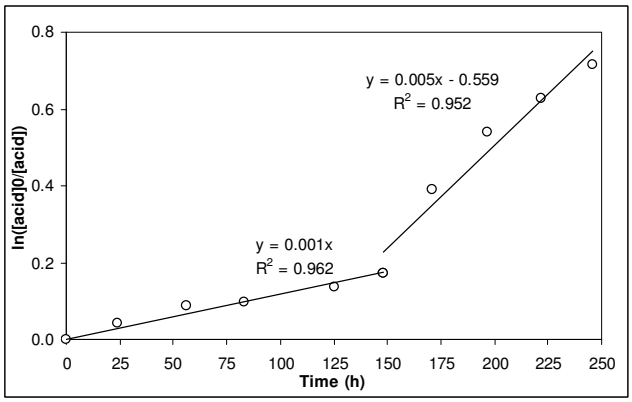
where k_s is the consumption rate constant (h^{-1}) and S, S_0 are the instantaneous and initial concentrations of substrates, respectively (mol/l). Figure 4.25 shows the first order consumption rate models for different organic acid runs.



(a)



(b)



(c)

Figure 4.25. The first order consumption rate models for a) malate (●) and butyrate (○) in single substrate runs, b) acetate in media containing mixture of acetate-butyrate-propionate. c) butyrate in media containing mixture of butyrate-propionate-acetate. Experimental data were shown as dots and model fits were shown as lines.

The substrate consumption rate was found to be first order with respect to malate concentration with a consumption rate constant of 0.026 h^{-1}. The R^2 value, which quantifies the dispersion of distribution from the mean, was used as a measure of the goodness of the fit. The R^2 value for the fit was 0.96 (Figure 4.25.a). This is quite comparable to those obtained in the previous studies (Eroglu et al, 1999 and Koku et al, 2003).

The butyrate consumption rate was also found to be first order with a consumption rate constant of 0.012 h^{-1}, indicating that butyrate was utilized slower than malate by the bacteria. The R^2 value for the fit was 0.94 (Figure 4.25.a).

On the other hand, the first and second order consumption rate equations do not fit to acetate consumption data well (R^2 value for the fits were 0.65 and 0.42, respectively), poorly defining the acetate consumption rate. That is most probably due to its yet unknown metabolic utilization pathway which is also supporting our experimental findings about the unstability of using acetate as the sole substrate.

In the case of substrate mixtures, only the consumption of main substrates was analyzed. The consumption rates were also first order but with poor fit to data (with R^2 values of 0.84 and 0.78 for butyrate and acetate media, respectively). On the other hand, the process could be split to two parts for analysis; from start to where the minor substrates were depleted, and to the end from that point onward. This is illustrated in Figure 4.25.b and 4.25.c. That way, the substrate consumption rates could be defined far more accurately. R^2 values were 0.96 and 0.95 for butyrate mixture media with consumption rate constants of 0.001 h^{-1} and 0.005 h^{-1} for the first and the second part of the batch, respectively (Figure 4.25.b). The consumption rate constant was lower than the sole-substrate case. In the case of the acetate mixture media, unlike sole-acetate case, equation fitted quite well to the acetate consumption data, possibly since it was supported by other minor substrates. R^2 values were 0.86 and 0.99. The consumption rate constants

were 0.002 h^{-1} and 0.024 h^{-1} for the first and the second part of the batch, respectively (Figure 4.25.c).

It is clearly shown that the consumption rate of the main substrate significantly increased when the minor substrates were depleted.

The malate and butyrate consumption rates were found to be first order with a consumption rate constant of 0.012 h^{-1}, whereas the first and second order consumption rate equations did not fit to acetate consumption data. In the case of substrate mixtures, it is found that the consumption rate of the main substrate significantly increased when the minor substrates were depleted.

4.6.4 Cell Growth Kinetics

It is given in the literature that the growth curves obtained for *R. sphaeroides* O.U. 001 could not be fitted to the Monod model or its modified versions but to the logistic model, on malate (Koku et al, 2003), acetate and lactate (Eroglu et al, 2007).

In this study, the obtained growth curves of *R. sphaeroides* O.U. 001 on different organic acids were fitted into the logistic growth model.

The growth rate for the logistic model is expressed as:

$$dX/dt=k_cX(1-X/X_{max}) \quad (4.11)$$

where k_c is the apparent specific growth rate (h^{-1}), X is the dry cell weight (g/L), and X_{max} is the maximum dry cell weight (g/L).

Integrating, the equation becomes:

$$X=X_{max}/[1+\exp(-k_c.t)(X_{max}/X_0-1)] \quad (4.12)$$

where X_0 is the initial bacterial concentration at the lag time (g/L) and t is the actual time minus lag time (h).

By using logistic model it is possible to model bacterial growth at exponential phase together with stationary phase. The predictive power of logistic model may be limited since it does not involve a substrate term, however, for the purposes of batch hydrogen production experiments, where the initial substrate

concentrations and the inoculation volume are kept constant, the logistic model is a fair approximation of the growth curve (Koku et al, 2003).

Figure 4.26 shows part of the growth curves (the period between the lag time until the cell death phase) that were fitted into the logistic model, experimental data were shown as dots and logistic model fits were shown as lines.

The model equations were given below for each of the substrates:

For malate: $X=0.99/[1+\exp(-0.183\cdot t)(0.99/0.034-1)]$ (4.13)

For acetate: $X=1.69/[1+\exp(-0.164\cdot t)(1.69/0.013-1)]$ (4.14)

For propionate: $X=1.02/[1+\exp(-0.083\cdot t)(1.02/0.038-1)]$ (4.15)

For lactate: $X=1.29/[1+\exp(-0.154\cdot t)(1.29/0.011-1)]$ (4.16)

For butyrate: $X=1.50/[1+\exp(-0.068\cdot t)(1.50/0.057-1)]$ (4.17)

In Table 4.12 the specific growth rate constants (μ_{max}), initial cell concentrations ($X_{0,e}$) and maximum cell concentrations ($X_{max,e}$) that were determined experimentally were compared to the obtained values from the models (k_c, $X_{0,m}$, $X_{max,m}$).

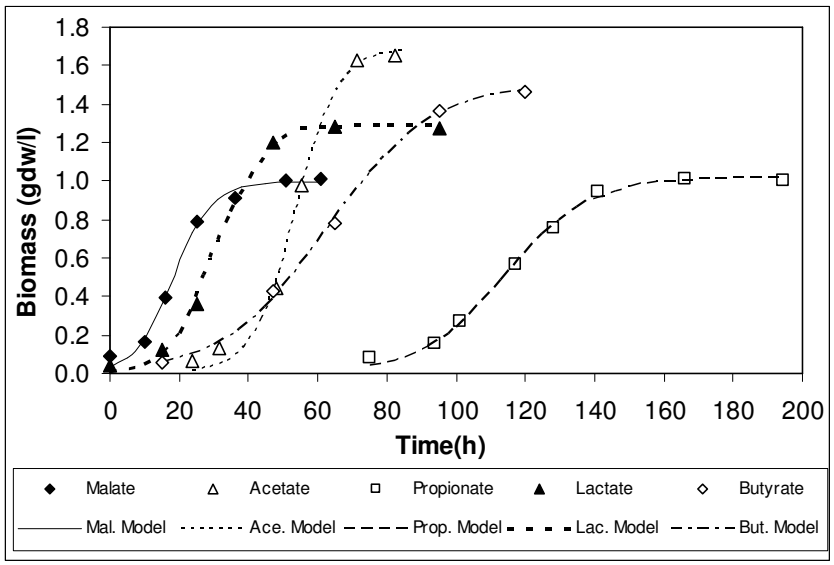


Figure 4.26. Effect of different carbon sources on growth curves (dots represents experimental data and lines represent logistic model fits)

Table 4.12 Growth modeling of *R. sphaeroides* in different organic acids

	C source				
	Malate	Acetate	Propionate	Lactate	Butyrate
$X_{max,e}$ ($g.l^{-1}$)	1.01	1.65	1.01	1.29	1.46
$X_{max,m}$ ($g.l^{-1}$)	0.99	1.69	1.02	1.29	1.50
$X_{0,e}$ ($g.l^{-1}$)	0.088	0.068	0.083	0.041	0.060
$X_{0,m}$ ($g.l^{-1}$)	0.034	0.013	0.038	0.011	0.057
μ_{max}	0.147	0.105	0.079	0.110	0.061
k_c	0.183	0.164	0.083	0.154	0.068

$X_{0,e}$: Experimental initial bacterial concentration at the growth lag time, (g/L)

$X_{0,m}$: Initial bacterial concentration obtained by logistic model, (g/L)

$X_{max,e}$: Experimental maximum bacterial concentration, (g/L)

$X_{max,m}$: Maximum bacterial concentration obtained by logistic model, (g/L)

μmax: Specific growth rate constant obtained by exponential model, (1/h)

k_c: Specific growth rate constant obtained by logistic model, (1/h)

The growth kinetics of *Rhodobacter sphaeroides* O.U. 001 in media with different organic acids were shown to fit into logistic model, the R^2 values for all of the logistic model fits were above 0.99.

The maximum bacterial concentration values that are determined by the logistic model are quite similar to that of experimental values however when X_0 values are considered logistic model does not give consistent results with that of obtained experimentally except butyrate media. For the specific growth rate constants, it can be seen that, exponential model give consistent results with that of obtained by logistic model for butyrate and propionate media only. According to the logistic model, highest and lowest specific growth rates were obtained when malate and butyrate were the carbon sources, respectively.

In a comparable study, Eroglu et al (2007) reported $0.06h^{-1}$ as the specific growth rate value obtained in 15 mM malate. Higher specific growth rates were obtained when acetate (30 mM) or lactate (20 mM) was the carbon source ($0.18\ h^{-1}$ and $0.28h^{-1}$, respectively). Koku et al (2003) also reported $0.098\ h^{-1}$ as the specific growth rate value obtained in 7.5 mM malate and 10 mM glutamate media.

CHAPTER 5

ANAEROBIC FLAT PANEL PHOTOBIOREACTORS

The construction and successful operation of the photobioreactors is of critical importance for photofermentative hydrogen production. The development of a successful panel/ tubular photobioreactor concept, which ensures simple design, low material and production costs and high utilization of light, is targeted.

Flat panel and tubular photobioreactors show highest efficiencies most probably due to their high illumination area and are commonly preferred (Akkerman et al, 2002, Sierra et al, 2007, Eroglu et al, 2007).

The photobioreactor type to be used within this thesis study was decided to be of flat-panel type.

5.1 Setting the Design Limitations for Anaerobic Panel Photobioreactor

Ideal anaerobic panel photobioreactor should:

- have low manufacture and operating cost,
- be resistant to outdoor conditions (i.e. wind, rain, temperature fluctuations),
- be made of a highly transparent material inert to bacteria
- should have a low maintenance cost

It has been targeted to fulfill these conditions by introducing several new aspects to the design. First of all, the design limitations were defined. Unfortunately, there are serious limitations in the design parameters:

i) The bioreactor should be made of a transparent material (i.e. common bioreactor construction materials such as stainless steel can not be used).

Appropriate materials have poor strength and this causes the bioreactor to be fragile and have low durability. Likewise, the maximum bioreactor size is also limited.

ii) As the bacteria create a self-shading effect, the depths of the bioreactors are limited by the light penetration. Consequently, bioreactors require large areas.

iii) Solar illumination should be preferred, artificial illumination increases cost dramatically due to the electric consumption. Since outdoor conditions are quite harsh compared to laboratory conditions, the bioreactors should be designed accordingly (i.e. wind, rain, temperature fluctuations should be considered).

iv) Due to the limited depth of the bioreactor and the nature of the construction material, mechanical agitation can not be used. Possible mixing methods are either recycling the medium or flushing the gas produced back.

v) The photobioreactor material should be inert to bacteria. i.e. it has been shown that iron affects hydrogen production considerably and stainless steel parts in the photobioreactor should be avoided. Similarly materials which promote cellular adhesion should be avoided.

vi) The photobioreactor must be completely sealed against gas leakage in and out due to two factors: First factor is the dependency of the process to anaerobic conditions, the bacteria is highly affected by the oxygen presence which inhibits the hydrogen production. Second factor is that the product gas, hydrogen, is a very diffusive gas and is hard one to store. Therefore the entire system must be completely leak-proof to air and H_2.

5.2 Construction Material of the Photobioreactor

Under the limitations stated in Section 5.1, the possible candidate materials were determined to be

- glass,
- polymethyl methacrylate (PMMA) which is commonly called acrylic sheet and sold by the tradename Plexiglas,
- polycarbonate

These materials were compared based on their cost, light transmission, durability and ease of manufacture.

The wholesale prices of these materials were obtained from local sellers:

Plexiglas (Acrylic sheet) (5mm thickness): 20 $ /m^2

Polycarbonate sheet (5mm thickness): 50 Euro /m^2

Glass (3mm thickness): 7.4 $ / m^2

As can be seen, among these materials, glass is the cheapest and polycarbonate is the most expensive one.

All three materials considered have high light transmittance: Acrylic transmits more light (up to 93% of visible light) than conventional glass, however unlike glass, acrylic does not filter ultraviolet (UV) light. Polycarbonate is highly transparent to visible light and has better light transmission characteristics than many kinds of glass. The choice of the bioreactor construction material should be made depending on other factors.

The density (which influences weight of the bioreactor), strength, hardness, elasticity, ease of shaping are the other factors to be considered. Glass is the hardest material to shape compared to the polycarbonate and acrylic. Acrylic and polycarbonate are more elastic than glass and deforms under stress, leading to the changes in the volume of the photobioreactor.

Acrylic is also lighter than glass; its density can range from 1150-1190 kg/m^3. This is less than half the density of glass which ranges 2400 to 2800 kg/m^3. Density of polycarbonate is 1200-1220 kg/m^3, which is very close to that of acrylic.

Acrylic and polycarbonate have higher impact strength than glass but both are softer and more easily scratched than glass.

In scope of these data, different bioreactors were manufactured during the progress of the thesis work. Some of the bioreactors were made of acrylic, some of glass, and some of them were composite bioreactors made of combination of different materials such as glass, acrylic and PVC (polyvinylchloride). The advantages and drawbacks of the bioreactors experienced were summarized in this part.

The acrylic bioreactors were manufactured at 8 L volume and utilized both indoor and outdoor (Figure 5.1). Although their performance indoor was satisfactory they could not endure the outdoor conditions; several bioreactors were cracked during outdoor tests (possible reasons are the temperature difference between cooling water and the bioreactor, temperature and pressure difference between day and night and thermal expansion difference between metal inlet parts and acrylic). Also, during indoor runs at large scales, it has been found that the bioreactor volume increases due to deforming of the acrylic plates under stress caused by the liquid pressure inside. This deforming is quite high (i.e. from 20L to 28L) and might lead to failure of the bioreactor at larger scales under self-weight.

The glass bioreactors used were actually bottles of different sizes (50 ml up to 0.5L). Those performed without any problem both indoor and outdoor (Figure 5.2). However shaping of glass is a problem and custom designs are extremely hard and expensive (due to the labor cost) to manufacture. The composite bioreactor design greatly overcame this problem.

One of the composite bioreactor was manufactured mainly from glass as it is the cheapest material. However as it is the most difficult one to shape, the top part of the bioreactor was made of acrylic which was easily shaped as needed and all the inlets-outlets are placed in this part. The cooling coil is made from glass tube. Silicon was used as the adhesive and sealant. Bioreactor inlet and outlet ports are made up of butyl rubber (Figure 5.3).

Another composite bioreactor type used was made of acrylic and PVC. These photobioreactors were manufactured in RWTH University Aachen, Germany. The frames of the bioreactors were made of PVC and the panels were made of acrylic sheets. Adhesives were not used; plates were screwed to the frame instead. Those photobioreactors proved to be quite strong and reliable but they suffered from the same deforming problem under the water pressure as in the case of acrylic bioreactors (Figure 5.4 and 5.5).

The pictures of the different photobioreactors (shape, material, scale) used within this thesis studies were given below in Figures 5.1-5.5 and their specifications were tabulated in a comparable form in Table 5.1.

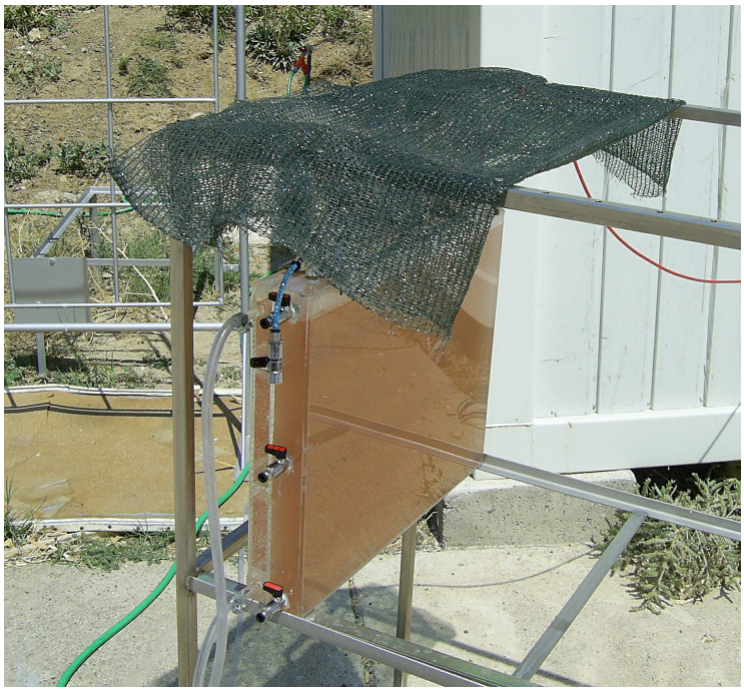


Figure 5.1. 8L acrylic photobioreactor (illuminated by sunlight).

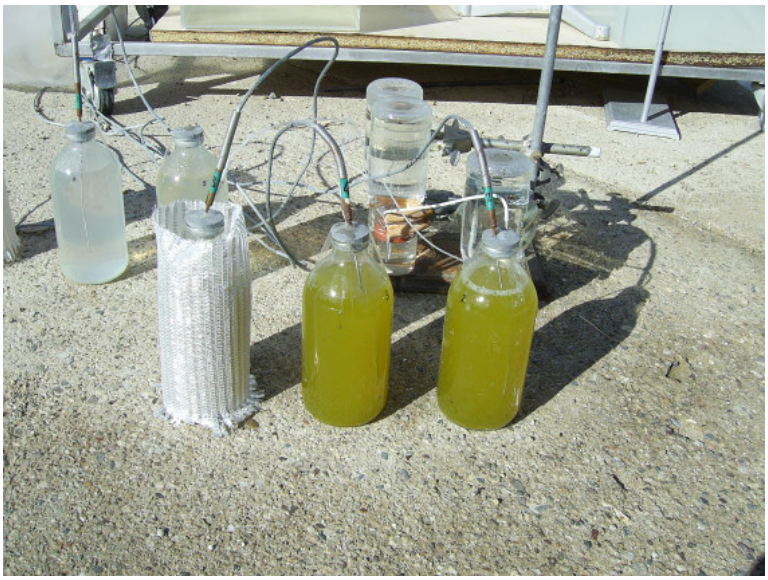


Figure 5.2. 0.5L glass photobioreactors (illuminated by sunlight).

Figure 5.3. 5.5L photobioreactor (illuminated by 2x75W tungsten lamps, bioreactor made of glass and acrylic (top part only)).

Figure 5.4. Four 5L photobioreactors connected in parallel (illuminated by 3x500W halogen lamps, frames of bioreactors are made of PVC, panels are from acrylic, the plates were inserted in a metal support).

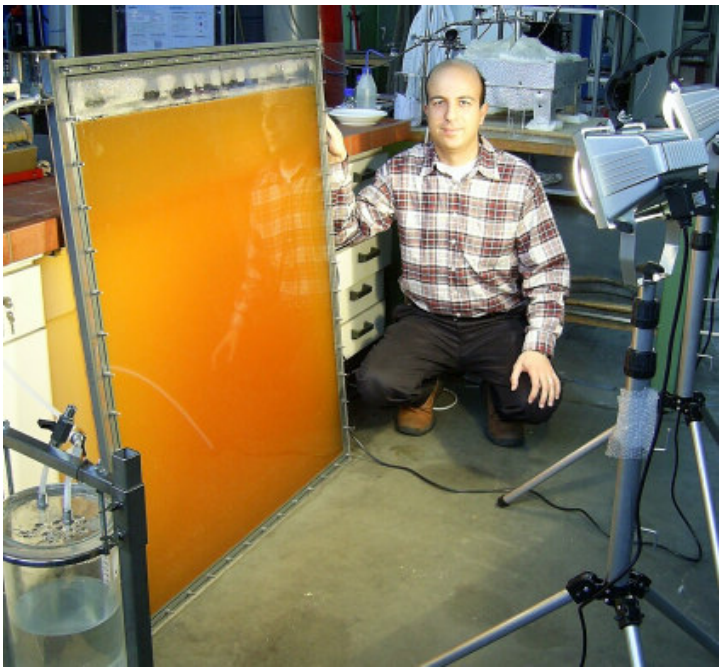


Figure 5.5. 25L photobioreactor (illuminated by 2x500W halogen lamps, frame of the bioreactors are made of PVC, side panels are from acrylic.

Table 5.1 Specifications of the photobioreactors used

Construction Material	Volume (L)	Dimensions (cm) (LengthxHeightxWidth) or (HeightxDiameter)
PVC (Frame), Acrylic (Panels)	5	50x50x2
PVC (Frame), Acrylic (Panels)	25	92x110x2
Glass	0.05 0.01 0.5	6x3.5 11x3.5 14x7
Acrylic	8	50x40x4
Acrylic (Top), Glass (Panels)	0.58 5.5	13x11x4.4 40x33x4.4

5.3 Introducing Novel Approaches to the Design of the Photobioreactor

5.3.1 The Geometry of the Photobioreactor

The flat-panel photobioreactors are used to have a rectangle-prism shape. Illumination is provided from the side panels, therefore the frame does not need to be transparent (as in the case of the PVC framed bioreactor). The inlets-outlets are placed on the frame, where required.

The depths of the bioreactors are between 2 to 5 cm. It may be possible to use deeper bioreactors in outdoor conditions with bright sunlight or in indoor conditions by illumination from both sides. Other dimensions of the bioreactors were determined by the volume desired; for 8L bioreactor length is 50cm and height is 40 cm.

One of the designs had a slight modification; top part was inclined towards the center (roof shape) to help hydrogen collection from the middle. A picture of this bioreactor was shown in Figure 5.6, showing the design, the manufactured photobioreactor and the photobioreactor during operation (filled by bacteria culture).

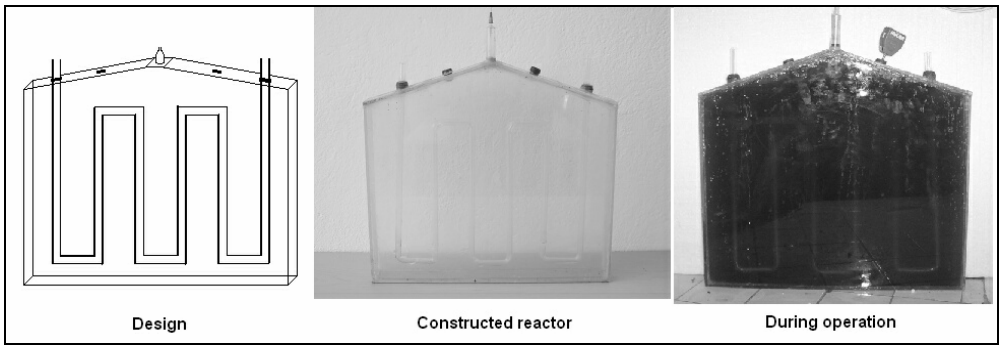


Figure 5.6. (From left to right) The design, the manifactured photobioreactor and the photobioreactor during operation (filled by bacteria culture) for a 5.5L photobioreactor.

5.3.2 Creating Anaerobic Conditions

5.3.2.1 Operating Bioreactor without Headspace

In most of the cases anaerobic conditions were created by flushing an inert gas such as argon through the culture medium, this is required since there is a 5-10% headspace in the bioreactors during the process. However in the roof-shaped design where the gas was collected from the top of the bioreactor, the top dead space is not necessary and the bioreactor could be filled completely with the culture medium. This is applicable as there is virtually no foaming in the process. In this design, if the dissolved oxygen in the medium and the air present in the capillary connection tube can be accepted to be negligible, the argon gas flushing could be removed from the system. This design has overwhelming advantages:

- Since there is no headspace, the efficiency of the bioreactor increases as the working volume is equal to the bioreactor volume.
- The need of using an inert gas (such as argon) to create anaerobic atmosphere was eliminated.

- Since the bioreactor does not have the top space containing the inert gas, the hydrogen collected has higher purity.
- Since argon gas is not used, the bioreactor is simplified and the operation cost is decreased (argon gas flushing equipment is removed, argon recycling equipment is not needed).

The applicability of this design was investigated in the next section.

5.3.2.2 Effect of Argon Gas flushing on Hydrogen Production

An experimental setup was designed in order to compare argon gas flushed and not flushed cases: 55ml bottle bioreactors were used, one bioreactor was filled completely without any headspace and assumed that anaerobic conditions were met, on the other hand, 90% of the other bioreactor was filled with the culture media and argon gas was flushed from the bioreactor as described in Materials and Methods section. *R. sphaeroides* O.U.001 was used as the bacteria. The growth, hydrogen production and pH changes were monitored during the batch and the results were given in the Figures 5.7 - 5.9 below.

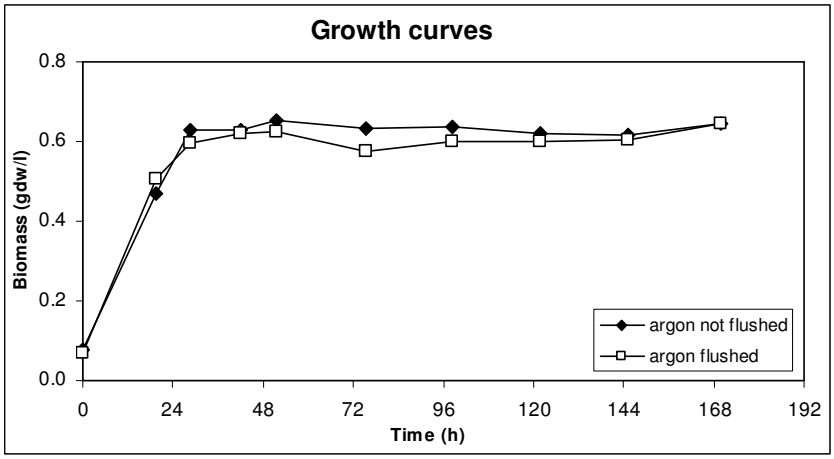


Figure 5.7. The growth of *R. sphaeroides* in argon flushed and not flushed 55ml bioreactors (C source was 15 mM of malate).

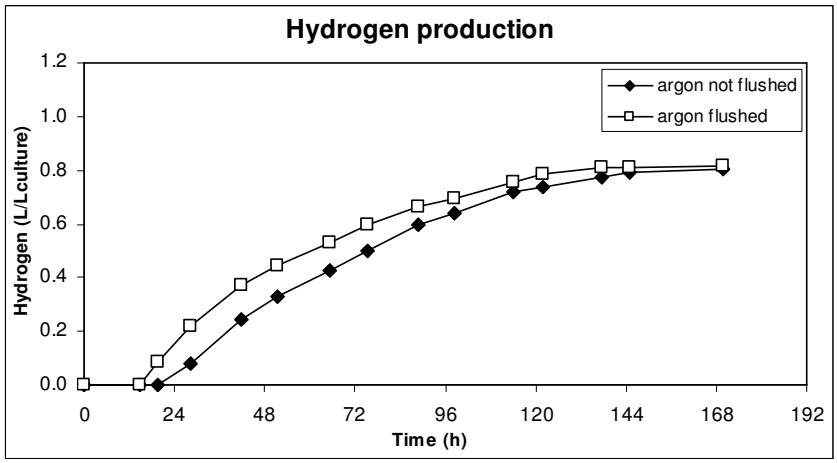


Figure 5.8. The H_2 production by *R. sphaeroides* in argon flushed and not flushed 55ml bioreactors (C source was 15 mM of malate).

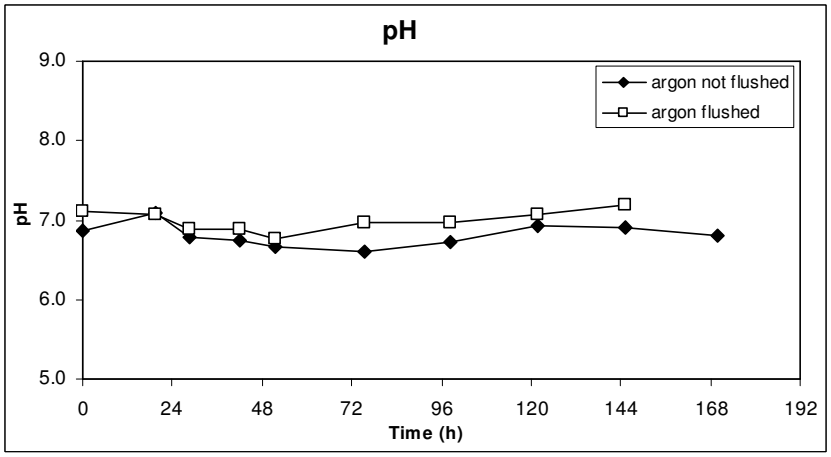


Figure 5.9. The pH change in argon flushed and not flushed 55ml bioreactors (C source was 15 mM of malate).

As can be seen from the figures, both bioreactors performed very comparably, the difference was insignificant considering that this is a bioprocess thus a degree of variety should be expected in the results. Most probably the small

amount of oxygen which may be present in the medium was consumed by the bacteria during the early stages of growth. The GC analyses of the produced gases were also made, the gas obtained from both bioreactors contained more than 98% H_2 and the rest was CO_2, on argon free basis.

Based on this result and considering the many advantages of working under non-headspace conditions, many of the studies within this thesis work were conducted under non-argon conditions.

5.3.3 Hydrogen Uptake by the Bacteria and Gas Collection System Design

R. sphaeroides is able to utilize back the hydrogen that it produces via its uptake hydrogenase enzyme. This consumption is known but not experimentally quantified in a working system where nitrogenase activity is also present. This uptake may reduce the hydrogen amount obtained or may not affect the process at all. Therefore the objective in this part was to show this uptake and to determine the appropriate gas collection system setup to prevent it in case of a significant reduction on the amount of the produced gas.

In order to show the hydrogen uptake, two different experimental setups were designed and applied. The working volumes of the bioreactors used were 55 ml. In the first setup, a piston was placed to the exit of the bioreactor, where the produced gas pushed the piston (Bioreactor 1). In this setup, the piston was able to move forward and backward to respond the hydrogen production/uptake. A second experimental setup was also used, in which the produced gas was collected in a glass cylinder (Bioreactor 3). However here, there was a control bioreactor for comparison in which the produced hydrogen was collected by a water trap and uptake was not possible (Bioreactor 2).

The experimental setup and the hydrogen production curve obtained is given in Figures 5.10 and 5.11:

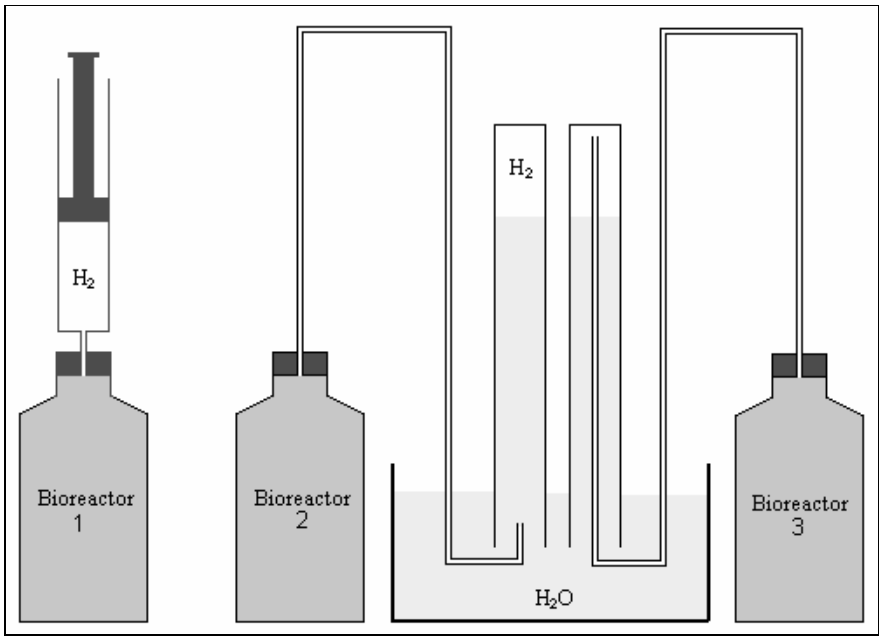


Figure 5.10. Setup to test hydrogen uptake

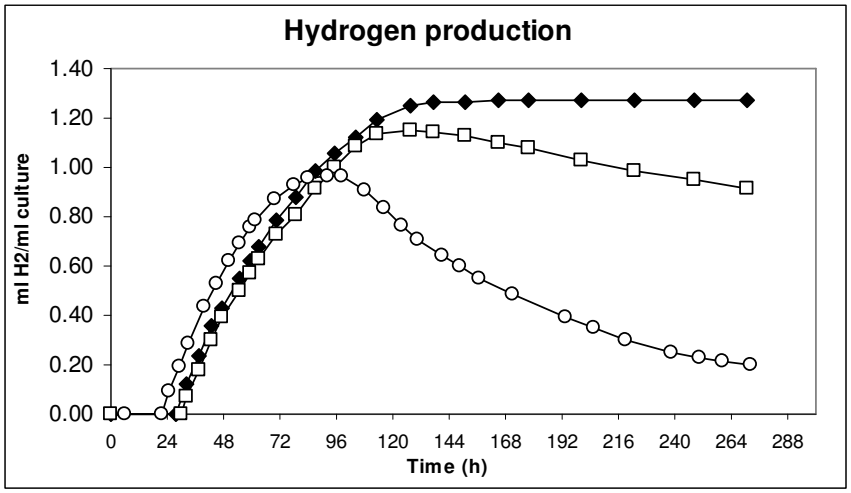


Figure 5.11. Demonstration of hydrogen uptake by *R. sphaeroides* in 55ml bioreactors (o: Bioreactor 1, piston system, uptake possible, ♦: Bioreoactor 2, uptake not possible, : Bioreactor 3, uptake possible). C source was 15mM of malate.

Hydrogen uptake by the bacteria was observed clearly after 96th hour in Bioreactor 1 and after 130th hour in Bioreactor 3. The difference in hydrogen amounts between Bioreactor 1 and 3 was possibly resulted from the higher hydrogen pressure in Bioreactor 1 due to friction forces affecting the piston movement. As a result, those two different setups showed hydrogen uptake by the bacteria, thus, revealing a key design feature of the photobioreactors; hydrogen collection tubes should be equipped by a water trap (our system) or one-way valves to prevent the hydrogen to go back into the bioreactor.

5.3.4 On-line Monitoring of the Hydrogen Production

An innovative approach has been made to monitor the hydrogen production online. The hydrogen produced from the bioreactors were collected in graded glass cylinders, a digital camera connected to a PC was placed in front of these cylinders. A software was developed for monitoring and recording the hydrogen production continuously. This allowed monitoring of the produced gas amount in real-time with high precision (0.1ml/h) and calculation of the hydrogen production rate very accurately during process. This system has been commercialized by the biotechnology company "Obitek Ltd. Şti." under the name "ObiGasMaster". The continuous hydrogen production data was obtained this way. The details of the commercial product were given in Appendix D.

5.4 Light Intensity Distribution in Indoor Applications

5.4.1 Light Intensity Distribution on the Surface of the Panel Photobioreactor

Light intensity distribution map on the surface of a flat panel photobioreactor has been constructed. 25L photobioreactor panel with 96x110 cm dimensions and 5L photobioreactor panel with 50x50 cm dimensions were used. The illumination was provided by 500W halogen lamps. The resulting maps were given in Figure 5.12 and 5.13

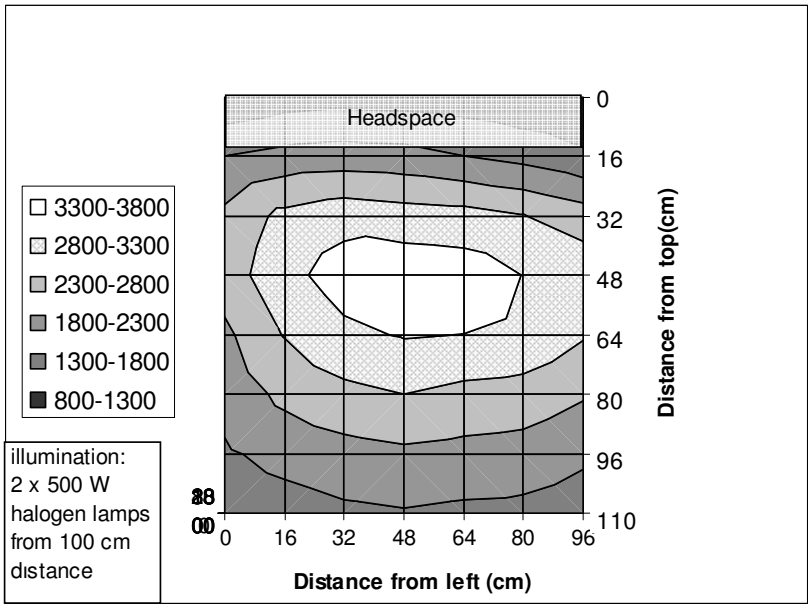


Figure 5.12 Light intensity distribution map on the surface of a 25L flat panel photobioreactor

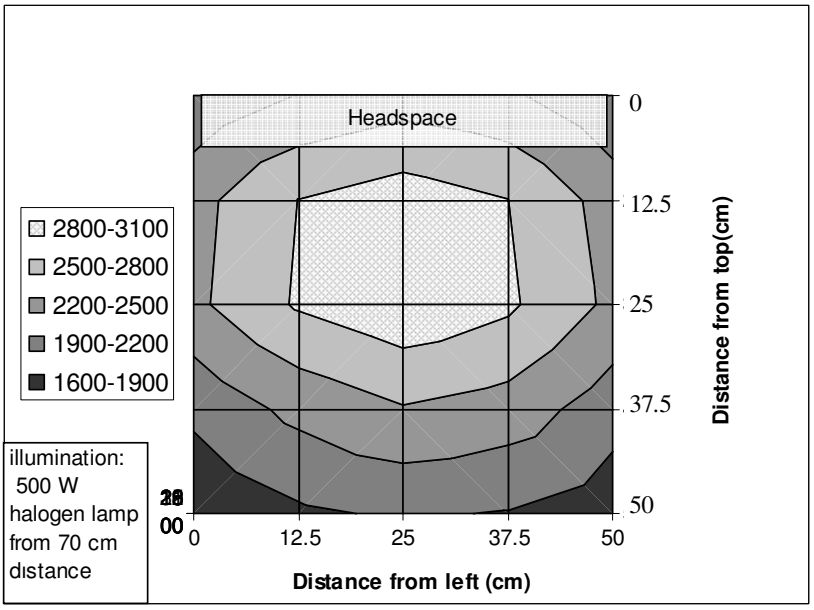


Figure 5.13 Light intensity distribution map on the surface of a 5L flat panel photobioreactor

It can be seen that the light distribution on the panel is far from being evenly distributed even if two lamps were used for illumination. This study provides feedback for indoor operations only as in the case of outdoor operations under sunlight, there will not be this uneven light distribution problem.

5.4.2 Light Intensity Distribution Inside the Panel Photobioreactor

The light intensity and wavelength change profiles in the photobioreactor with respect to depth was analyzed. The experimental setup shown in Figure 5.14 had been designed for collecting the data for analysis:

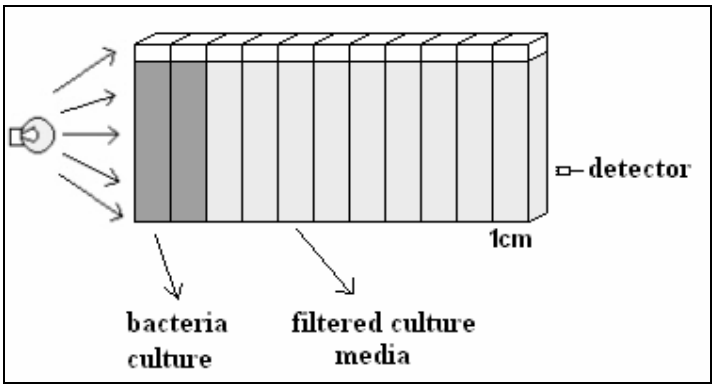


Figure 5.14 Experimental setup to analyze the change of the light intensity and wavelength inside a photobioreactor.

In this setup, the compartments were filled with the culture media which was not containing bacteria initially, and then the compartments were filled with the bacterial culture at OD 1.0 (which corresponds to the cell concentration during the stationary phase in a typical batch run) one by one. The change in the light spectra and the absorbance obtained after each change was recorded by a

spectroradiometer. Data were taken up to 10 cm depth by 1 cm intervals. The incident light intensity was 10klux. The result is given in Figure 5.15 and 5.16.

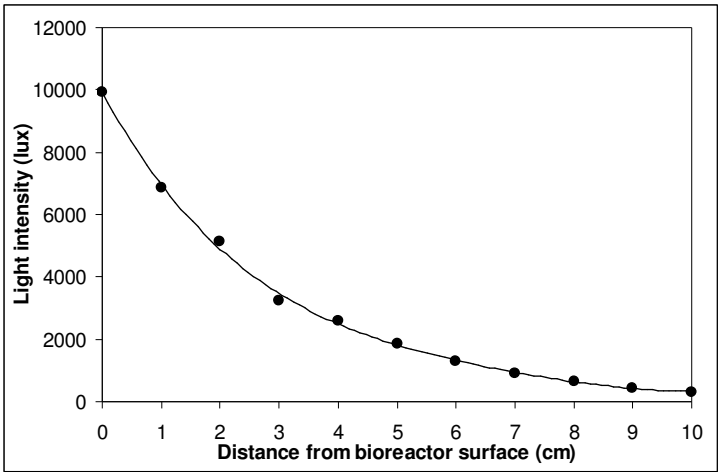


Figure 5.15 Change of light intensity inside a flat panel photobioreactor containing grown *R. sphaeroides* culture (0.6g/l).

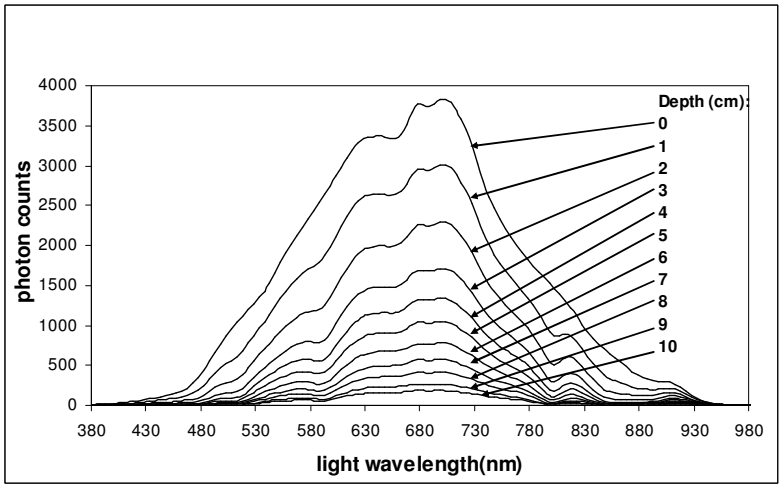


Figure 5.16 Change of light wavelength inside a photobioreactor containing grown *R. sphaeroides* culture (0.6g/l).

It can be concluded that the light was rapidly absorbed by the bacteria within the photobioreactor, limiting the maximum depth of the photobioreactor that can be used for an efficient process: 31%, 49% and 68% of the incident light was absorbed by the first 1, 2, 3 and 4 cm of the bacteria culture, respectively. At that point the light intensity drops below the optimum value suggested by the light intensity studies (3500 lux) given in Section 4.4.4. That means the photobioreactor depth should be limited to 3 cm to obtain the highest hydrogen production rate, in case of a 10000 lux surface illumination. However, the indoor photobioreactor can be illuminated from both sides to double the photobioreactor depth, but in such a setup the cooling requirements should also be considered due to the increased radiation received.

In the case of the outdoor bioreactors, the light intensity received by the bioreactor is much higher (up to 100 klux) and the depth of the photobioreactor can be up to 10 cm based on these data: at 10^{th} cm the 96.7% of the incident light would be absorbed dropping the light intensity to 3230 lux. However, it should be noted that this peak value is valid for a small time interval and the light intensity varies greatly during the daytime. The maximum allowed depth for a solar bioreactor should actually be much less.

Availability of mixing and the photoinhibition of the cells at high light intensities should also be considered while setting the bioreactor depth.

5.5 Temperature Distribution in Indoor Applications

5.5.1 Temperature Distribution on the Surface of the Panel Photobioreactor

Temperature map on the surface of flat panel photobioreactors have been constructed by using an infrared thermometer. 25L photobioreactor panel with 96x110 cm dimensions and 5L photobioreactor panel with 50x50 cm dimensions were used. The illumination was provided by 500W halogen lamps. Ambient temperature was 23.5 ºC. The obtained maps were given in Figure 5.17 and 5.18.

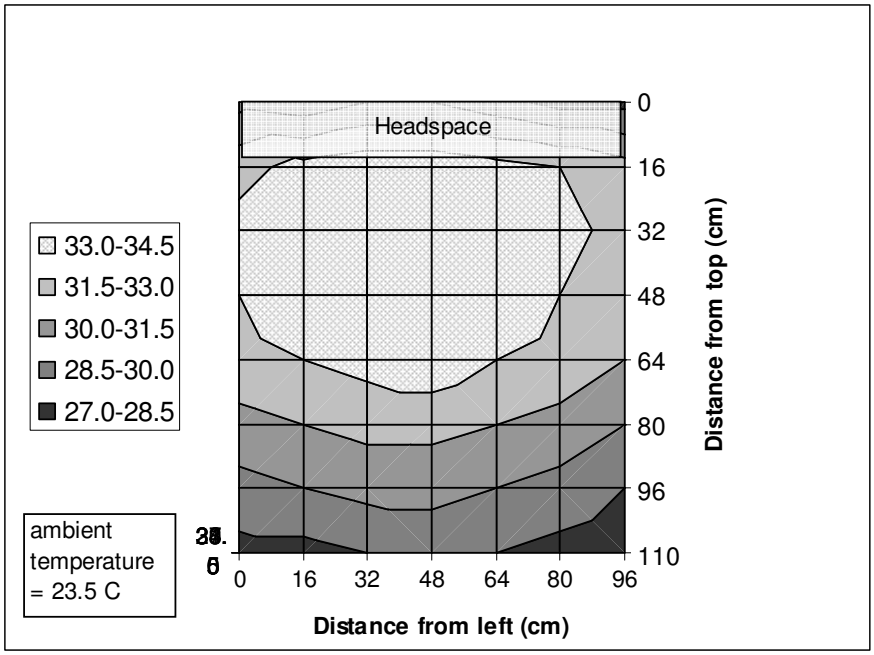


Figure 5.17 Temperature distribution map on the surface of a 25L flat panel photobioreactor

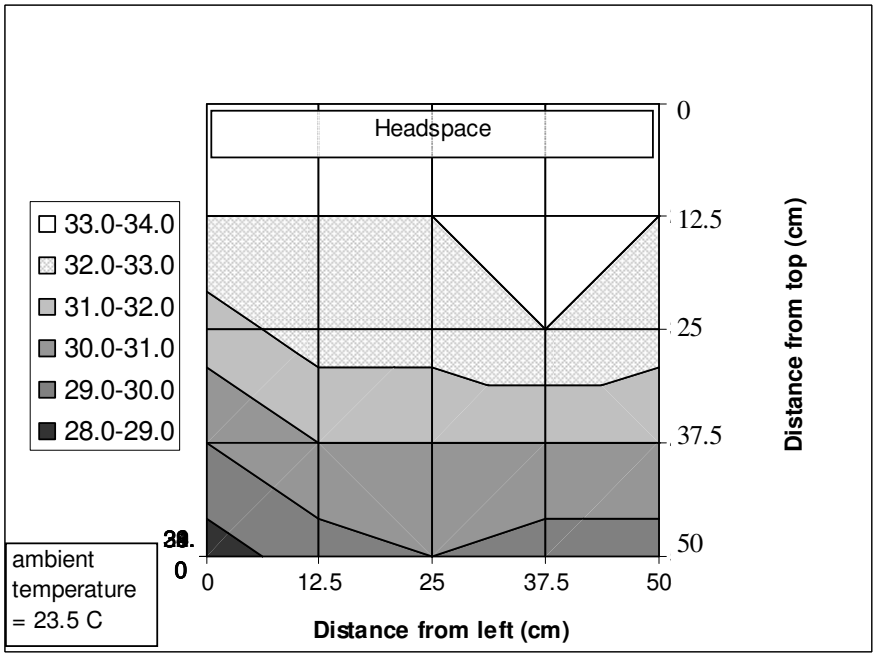


Figure 5.18 Temperature distribution map on the surface of a 5L flat panel photobioreactor.

It can be seen that the hottest part of the panel photobioreactor is the top part and the coolest part is the bottom part, due to the movement of the hot liquid. The temperature difference within the panel is up to 6ºC. The temperature at the coolest part is 4-5ºC higher than ambient air temperature, and this difference reaches up to 10ºC for the top part of the panel. This temperature increase is attributed to the heat generation by the metabolism of the bacteria and the absorption of the red-infrared light. The temperature difference between the bioreactor and the ambient air is more in outdoor conditions, due to the high intensity of the sunlight (Section 6.2 gives a detailed analysis of temperature increase in outdoor bioreactors).

5.5.2 Temperature Distribution Inside the Panel Photobioreactor

In order to document the temperature distribution inside the panel photobioreactor, a 5.5 liters flat panel photobioreactor with 45 mm depth was used. The bioreactor filled with the bacterial culture at OD 1.0 (which corresponds to the cell concentration during the stationary phase in a typical batch run). The photobioreactor was illuminated and temperature data were taken at different points inside the bioreactor to obtain the temperature distribution profile. The photobioreactor was cooled during testing by circulating water inside a coil system. Two cases were studied; in the first one the cooling water was 20ºC and in the second one it was 24ºC. The ambient temperature was 22ºC. The artificial illumination provided was 70klux, which is equal to the sunlight intensity in July at around 10:00 am. The schematic view of the photobioreactor and the obtained results were given in Figures 5.19 and 5.20.

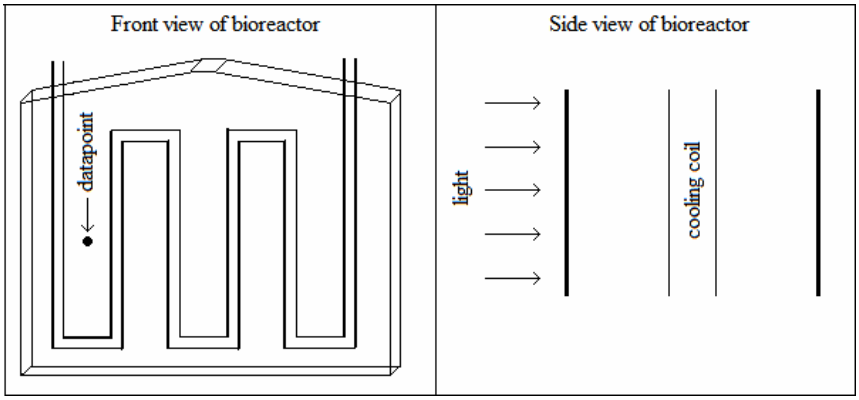
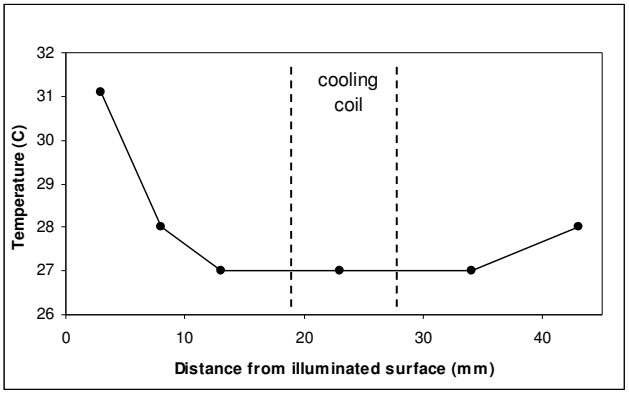
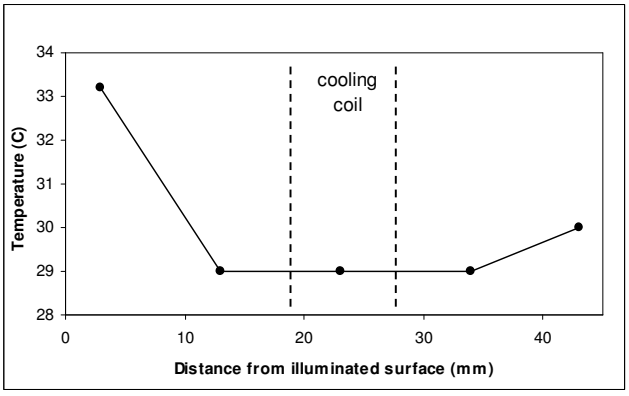


Figure 5.19 The schematic view of the photobioreactor



(a)



(b)

Figure 5.20. Temperature profile inside the bioreactor with cooling a) cooling water temperature = 20ºC, b) cooling water temperature = 24ºC

Since data were taken at moderate ambient temperature, cooling was enough to keep the temperature between 27 - 33°C, which is the optimum range for hydrogen production process. The hottest part of the bioreactor is the region next to the illuminated surface, the temperature drops to minimum around the coil and then increases again next to the other surface of the bioreactor.

5.6 Scale-up

5.6.1 Scaling up to 0.58 L and 5.5 L (*R. sphaeroides*)

The applicability of the bioreactor designs to larger scales was investigated by analyzing the growth and hydrogen production performances.

By using the roof-shaped composite design made of glass and acrylic, two bioreactors were manufactured with culture volumes of 585 and 5550 ml (scale up factor was 10.6 and 100.9 respectively, comparing to the small bottles with 55 ml volume used in most of the physiological studies). A picture of the two bioreactors and the small bottle is given below.

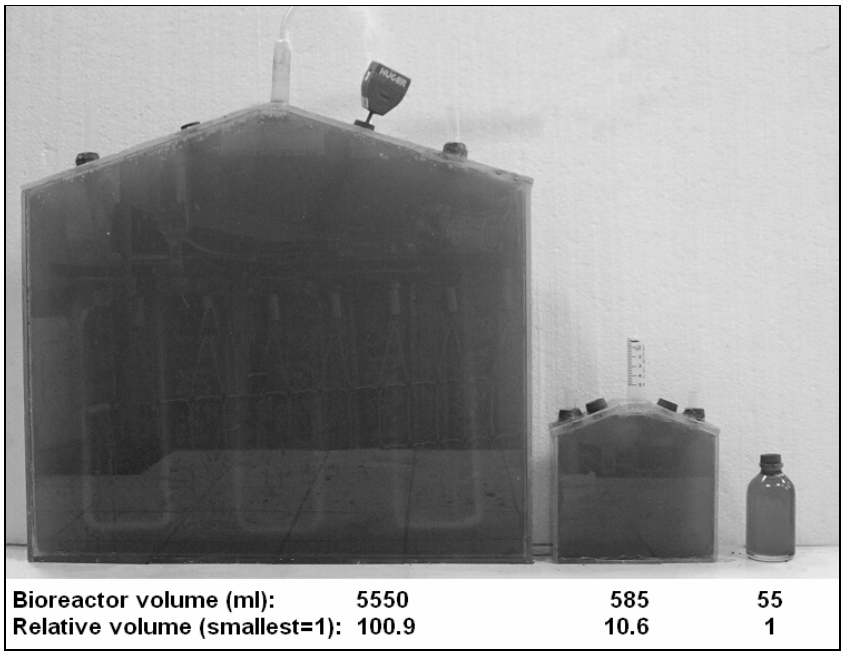


Figure 5.21 Flat-panel photobioreactors of different scales containing grown *R. sphaeroides.*

The bioreactors were tested indoor with defined media containing 15mM malate and 2mM glutamate as C and N source. *R. sphaeroides* was used as bacteria and 10% (v/v) inoculation was made. The bioreactors were completely filled with the culture media and argon gas was not used. Hydrogen production, biomass and pH changes were recorded throughout the runs and the results were given below.

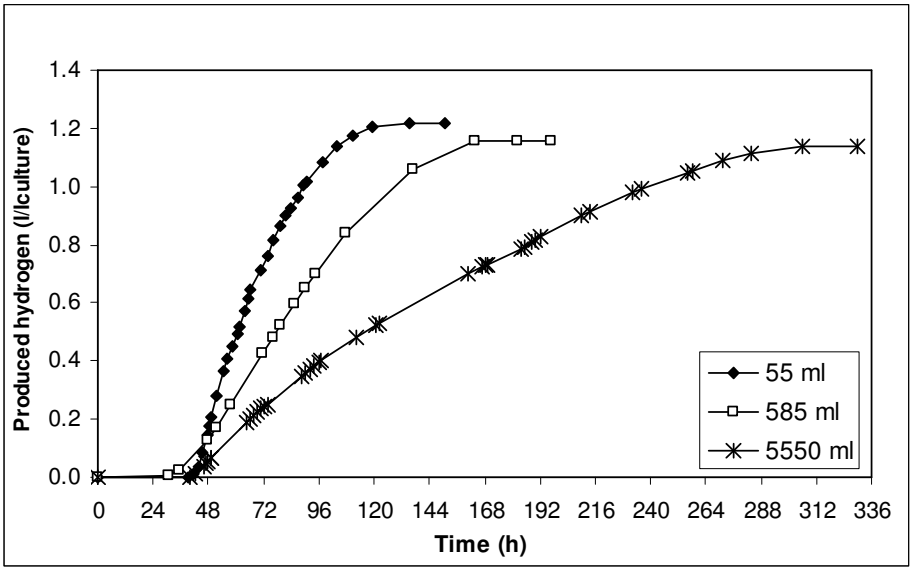


Figure 5.22 Hydrogen productions by *R. sphaeroides* in photobioreactors of different scales containing 15mM malate medium.

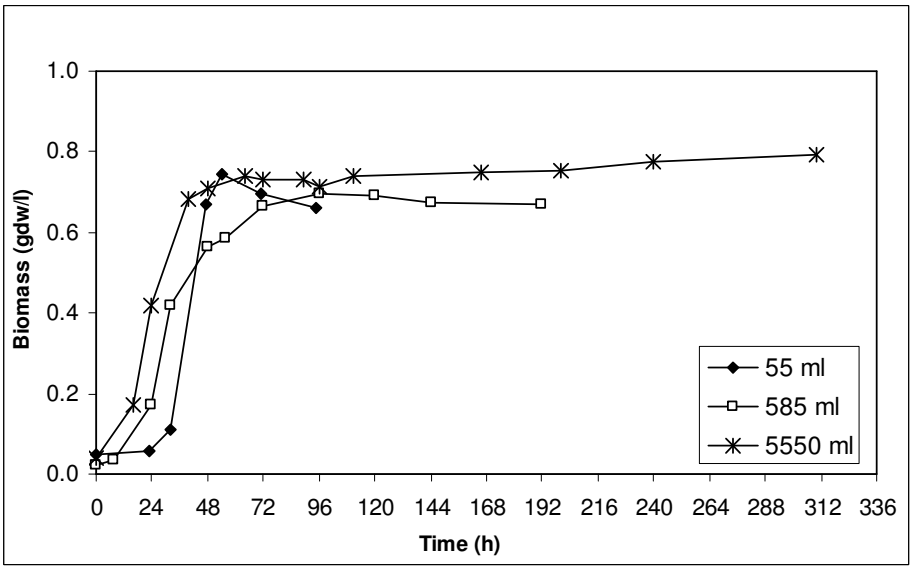


Figure 5.23 Biomass growths of *R. sphaeroides* in photobioreactors of different scales containing 15mM malate medium.

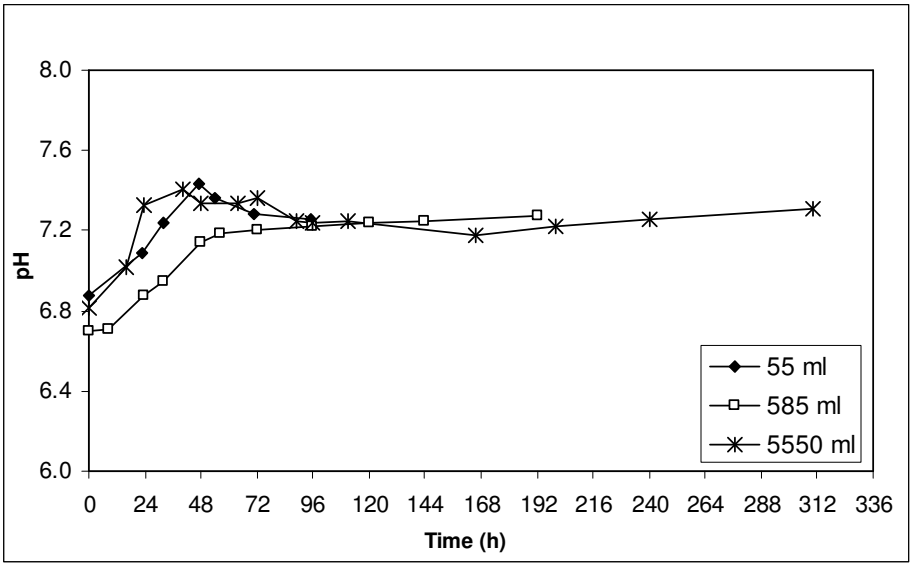


Figure 5.24 pH changes in photobioreactors of different scales containing 15mM malate medium.

The results show that the total amount of hydrogen produced and the maximum biomass amount obtained from the three runs were very similar. Time dependent pH and growth curves show very similar patterns too. Only noticeable difference is the rate of the hydrogen productions. The lower rate of hydrogen production observed in large reactors are mostly due to the low light intensity received due to the large surface area as the illumination was provided by a single lamp and distributed unevenly on the large panels.

It can be concluded that panel photobioreactor is applicable to large scales.

5.6.2 Scaling up to 5L, 20L and 25L (*R. capsulatus*)

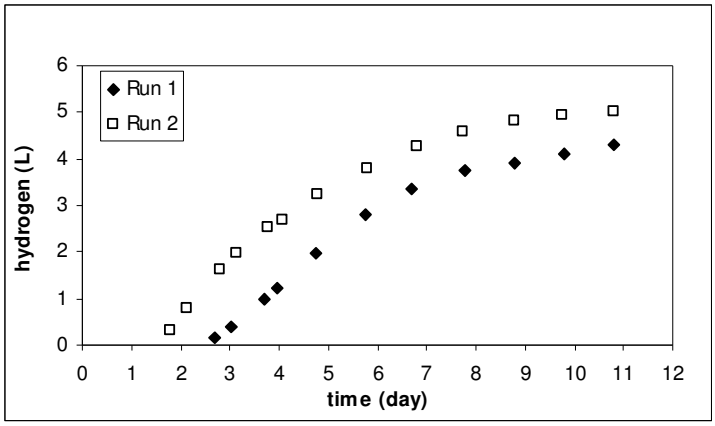
Physiological studies showed that hydrogen production by *R. sphaeroides* is not as stable and reproducible as *R. capsulatus* in media where acetate or lactate/acetate mixtures were used as the carbon source(s). Based on these findings and the results of the other studies performed within the Hyvolution

project (Deliverable Report 3.2, 2006), *R. capsulatus* was chosen as the microorganism to be employed in the large scale processes.

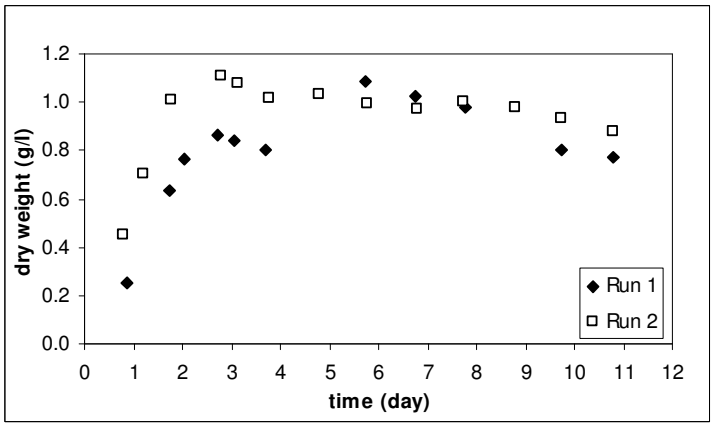
In this study, scale-up studies were conducted with *R. capsulatus* (DSM 155) in defined media containing 40 mM of acetate and 7.5 mM of lactate as the C source, and 2 mM of glutamate as the N source. The studies were carried out in RWTH labs, Aachen-Germany. Rectangular-prism shaped photobioreactors made of acrylic (side panels) and PVC (frame) were used with culture volumes of 5 L and 25 L.

In the first run, 5 L bioreactor was tested, illuminated by a 500W halogen lamp at a distance of 90 cm. The run was carried out twice and results of both runs were plotted in Figure 5.25. The results of the media analysis by HPLC were presented in Figure 5.26.

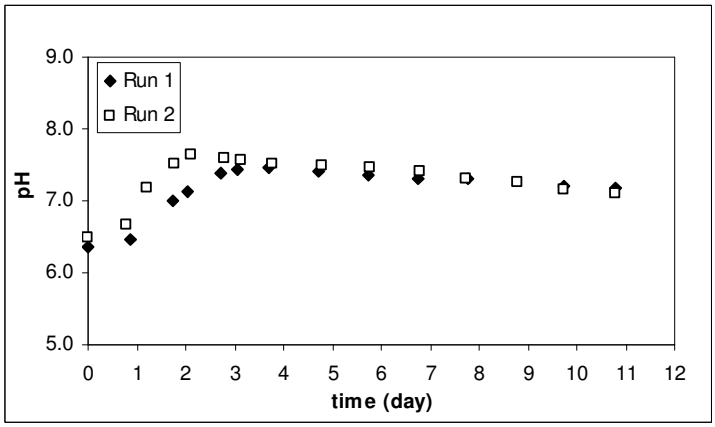
As can be seen in the hydrogen production graph (Figure 5.25a), hydrogen was still slightly produced when the Run 1 had to be stopped in 11th day due to an illumination failure, so the final amount would be higher if it continued a few days more. Also there is a problem with the growth curve data of Run 1; the 3rd and 4th day data are probably not correct. However Run 2 was a fine experiment. The growth, pH and hydrogen production patterns were comparable for both runs. Run 2 had shorter lag time. Substrate consumption curve shows that both lactic and acetic acids were consumed by the bacteria. GC analysis of the produced gas showed that the gas obtained in Run 1 was composed of 96.0% H_2 and 4.0% CO_2 and the gas obtained in Run 2 was composed of 97.3% H_2 and 2.7% CO_2.



(a)



(b)



(c)

Figure 5.25 Hydrogen productions (a), biomass growths (b) and pH changes (c) by *R. capsulatus* in 5L panel photobioreactor containing 40 mM acetate and 7.5 mM lactate as the C sources.

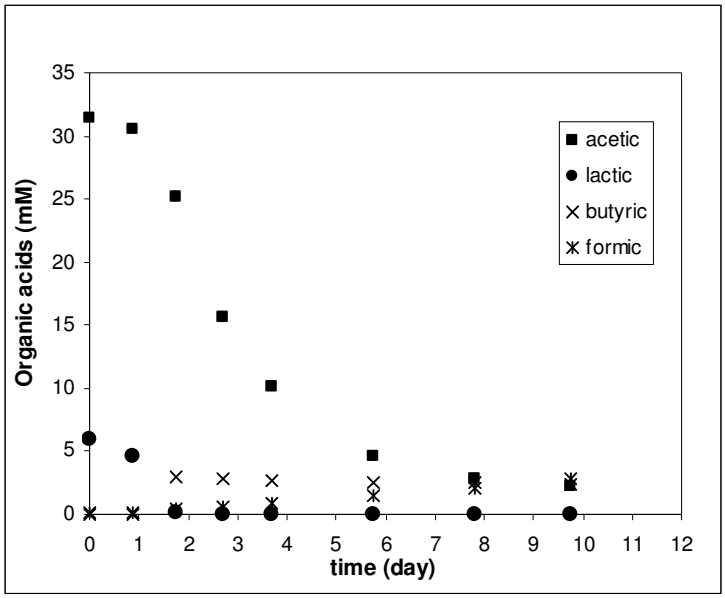
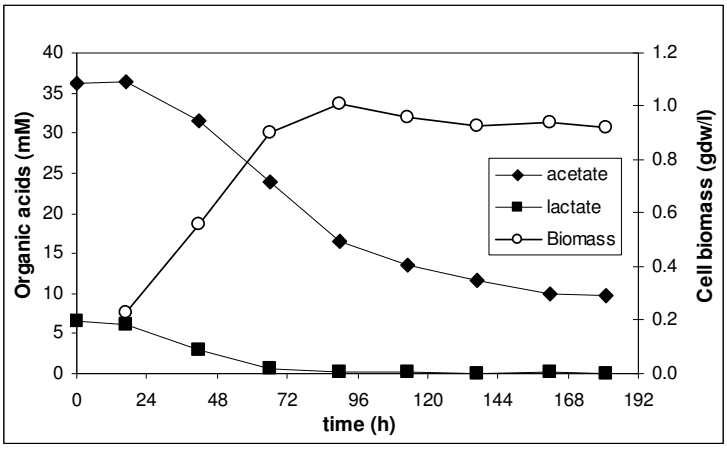
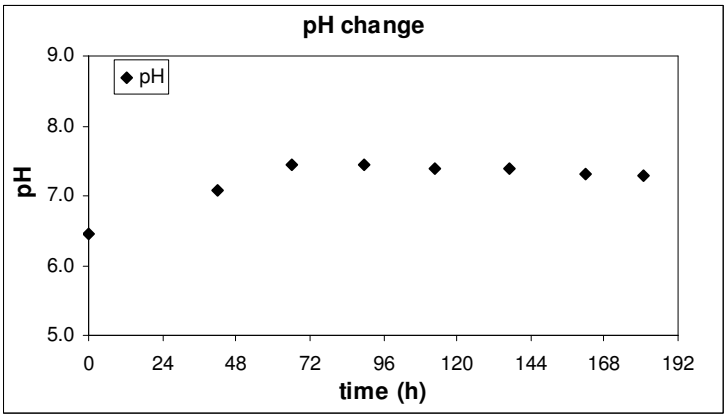


Figure 5.26 Change in organic acid concentrations in 5L panel photobioreactor containing *R. capsulatus* and 40 mM acetate and 7.5 mM lactate as the C sources.

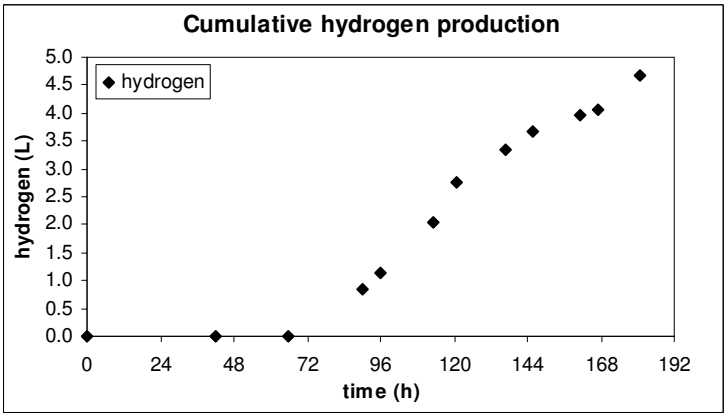
In the second phase, 20L bioreactor was tested, this was a modular design constructed by connecting gas outlets of four 5L panels running in parallel. The system was illuminated by three 500W halogen lamp. The results were given in Figure 5.27.



(a)



(b)



(c)

Figure 5.27 Biomass growth and substrate consumptions (a), pH change (b), H_2 productions (c) by *R. capsulatus* in 20L (4x5L) panel photobioreactor containing 40 mM acetate and 7.5 mM lactate as the C sources.

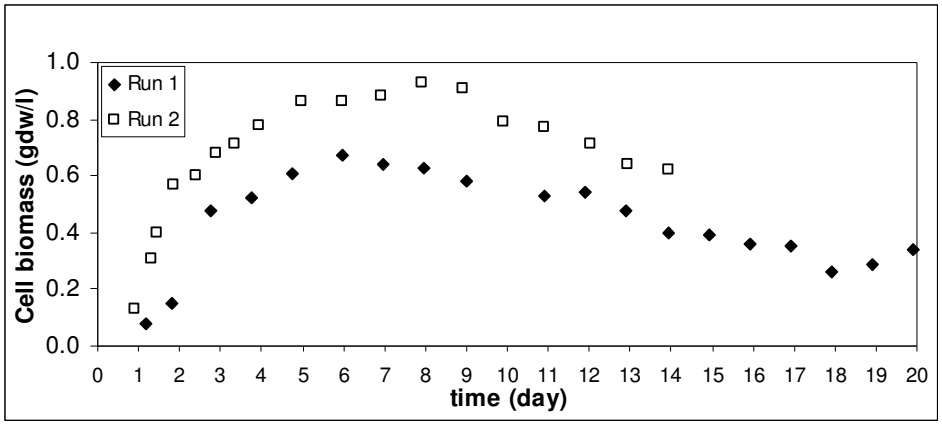
There was a leakage out of gas collecting apparatus and only fraction of the produced gas could be collected, therefore the hydrogen production result was not accurate and it was far less than expected. Bacteria grew without problem, substrate consumption curve shows that both lactate and acetate were consumed by the bacteria. GC analysis of the produced gas resulted in 94.9% H_2 and 5.1 % CO_2.

In the third phase, 25L photobioreactor was tested. The panel was illuminated by two 500W halogen lamp. The run was carried out twice and results of both runs were given below in Figure 5.28.

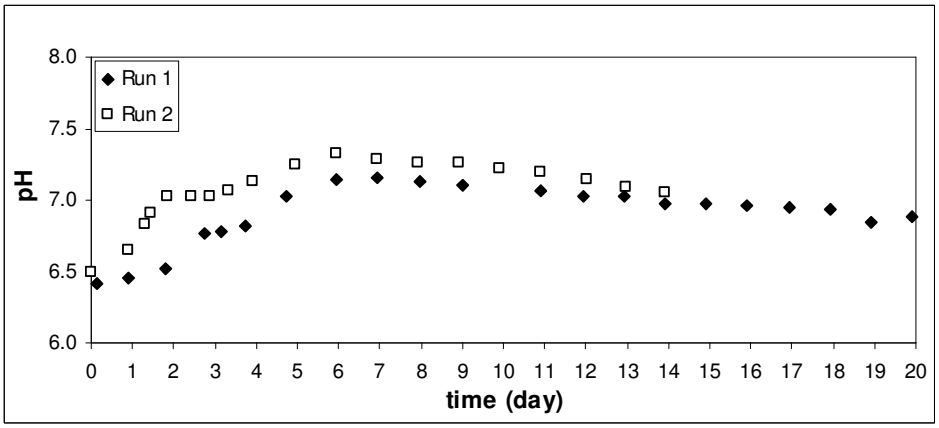
In Run 1, contamination was observed in 19th day; results after day 18 should be discarded. 25.9 and 28.7 L of gas were produced in both runs, respectively. The pH changes were comparable, growth patterns were also similar in both runs but biomass density was higher in the second run compared to the first run. The process continued in a tri-phasic manner in Run 1; three separate waves can be distinguished. In the second run such a pattern was not observed. The reasons behind this are not clear and it could not be replicated, it might be attributed to non-homogenous light and substrate distribution.

GC analysis of the produced gas showed that the gas obtained in Run 1 was composed of 90.9% H_2 and 9.1% CO_2 and the gas obtained in Run 2 was composed of 95.5% H_2 and 4.5% CO_2. That is, the gas obtained in the first run had lower hydrogen compared to the gas obtained in the second run.

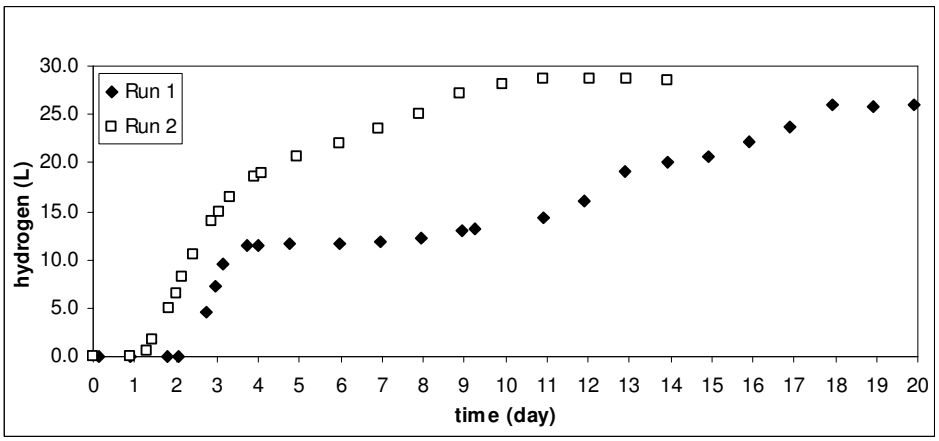
The hydrogen production, biomass and pH change data obtained in the 5L (Run 2) and 25L (Run 2) photobioreactors were compared below in Figure 5.29.



(a)

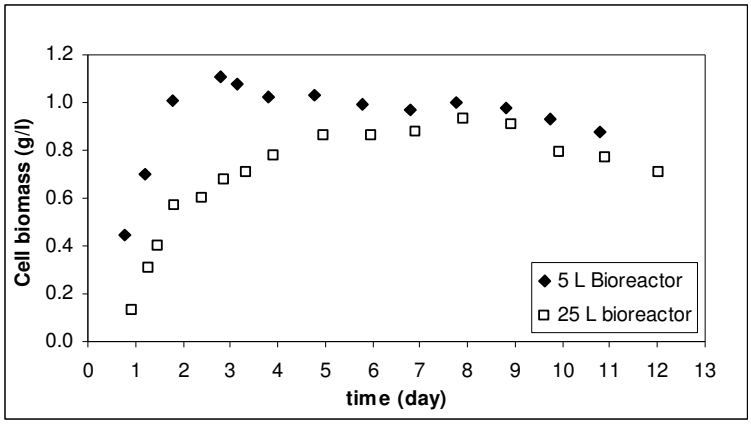


(b)

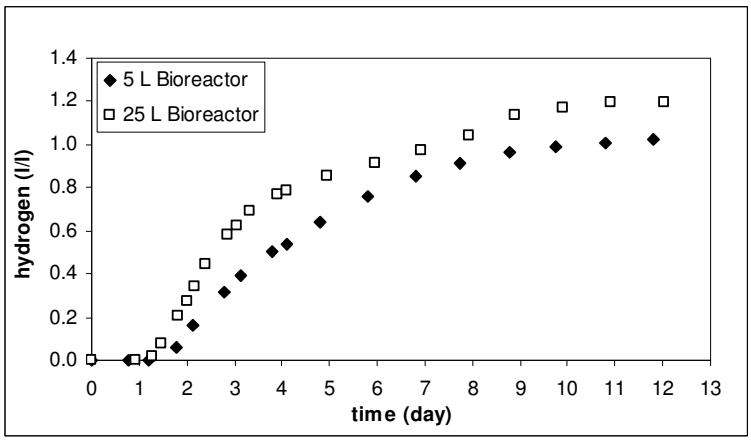


(c)

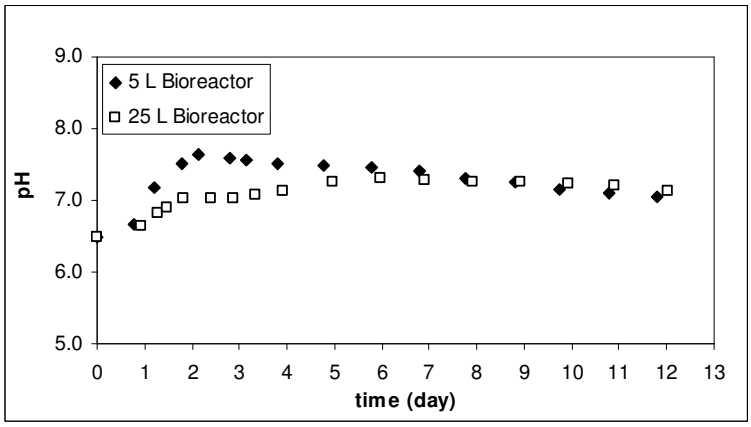
Figure 5.28 Biomass growths (a), pH changes (b) and hydrogen productions (c) by *R. capsulatus* in 25L panel photobioreactor containing 40 mM acetate and 7.5 mM lactate as the C sources.



(a)



(b)



(c)

Figure 5.29 Comparison of the biomass growths (a), hydrogen productions (b) and pH changes (c) of *R. capsulatus* in 5L and 25L panel photobioreactors containing 40 mM acetate and 7.5 mM lactate as the C sources.

The results show that the total amount of hydrogen produced and the maximum biomass amount obtained from both bioreactors have similar patterns and comparable values; the hydrogen production in 25L bioreactor was more than the hydrogen production in 5L bioreactor whereas biomass amount was higher in the 5L bioreactor compared to the other.

The runs with these large bioreactors were successful showing that the bioreactor designs and the photobiological hydrogen production process is applicable to large scales, with both *R. sphaeroides* and *R. capsulatus* species.

5.7 Evaluation of Panel Photobioreactor Studies

The photobioreactor parameters that may affect the hydrogen production performance are:

- The depth of photobioreactor: it means the light path and is a very important parameter for the design since the light intensity decreases inside the photobioreactor rapidly due to the absorbance by bacteria.
- The height of photobioreactor: it means the maximum hydrogen bubble travel distance, since a formed hydrogen bubble at the bottom of the photobioreactor has to travel all the way up through medium, where it may be absorbed back by the bacteria via uptake hydrogenase enzyme. Moreover, increasing the height of photobioreactor increases the pressure drop and the bacteria may be sensitive to high pressure.
- The surface area/volume ratio for the photobioreactor may be important as well, during the runs, it was visually observed that hydrogen bubbles tend to form next to surfaces, so increasing the surface area may promote desorption of hydrogen from media.

The hydrogen production rates, yields and conversion efficiencies for the reported runs carried out in large scale (0.5 - 25L) flat panel photobioreactors were calculated and tabulated in Table 5.2. The photobioreactor parameters were also listed in the Table and used for evaluation of the results.

Table 5.2 Results obtained in large scale (0.5 - 25L) flat panel photobioreactors

Bioreactor parameters	Panel material	Glass	Glass	Acrylic	Acrylic
	Culture volume (L)	0.58	5.5	5	25
	Light path (depth) (cm)	4.5	4.5	2	2
	Surface area/Volume	0.49	0.48	1	0.81
	Height (cm)	11	32.5	50	110
Experimental parameters	Bacteria used	*R. Sph.*	*R. Sph.*	*R. Caps.*	*R. Caps.*
	C source used	Malate	Malate	Ace+Lact	Ace+Lact
	Initial C source conc. (mM)	15	15	40 + 7.5	40 + 7.5
	C/N ratio (mol/mol)	60/2	60/2	110/2	110/2
Results of the test runs	Max. Cell concentration (g/L)	0.69	0.79	1.11	0.93
	Final H_2 produced ($l/l_{culture}$)	1.15	1.14	1.02	1.19
	Hydrogen prod. duration (h)	128	229	166	183
	Aver. H_2 prod. rate ($ml/l_{culture}.h$)	9.0	5.0	6.1	6.5
	H_2 productivity (mg $H_2/l_{culture}.h$)	0.74	0.41	0.50	0.54
	H_2 yield (g H_2/g substrate)	0.047	0.047	0.027	0.032
	Substrate conv. eff. (%)*	57	56	22	26
	Light conv. eff. (%)	0.49	0.27	0.34	0.35

* It was assumed that all of the substrates were consumed by the bacteria

By comparing the results obtained in glass bioreactors in which *R. sphaeroides* was used as bacteria in malate media, it can be concluded that the height of the bioreactor did not affect the hydrogen yield but the productivity decreased. Another noticeable difference is the poor light conversion efficiency in the bigger bioreactor compared to the smaller one although both bioreactors approximately received the same light intensity ($150W/m^2$). This might be due to the fact that the light distribution on the bioreactor panel becomes more non-uniform as the panel size increases, this results in part of the bioreactor receiving poor illumination where another part receiving more light than the saturation level. This is reflected as decreased overall gas production rate and light conversion efficiency to the results.

In case of the acrylic bioreactors which were ran with *R. capsulatus* in acetate + lactate media, one can conclude that the height of the bioreactor up to 1.1 m is not affecting the hydrogen production negatively, the yield, rate of production and conversion efficiencies were comparable for both bioreactors.

In the 25L acrylic bioreactor, two lamps were used for illumination to create a more uniform illumination profile on the panel (Figure 5.12) and decrease in rate and light conversion efficiency was not observed unlike the glass bioreactors case.

The glass and acrylic bioreactor runs reported are different in terms of bacteria used and culture media, therefore more research would be necessary to comment on the effect of the depth and surface/volume ratio of photobioreactor on hydrogen production. However it should be noted that final amount of hydrogen produced and hydrogen production rate are in comparable ranges for all of the runs: 1.02-1.19L/$L_{culture}$ and 5-9 ml H_2/$L_{culture}$.h, respectively.

CHAPTER 6

PARAMETERS AFFECTING HYDROGEN PRODUCTION IN OUTDOOR APPLICATIONS

Photobacterial growth and hydrogen productivity of photosynthetic bacteria in an outdoor reactor system is strongly affected by fluctuations in temperature and light intensity due to the day-night cycle and due to seasonal, geographic and climatic conditions (Hyvolution Project Deliverable Report 3.1, 2006).

In this chapter the parameters affecting the outdoor process are identified. For this purpose climatic data as well as operational parameters were collected and evaluated. Then, the growth and hydrogen production of the bacteria were investigated. Finally, an assessment of the obtained results was made.

6.1 Analysis of the Outdoor Parameters That Affect Solarbioreactors

The parameters in outdoor conditions that may affect the photobiological hydrogen production process were determined to be the sunlight properties (intensity, wavelength), diurnal (day-night) cycle and air temperature. Those parameters can not be controlled. These parameters were monitored and documented first, then the response of the system to the changes in these parameters was investigated.

6.1.1 Sunlight Spectrum and Intensity

The outdoor experiments were carried out in Ankara, which is situated on 40^{th} parallel. The properties of the sunlight that Ankara receives were determined.

6.1.1.1 Daily Changes in Sunlight Spectrum and Intensity

During summer, day length is approximately 14 hours and night lasts for 10 hours: Sun rises at around 6:00 and sets around 20:00. The sunlight intensity increases rapidly after sunrise and reaches over 150W/m^2 on the ground one hour after sunrise, this is the minimum light intensity value suggested by indoor studies (Section 4.4.4). The light intensity is maximum at 13:00 (actually it is 12:00 but daylight saving time is taken into account during summer) and reaching over 800 W/m^2. Then the intensity starts to decrease as the sun gets close to the horizon and drops down to 150W/m^2 at 19:00, one hour before sunset. The daily light intensity pattern obtained in August 10th, 2007 is given in Figure 6.1:

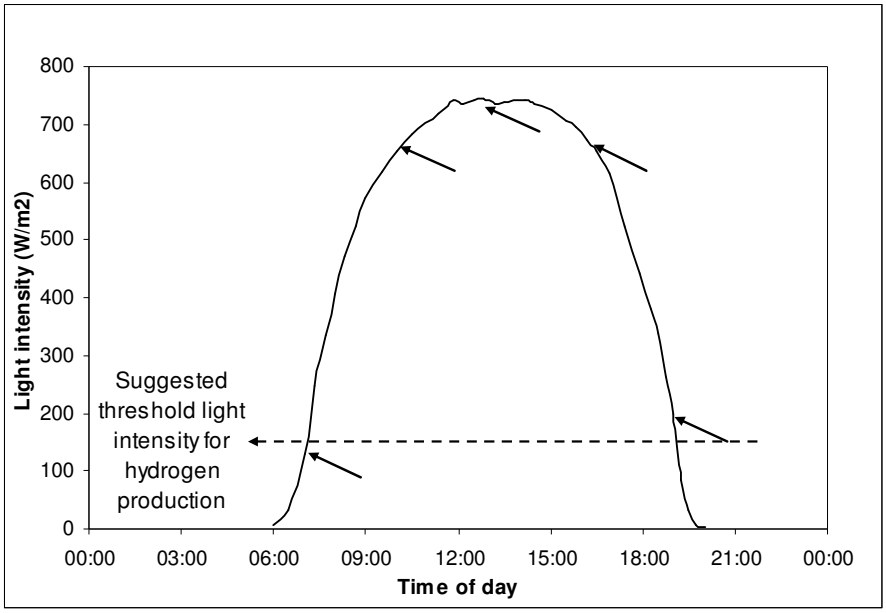


Figure 6.1 Daily change in the sunlight intensity

The light spectra are also affected by the time of the day, as the sunlight travels longer distance inside the Earth's atmosphere when the sun is close to the horizon. The sunlight spectra was analysed at 7:00 (one hour after sunrise), 10:00, 13:00 (when the sun is at zenith), 16:00 and 19:00 (one hour before sunset). Those times were marked in Figures 6.1 by arrows. The data obtained was given in Figure 6.2:

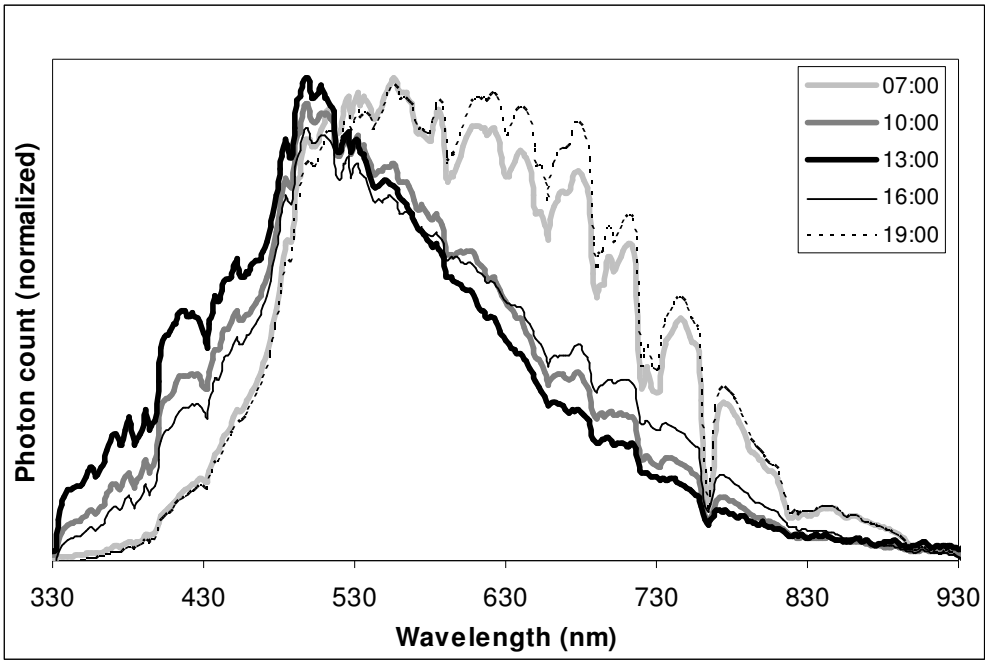


Figure 6.2 Change in sunlight spectra during day

As can be seen, the spectra changes during day, due to the changing atmosphere thickness between the sun and the observer. The spectra are shifted to red during sunrise and sunset.

6.1.1.2 Seasonal Changes in Sunlight Spectrum and Intensity

Another measurement was made to observe how sunlight intensity changes within a longer period. Sunlight intensity was recorded in 1st of October and compared to the data obtained in 10th of August. The comparison was given in Figure 6.3 below:

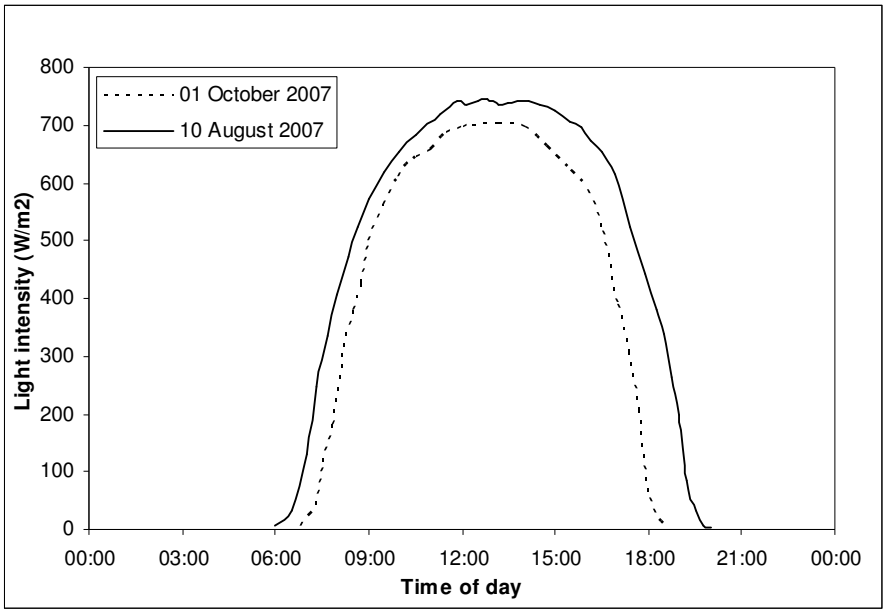


Figure 6.3 Change in daily sunlight intensity from August to October.

As can be seen, the day length and the maximum light intensity decrease towards winter due to the sun approaching to the horizon.

In order to see the longer term change in the sunlight intensity, data for Ankara for the year 2006 was obtained from National Meteorology Institute of Turkey and the change in the received daily light intensity was plot in Figure 6.4.

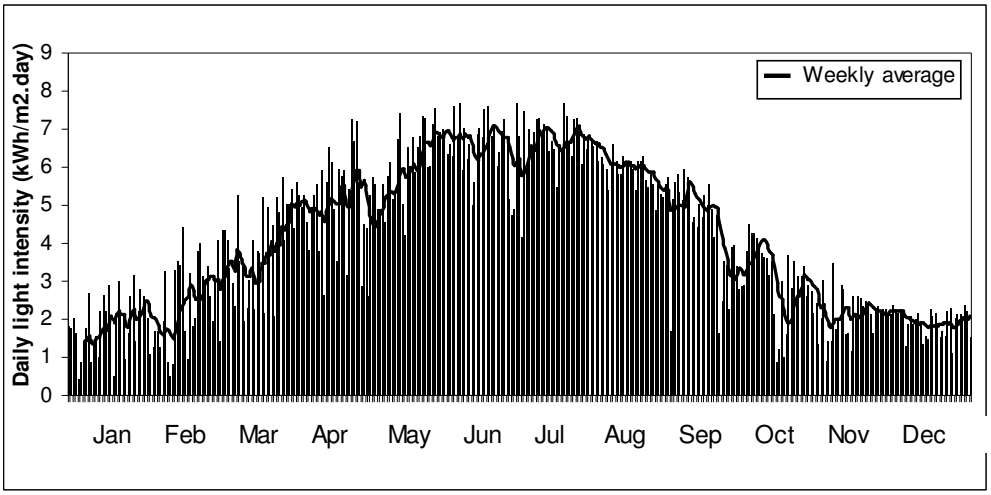


Figure 6.4 Change in sunlight intensity throughout year 2006 for Ankara

As can be seen, the light energy received was maximum during summer (more than 6 kwh/m^2.day between May-August) and decreased below 2.5 kwh/m^2.day during winter (between November-February).

6.1.1.3 Cloud Effect on Sunlight Spectrum and Intensity

The clouds decrease light intensity and change the spectrum of the sunlight. Several measurements were made under different weather conditions to document the change in the sunlight intensity. The data of the clear and sunny days were compared to cloudy and rainy days. In order to eliminate the seasonal and

daily change in the parameters, the data were taken at the consecutive days at the same hour and tabulated in Table 6.1

Table 6.1 Effects of the clouds and rain on the sunlight intensity

Date-time:	**Weather:**	**Light intensity (W/m^2):**
Effect of clouds:		
20 – 08 – 2007 at 9:45	Overcast	116
21 – 08 – 2007 at 9:45	Clear and sunny	605
Effect of clouds II :		
28 – 08 – 2007 at 9:45	Overcast (dark rain clouds)	47
29 – 08 – 2007 at 9:45	Clear and sunny	578
Effect of clouds III :		
02 – 10 – 2007 at 17:15	Clear and sunny	310
03 – 10 – 2007 at 17:15	Overcast	88
Effect of rain :		
28 – 08 – 2007 at 14:00	Raining heavily	24
29 – 08 – 2007 at 14:00	Clear and sunny	718

The data show how the light intensity depends on the cloud type and density. Clouds cause a significant decrease in the light intensity: For the three cases analyzed, the decrease in the light intensity caused by the clouds was between 72 - 92%. Another measurement showed that during heavy raining, light intensity was as low as 24 W/m^2, at 14:00 in August 28th. The next day again at 14:00 the light intensity was measured to be 718 W/m^2 under clear sky. This gives a 96.6% reduction in the sunlight intensity caused by the heavy rain.

The change in light spectrum data was obtained in late March around noon and illustrated in Figure 6.5.

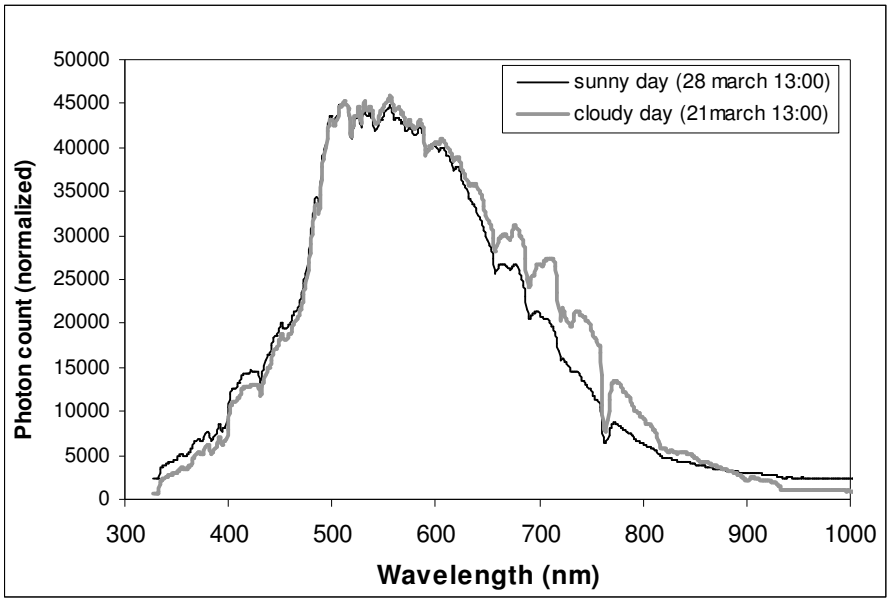


Figure 6.5 Change in sunlight intensity throughout year 2006 for Ankara

It can be concluded that clouds slightly alter the spectrum of the sunlight; absorbing less light at the 650-850nm region, however the change is negligible, the decrease in light intensity is much more drastic.

6.1.2 Air Temperature

Another outdoor parameter which can not be controlled is the air temperature. The air temperature affects the process; at high temperatures the bioreactors need cooling to prevent bacteria dieing, at low temperatures the bioreactors need heating to prevent bacteria from freezing.

6.1.2.1 Daily Changes in Air Temperature

The daily change in the air temperature in August 9th, 2007 was given in Figure 6.6.

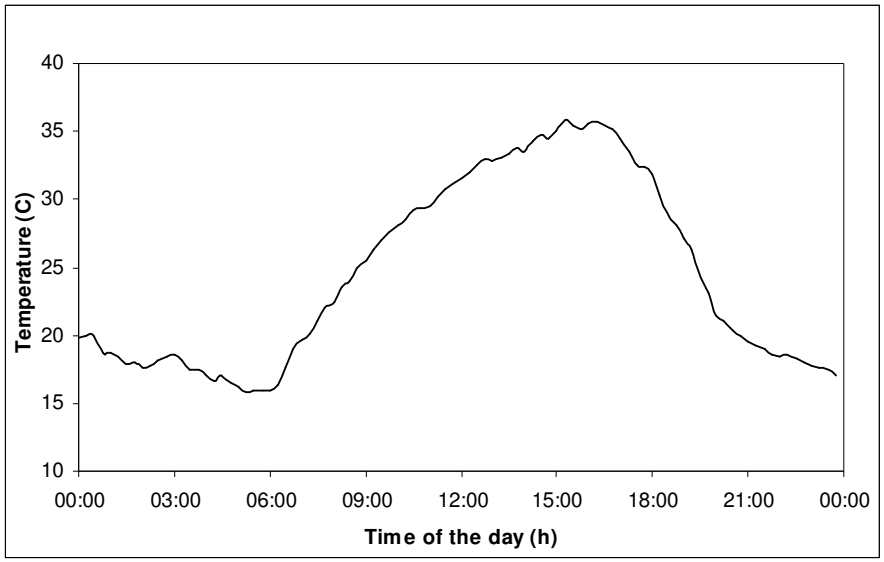


Figure 6.6 Change in the air temperature during a day (August 9th, 2007)

As can be seen in the Figure, air temperature starts to increase after the sunrise until the afternoon, after when it starts to drop again until the next sunrise.

6.1.2.2 Seasonal Changes in Air Temperature

Another aspect is the long term change in the air temperature. The data for Ankara for the years 2003-2005 was obtained from National Meteorology Institute of Turkey and the change in monthly average air temperature was given in Figure 6.7.

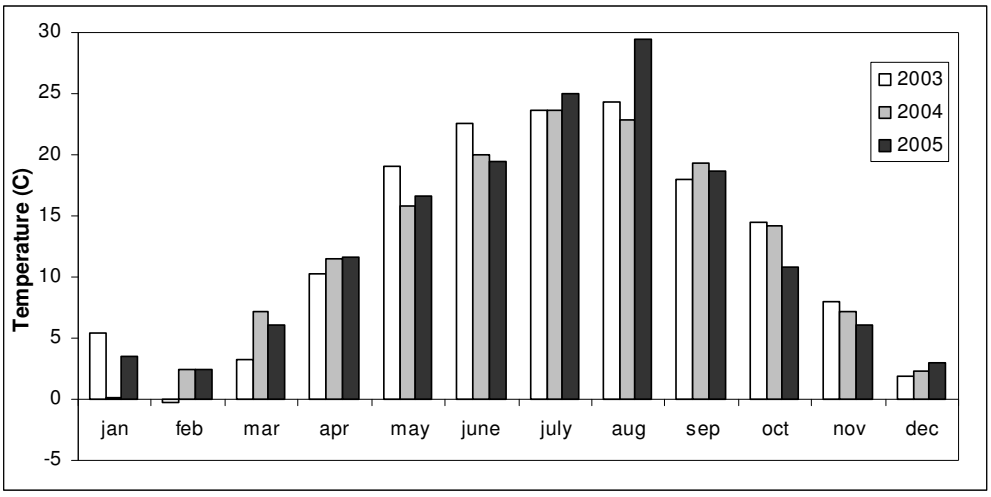


Figure 6.7 Change in the average monthly air temperature for Ankara in 2003-2005

As can be seen from the Figure, the air temperature is at maximum during July-August, during when the cooling of the photobioreactor becomes critical, and the air temperature is minimum during December-February where the freezing of the culture may occur and the heating of the photobioreactor becomes important.

6.1.3 Orientation of the Solarbioreactor

The orientation (position) of the outdoor photobioreactor panel is a configurable parameter that affects the sunlight received by the bioreactor plate. In this part of the study, the effect of the solarbioreactor orientation on the received sunlight intensity was investigated for three different orientation configurations:

i. South-facing (one side of the reactor faces south with 40º to horizontal for maximizing the sunlight expose). In this configuration, light intensity on the panel is low during sunrise and sunset but is maximum around noon.
ii. East-west facing (one side of the panel facing east, other side facing west). In this case the light intensity on the panel is high during sunrise and

sunset, and the bioreoactor is practically not receiving any direct light around noon (receives only scattered and reflected light during that time).

iii. Sun-tracking (one side of the panel always facing sun; the bioreactor position was changed manually during the day to keep sun at the normal of the panel). In this case, one side of the panel receives maximum possible light intensity during the day.

6.1.3.1 Effect of Orientation on the Received Sunlight

The data were collected in August 10th, 2007, where the sunrise was at 05:52 and the sunset occurred at 19:56. It was a clear day without any clouds in the sky. The panels placed and positioned outdoor under sunlight as mentioned above and the light intensity data on the panels were collected continuously during the day. The results were given in Figures 6.8-10 below:

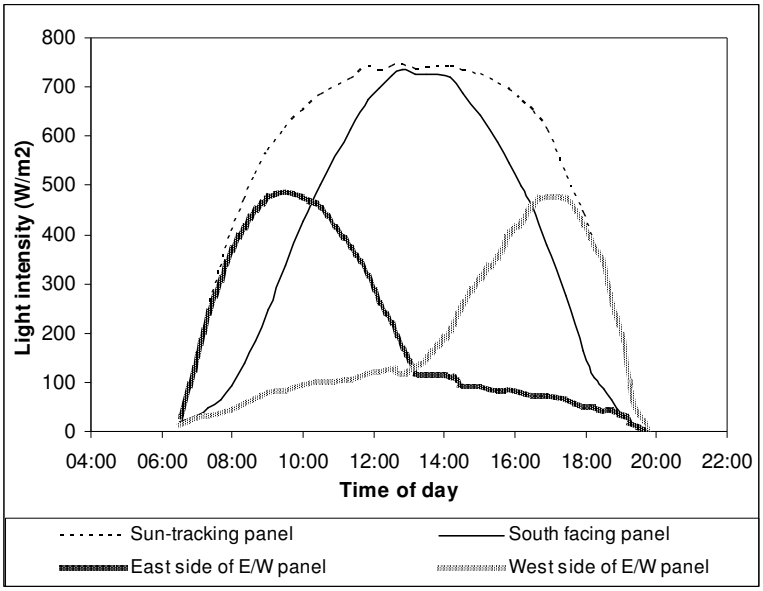


Figure 6.8 Light intensities on the panels for different bioreactor orientations

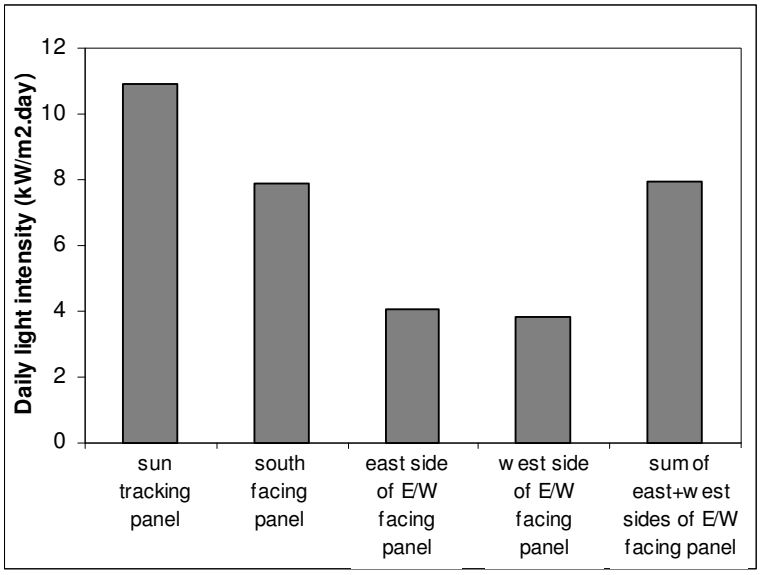


Figure 6.9 Total daily sunlight energy received by the panels for different solarbioreactor orientations.

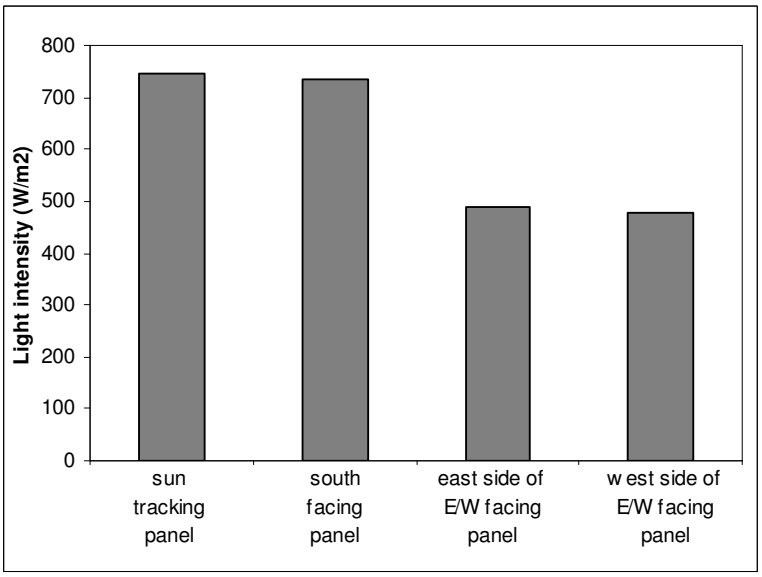


Figure 6.10 The maximum sunlight intensities measured on the panels for different solarbioreactor orientations.

The light profiles given on the panels (Figure 6.8) were as expected. However it should also be noted that during the day, the side of the east/west oriented panel which is not receiving direct sunlight received a considerable amount of indirect (scattered-reflected) light (up to 100W/m^2).

In Figure 6.9, it can be seen that the sun-tracking bioreactor received more light energy (10.9 kwh/m^2.day) than the other two stationary bioreactors, which received almost the same amount of light energy (7.9 kwh/m^2.day for both).

The maximum light intensity measured on the sun-tracking and south facing panels were comparable at around 740 W/m^2, however the maximum light intensity on east-west facing panel did not exceed 490w/m^2 since considerable part of the light energy is absorbed by the thick atmosphere around sunset and sunrise time, when the sun is at normal of the panel (Figure 6.10). However, providing maximum light intensity to the bioreactor is a questionable effort, as may cause photoinhibition on the bacteria, as well as increasing the cooling requirement. On the other hand, sun-tracking the bioreactor requires energy, which decreases its appeal over stationary reactors.

6.1.3.2 Effect of Orientation on the Solarbioreactor Temperature

In this part of the study, the effect of the orientation of the solar bioreactor on the internal reactor temperature was investigated. The three orientation configurations investigated were: south-facing (one side of the reactor faces south at an angle of 40º with horizontal to maximize the sunlight expose). east-west facing (one side of the panel facing east, other side facing west). sun-tracking (one side of the panel facing sun always; the bioreactor position was changed manually during the day to keep sun at the normal of the panel).

The light intensity profiles obtained on these bioreactors were given and discussed in the previous section.

The experiment was performed in August 9^{th}-10^{th}, 2007. Six flat panel photobioreactors (volume=750 ml, depth (light path)=5 cm) made of PVC were

used, three of the bioreactors were filled with distilled water (controls), other three with fully grown bacteria at stationary phase (0.6gdw/l) which represents the average cell concentration of an actual batch run. The bacteria used were *R. capsulatus*. Those photobioreactors were placed outdoor under sunlight. There were no cooling. The solar bioreactors were kept for one full day under these conditions. The temperature changes inside the bioreactors and the light intensity on the panels were recorded continuously. The results obtained for the bioreactors were given in Figures 6.11-13, and the overall results for all three bioreactors in a comparable form were given in Figure 6.14 and in the Table 6.2 below:

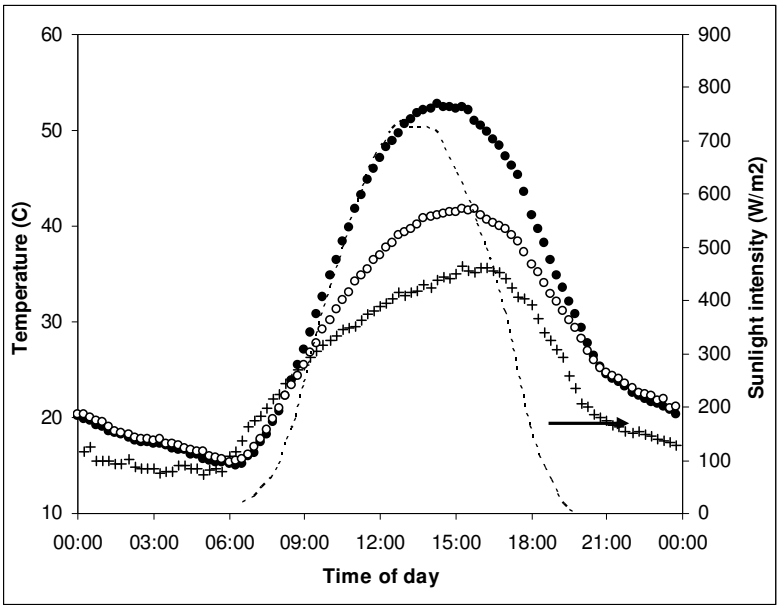


Figure 6.11 The temperature change in south facing solarbioreactor.
(++++): air temperature, (••••): bioreactor containing bacteria, (○○○○): bioreactor containing water, (----): sunlight intensity on the panel.

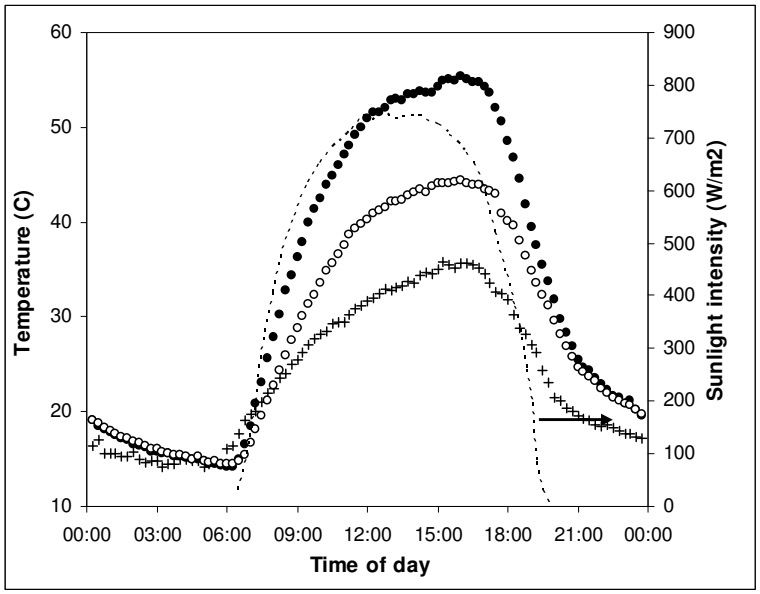


Figure 6.12 The temperature change in sun tracking solarbioreactor.
(++++): air temperature, (••••): bioreactor containing bacteria, (○○○○): bioreactor containing water, (----): sunlight intensity on the panel.

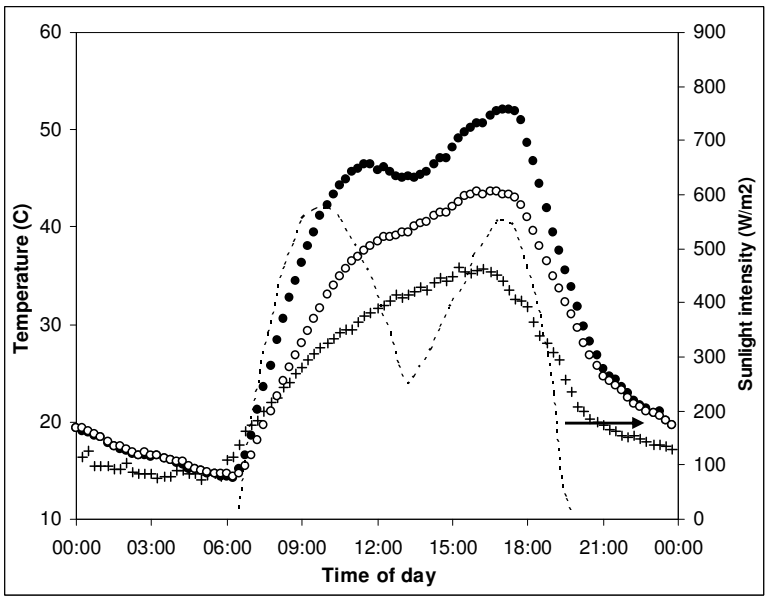


Figure 6.13 The temperature change in east-west facing solarbioreactor.
(++++): air temperature, (••••): bioreactor containing bacteria, (○○○○): bioreactor containing water, (----): sunlight intensity on the panel.

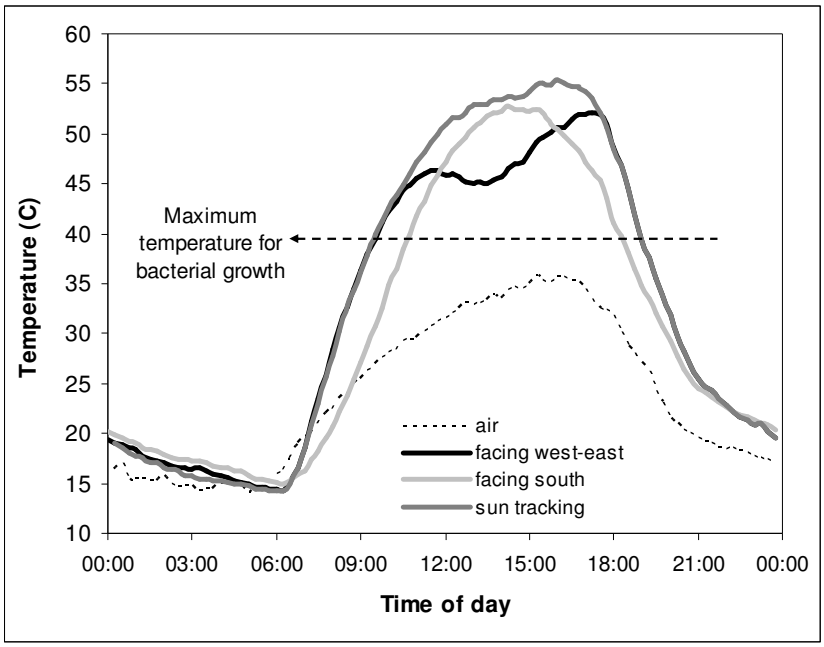


Figure 6.14 Comparison of the temperature profiles for different solarbioreactor orientations

Table 6.2 Comparison of different solarbioreactor orientations in terms of the light intensity received and the inner temperatures

Bioreactor orientation	South, 40º inclined	East-West	Sun-tracking
Max. light intensity received (W/m^2)	736	490	744
Total light intensity received ($kW/m^2.day$)	7.87	7.93	10.92
T_{max} during day	52.5	52.1	55.4
ΔT_{max} between air and bioreactor	18	19.3	19.8
Duration at T> 40ºC (h)	7.5	9.5	9.5

The heat generation by bacteria can be easily seen in these graphs, this generation causes bioreactor temperature to raise 10-12ºC more compared to the water-containing bioreactors, and 18-20ºC more compared to the air temperature.

Also from these data, it can be suggested that the orientation of the bioreactor does not affect the maximum temperature of the bioreactor significantly; more than 52ºC was reached in all three cases.

The south facing bioreactor stays over 40ºC (which is the critical threshold for the bacteria to survive) for a shorter period of time than the others (7.5 hours compared to 9.5 hours of east-west facing and sun-tracking bioreactors).

It can be concluded that manipulating the orientation of the bioreactor is not enough to keep the temperature under control, but proper positioning of the panel may decrease the cooling requirement and duration.

6.2 Cooling the Solarbioreactor

6.2.1 Heat Generation by the Bacteria

The bacteria generate heat due to their metabolic activities, the absorbed light at red-infrared region increases this heat generation even more. As a result of this generated heat, the temperature inside the photobioreactor increases and solar bioreactors requires cooling systems to ensure the maximum temperature that the bacteria can survive is not exceeded. The bacteria used in these studies (*R. capsulatus* and *R. sphaeroides*) are of mesophilic type and can not grow over 39ºC but they can survive temperatures over 40ºC for short time periods.

In this study, it is targeted to find out the requirement of a cooling system for the solar bioreactors. In order to do this, the temperature increase due to the heat generation by the grown bacteria inside a solar bioreactor needs to be shown. The data was also required for planning the type and the performance of the cooling system to be employed.

The experiment was performed in 4th of August 2007, in a very hot summer day. Grown *R. capsulatus* culture at stationary phase were prepared at cell

concentration of OD 1.0 (corresponding to 0.6 gdw/l, which represents the stationary phase cell concentration of an actual run) by manipulating the glutamate (N source) concentration in the media. Two flat panel photobioreactors (volume=750 ml, depth (light path) =5 cm) made of PVC were used, one bioreactor was filled with distilled water (as a control), another with bacteria at a concentration of 0.6gdw/l. Those two photobioreactors were placed in outdoor under sunlight. They are oriented in east-west direction (one side of the panel facing east, another side facing west). There was no cooling. The solar bioreactors were kept for one full day under these conditions and the temperature changes inside the reactors were recorded continuously. The results were given in Figure 6.15 below:

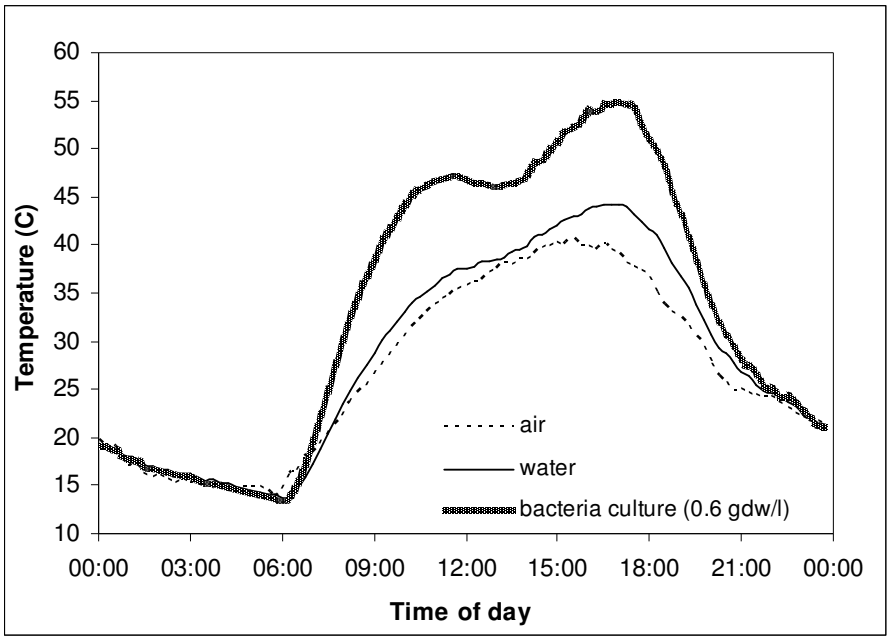


Figure 6.15 Daily temperature profile of the solarbioreactors that contain pure water and cell culture.

As can be seen in the Figure 6.15, air temperature started to increase after sunrise (6:00 am) up to 40ºC in the afternoon, after when it started to drop again until next sunrise.

The temperature of the reactor which contained water was followed a similar pattern compared to the air temperature and reached more than 44ºC around 17:00.

The temperature profile in the bioreactor which contained bacteria followed a different pattern however: it increased much faster following the sunrise, reached a maximum before noon, where it delayed for around two hours as it did not receive any direct light, then as the panel started to receive light from west side, the temperature increased again to a new maximum at around 55ºC, than it started to drop during the night until next sunrise.

The maximum temperature difference between the grown bacteria containing reactor and the water containing reactor was 11ºC, showing the considerable heat generation by the bacteria.

As a result, the heat generation by the bacteria demonstrated. Also it can be concluded that a cooling system is a must, as the temperature reached up to 55ºC in the bioreactors, where the bacteria can not survive. The cooling alternatives were studied and discussed in the next sections.

6.2.2 Shading

Shading (blocking part of the sunlight) the solarbioreactors in order to decrease the cooling requirement was investigated. Shading is a passive (non-energy consuming) method which may decrease the temperature inside the solarbioreactor. It is assumed that the sunlight is already in excess amount for the process and may be decreased without sacrificing from process efficiency.

Different materials were tested and used for the shading of the solarbioreactors.

First, white filter papers were utilized to create shade on bioreactors. More than one layer of paper sheets were used to increase the blocked light amount. The

light spectra and light intensity of the sunlight after passing through the filters were determined first. The results were given in Figure 6.16 and 6.17.

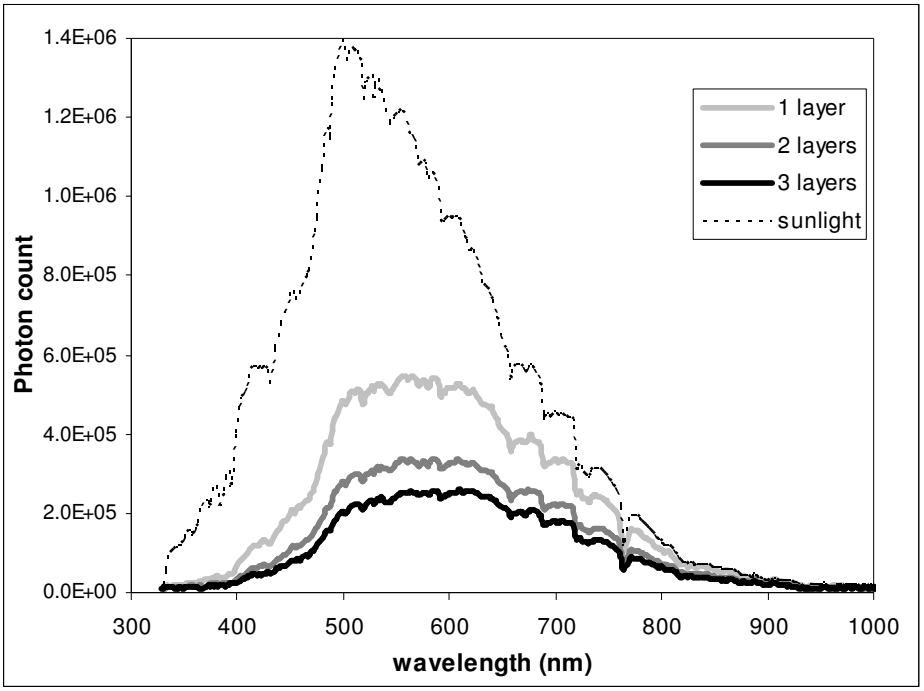


Figure 6.16 Change in the sunlight spectra caused by shade material

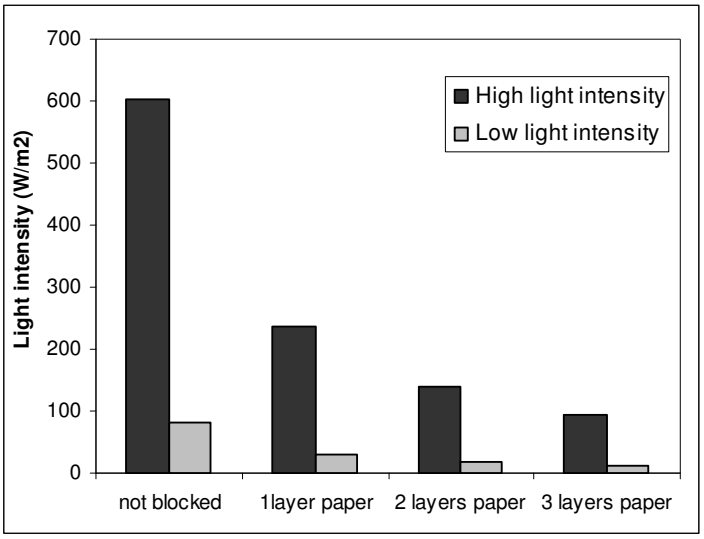


Figure 6.17 Change in the sunlight intensity caused by shade material

It can be seen that the material is passing light at wavelength between 400-900 nm and not blocking a particular wavelength region, on the contrary the absorbance is rougly even throughout this wavelength range.

By using luxmeter, the light intensity decrease was also determined. It was found that the light intensity drops by 60% if one layer of paper were used, 77% if two layers of papers were used and 85% if three layers of papers were used. The block percentages were not dependent on light intensity; the measurements were done at high and low light intensities and same blocking ratio was obtained.

After documenting the shading properties of the filter material, second part of the study was carried out. The experiment was performed in August 17th, 2007. Four flat panel photobioreactors (volume=250 ml, depth (light path)=32 mm) made of PVC were used, three of the bioreactors were coated by filter paper to create shade (one, two and three layers of paper were used for three bioreactors), last bioreactor was not shaded (control). The bioreactors were filled with fully grown bacteria at stationary phase (0.6gdw/l) which represents the average cell concentration of an actual batch run. The bacteria used were *R. capsulatus*. Those

photobioreactors were placed outdoor under sunlight in south-facing orientation (40 inclined from the ground) and were kept for one full day. The temperature changes inside the bioreactors were recorded continuously. The results were given in Figure 6.18.

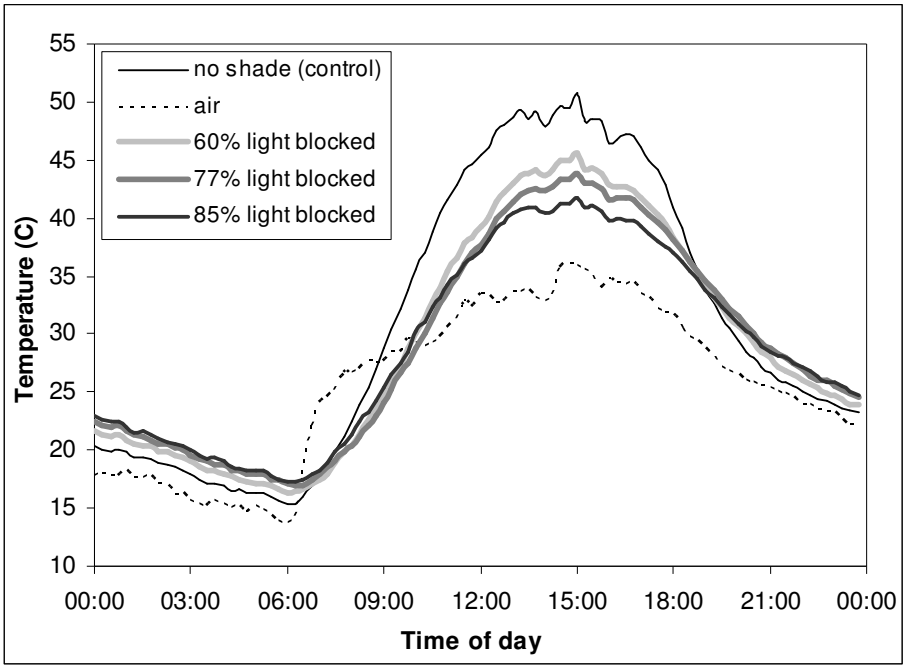


Figure 6.18 Effect of shading on bioreactor temperature

It can be seen that in the bioreactor which is not shaded, the temperature increased up to 50°C, which is very comparable to the previous study. The shading decreased the maximum temperature achieved on the bioreactors. The results obtained in this study suggests that shading the bioreactors decreases cooling requirement, however the minimum light intensity required for the process should also be considered in order to not decrease the hydrogen production efficiency.

During the studies in outdoor, it was observed that the shading material used (white filter paper) was not appropriate for outdoor conditions (i.e. it is not resistant to water), therefore greenhouse shading material specifically designed and manufactured for this purpose was also employed in some of the late studies. The light spectra and light intensity of the sunlight after passing through this material was also determined and given in Figure 6.19 below:

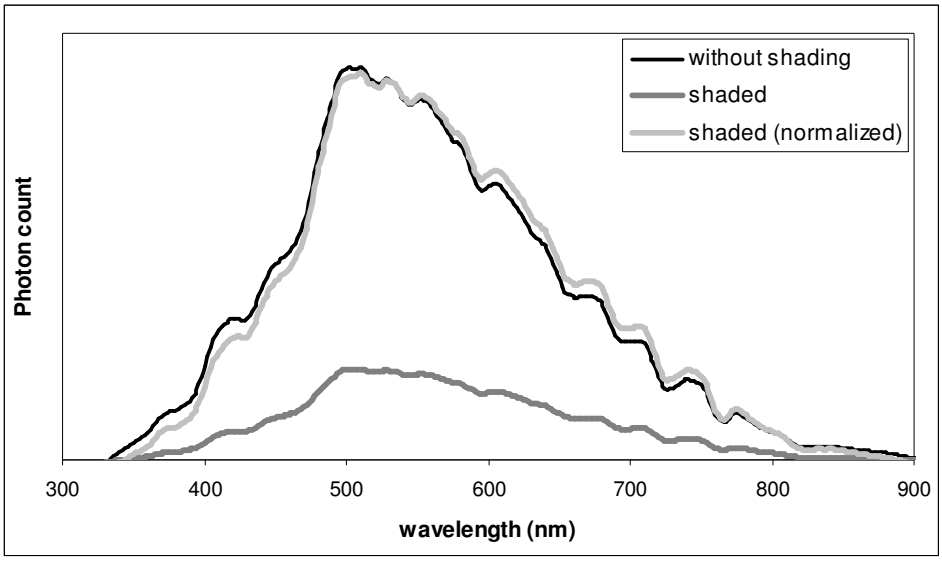


Figure 6.19 Change in the sunlight spectra by greenhouse shade material

It can be seen that the material is passing light at wavelength between 400-900 nm and not blocking a particular wavelength region, on the contrary the absorbance is rougly even throughout this wavelength range.

By using luxmeter, the light intensity decrease was also determined. It was found that the light intensity drops approximately by 60%. The block percentage

was not dependent on light intensity; the measurements were done at high and low light intensities and same blocking ratio was obtained.

6.2.3 Water Spraying

Spraying of the tap water on the panel of a solarbioreactor may be argued to be a passive (non-electricity consuming) cooling method, as the energy is not consumed *in situ*, however tap water is also pumped somewhere else and electricity is used anyway. Moreover, in case of recycling the used water for re-spraying, a pump would be necessary. Therefore it is more convenient to accept that water-spraying is an energy consuming cooling method, unlike the shading.

The experiment was performed in August 17th, 2007 in conjunction with the passive cooling (shading) methods. Two flat panel photobioreactors (volume=250 ml, depth (light path)=32 mm) made of PVC were used, one bioreactor was cooled by water-spraying on the south-face of the panel. The other bioreactor was not cooled (control).

The bioreactors were filled with fully grown bacteria at stationary phase (0.6gdw/l) which represents the average cell concentration of an actual batch run. The bacteria used were *R. capsulatus*. Those photobioreactors were placed outdoor under sunlight in south-facing orientation (40 inclined from the ground) and were kept for one full day.

The water spraying was done between 10:00-16:00 only (6 hours/day). The temperature changes inside the bioreactors were recorded continuously. The results were given in Figure 6.20.

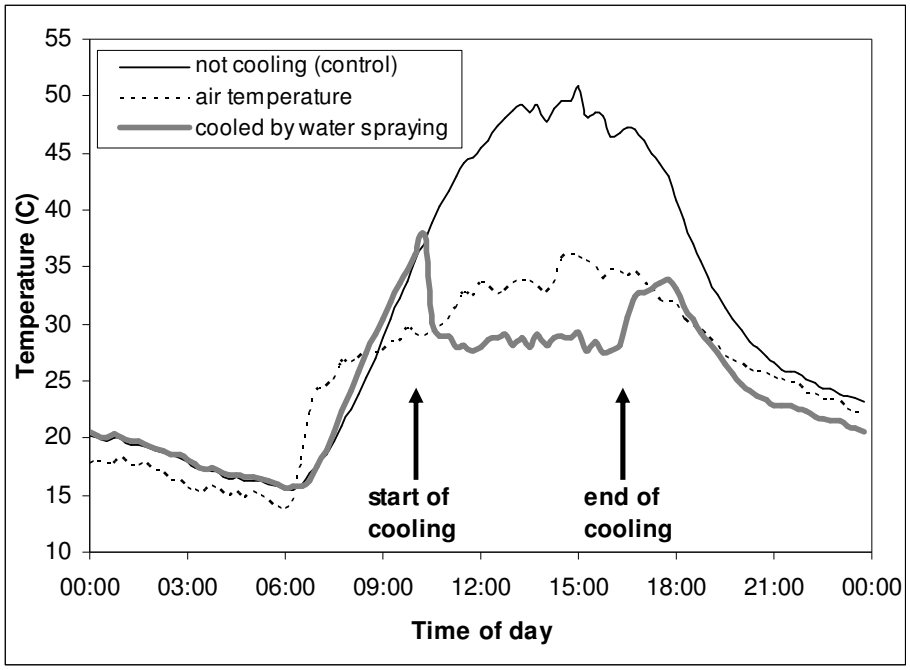


Figure 6.20 Effect of water cooling on the temperature of the solarbioreactor.

It can be seen that water spraying was an effective method for temperature controlling and kept the temperature far below the threshold (40ºC) during cooling. The temperature inside the bioreactor decreased from 37.8ºC to below 30ºC after cooling started at 10:00 and did not exceed 30ºC during the cooling, after the cooling was stopped at 16:00, it started to rise again but as the sunset was near, the temperature did not exceed 34ºC.

6.2.4 Other Cooling Methods

Other alternatives for cooling the solarbioreactors exist, but they require electricity to operate: one alternative is using a thermoelectricial element (Peltier cooler). Cooling by a Peltier device was investigated and found to be ineffective compared to water cooling by the initial tests (results not given), however the

efficiency of this method depends on many factors such as the electrical current applied and the effectiveness of heat removal at the hot side of the device and requires a more detailed study for optimization. In any case, this method is electricity-intensive and will not be economical to use.

Another alternative may be to use fans to increase the convection on the panel to increase heat loss; this method also requires electricity for cooling and was not further studied.

6.2.5 Evaluation of Cooling Methods

The results obtained were compared in the Table 6.3:

Table 6.3 Comparison of cooling methods

	Control (not cooled)	Shaded by one layer of filter paper	Shaded by two layers of filter paper	Shaded by three layers of filter paper	Water spraying
Light blocked (%)	-	60	77	85	-
T_{max} during day (ºC)	50.8	45.6	43.9	41.8	37.8 (29.3)*
ΔT_{max} between air and bioreactor (ºC)	14.9	9.7	8.0	5.9	-6.7
Duration at T > 40ºC (h)	7.25	5.25	4.75	3.0	0

*T_{max} during water spraying

From the table, the best cooling method seems to be water-cooling, however partial shading can be used in addition to water cooling too, that would decrease water flow rate and/or duration of cooling. The obtained results are utilized to cool the bioreactors in hydrogen production experiments, given in the next section.

Electricity-consuming cooling methods such as using thermoelectrical elements or fans were not considered further for investigation due to their uncomparable energy cost compared to the shading and water cooling methods.

6.3 Hydrogen Production in Solarbioreactors

Obviously, the ultimate goal of biohydrogen research with the photosynthetic bacteria is the operation of large-scale bioreactors under natural sunlight (Koku et al, 2002). Therefore, outdoor application of the photobiological hydrogen process is a critical step. In this part of the study, it was targeted to investigate the growth and hydrogen production of the bacteria (*R. capsulatus* and *R. sphaeroides*) in outdoor conditions.

6.3.1 Effect of Indoor and Outdoor Conditions on Growth and Hydrogen Production of *R. sphaeroides*

In order to compare the effect of indoor and outdoor conditions, a run was carried out between 21.10.2003 - 10.11.2003 (Run 211003). Two 55 ml glass bottles were used as photobioreactors. One of the bottles was placed in indoor as a control and the other one was placed in outdoor conditions. Temperature was not controlled. *R. sphaeroides* OU001 was used as the bacteria. 10% inoculum was made. The media contained 15 mM of malate as the carbon source and 2 mM of glutamate as the nitrogen source.

The growth and hydrogen production graphs of the two cases were given below for comparison:

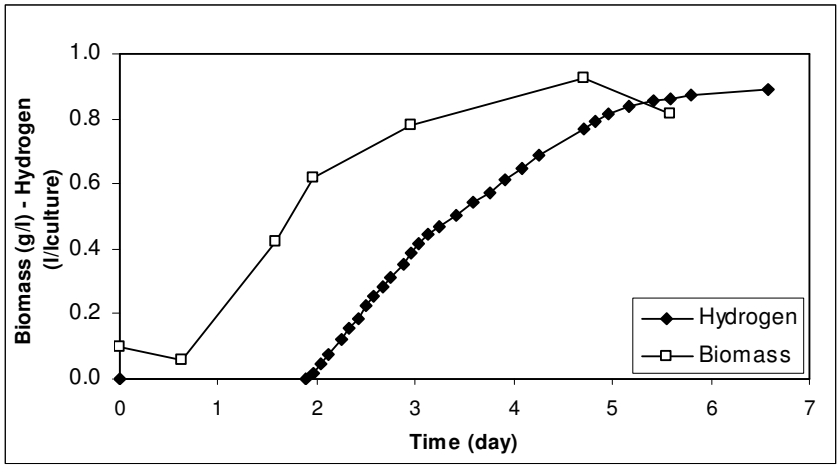


Figure 6.21 Growth and hydrogen production in indoor bioreactor

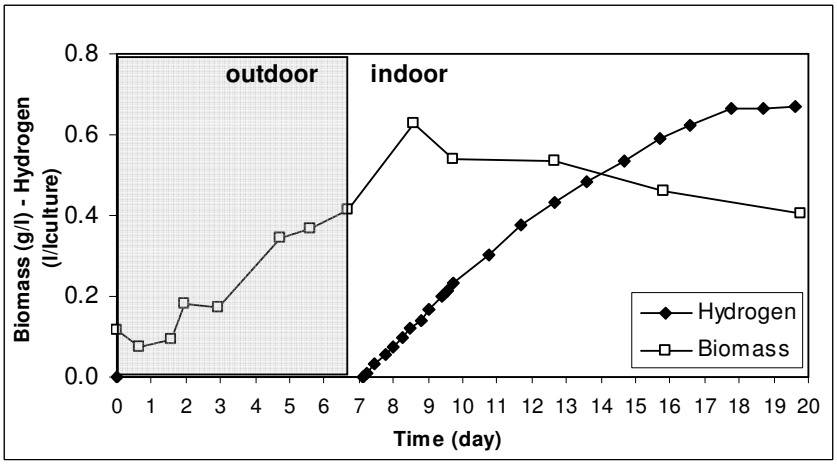


Figure 6.22 Growth and hydrogen production in outdoor bioreactor (the bioreactor was taken indoor at 161th hour; shaded region corresponds to outdoor part, white region corresponds to indoor part)

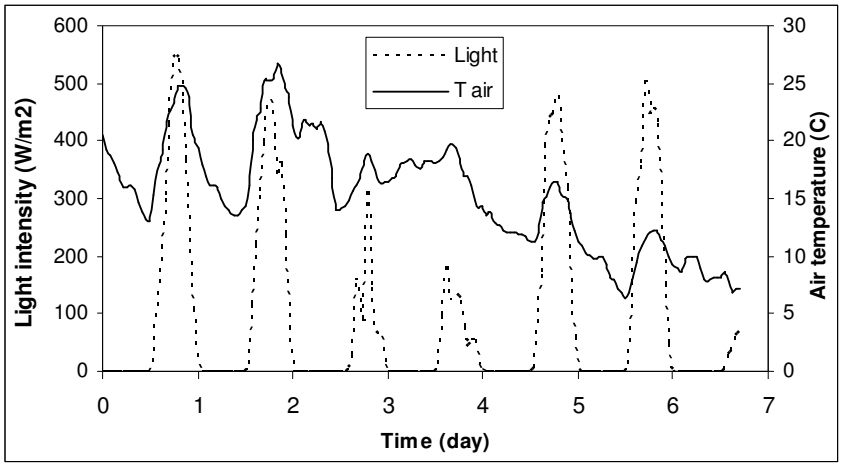


Figure 6.23 Change in sunlight intensity and air temperature during the outdoor part of the run (Run 211003).

The indoor bioreactor process ran successfully, bacteria grew and 0.9 $l_{hydrogen}/l_{culture}$ hydrogen gas was produced in one week. The outdoor twin however, did not perform well, probably due to the bad weather conditions. It was cold and the sky was cloudy during the batch. The outdoor conditions (sunlight intensity and air temperature) were given in Figure 6.23. The outdoor bottle was taken in indoor at the 161th hour. Hydrogen production started in 173th hour and continued for 11 more days, 0.66 $l_{hydrogen}/l_{culture}$ was produced at the end. This final amount is lower compared to the one obtained from indoor bottle, this is expected as bacteria spent some of the available substrate in the media for surviving the first week. The pH was between 6.8 - 7.5 during the process in both of the bioreactors.

This experiment shows that the bacteria can survive harsh outdoor conditions and when the temperature-illumination conditions restored they start to produce hydrogen.

Another run was carried out between 08.09.2005 - 13.09.2005 (Run 080905). *R. sphaeroides* OU001 was used as the bacteria. 10% inoculum was made. The media contained 15 mM of malate as the carbon source and 2 mM of glutamate as the nitrogen source. 585 ml photobioreactor made of acrylic and glass

was used. Temperature was controlled by continuously circulating water at 25ºC from the inner cooling coil of the bioreactor.

The run was started in indoor, the bacteria grew without any problem. When it started to produce hydrogen in a stable manner (second day), the setup was carried to outdoor in the morning and the batch continued in outdoor conditions. The hydrogen production continued without any problem for three more days and totally 1.2 $l_{hydrogen}/l_{culture}$ was obtained.

The growth, hydrogen production and outdoor conditions (sunlight intensity and air temperature) were given in Figures 6.24 and 6.25 below, arrows indicate the indoor and outdoor periods:

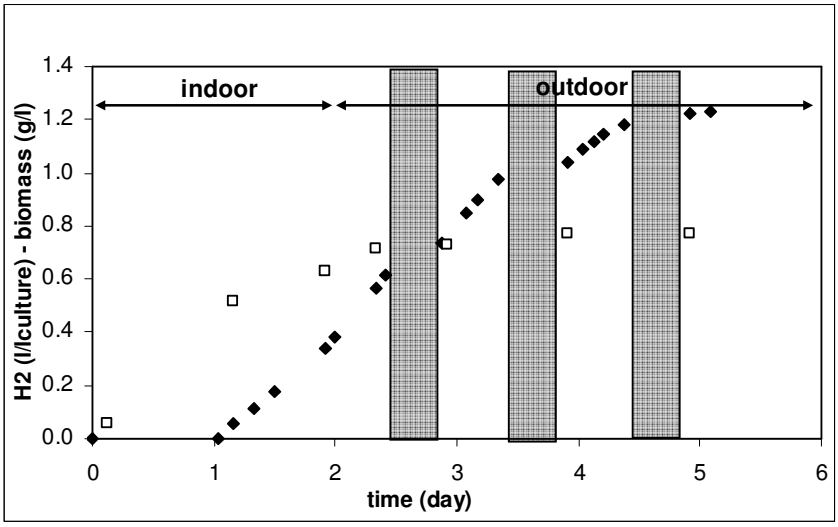


Figure 6.24 Cell growth and hydrogen production in the bioreactor (shaded regions correspond to nights).

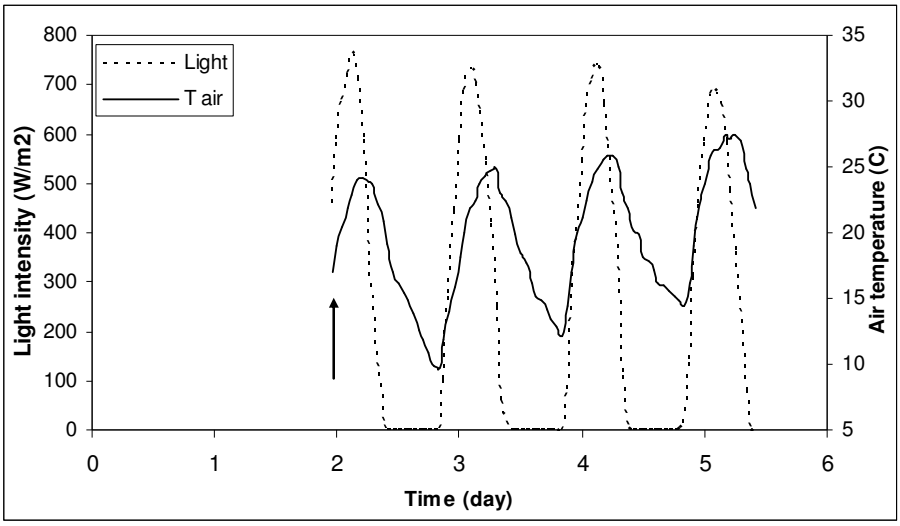


Figure 6.25 Change in sunlight intensity and air temperature during the outdoor part of the run (Run 080905).

The pH was between 6.9 - 7.4 during the process in the bioreactor.

It can be said that once the bacteria establish themselves in the bioreactor at a stationary growth phase and start to produce hydrogen, they are less susceptible to change in the process conditions and can continue hydrogen production in outdoor without being affected negatively.

From the results obtained, it was observed that a "critical biomass concentration" should be reached in the bioreactor for the start of hydrogen production. Evaluating both runs, this biomass concentration is approximately 0.4 g/l. In case bad outdoor conditions (temperature and light/dark cycle) slow down the growth of bacteria, hydrogen production do not start until this critical biomass concentration is reached. Therefore it is suggested to use higher inoculum amount to exceed this threshold bacteria concentration quickly.

6.3.2 Effect of Outdoor Conditions on Growth and Hydrogen Production of *R. capsulatus*

6.3.2.1 Effect of Shading on the Growth and Hydrogen Production

The run was carried out between 14.08.2007 – 25.08.2007 (Run 140807). Three flat-panel bioreactors made of glass were used (320ml volume). 20% inoculation was made, the bacteria used were *R. capsulatus*. The media contained 30 mM of acetate and 7.5 mM of lactate as the carbon source. The photobioreactors were placed outdoor under sunlight in south-facing orientation (40 inclined from the ground). Two of the photobioreactors were shaded by one and two layers of white filter paper (blocking 60% and 77% of the incoming light, respectively), the third photobioreactor was not shaded (control). The growth, bioreactor temperatures and sunlight intensity curves obtained were given in Figures 6.26 - 6.31 below:

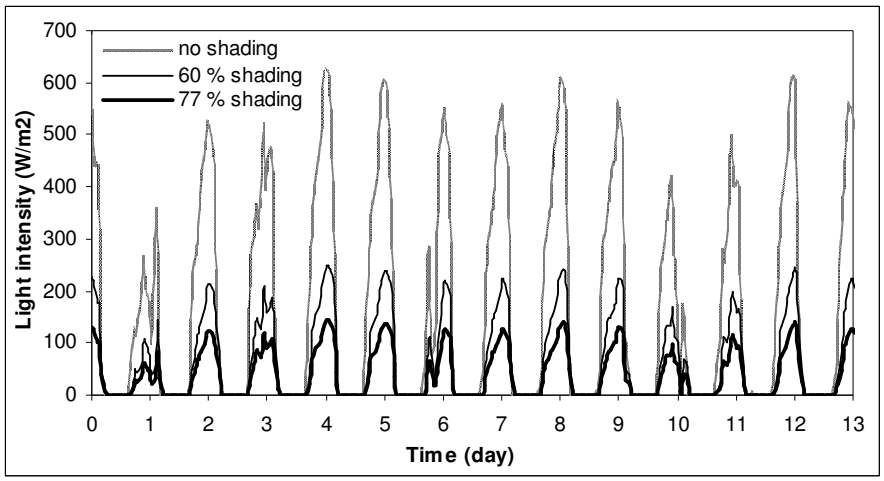


Figure 6.26 Sunlight intensity received by bioreactors during the run (Run 140807).

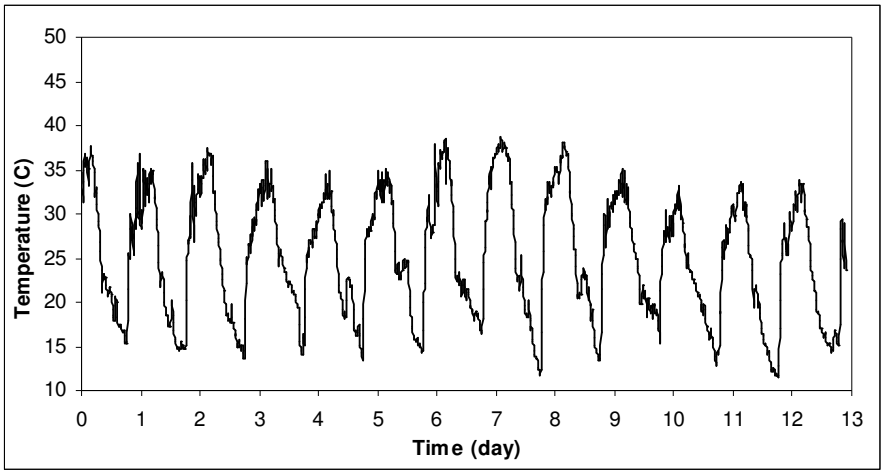


Figure 6.27 Air temperature during the run (Run 140807).

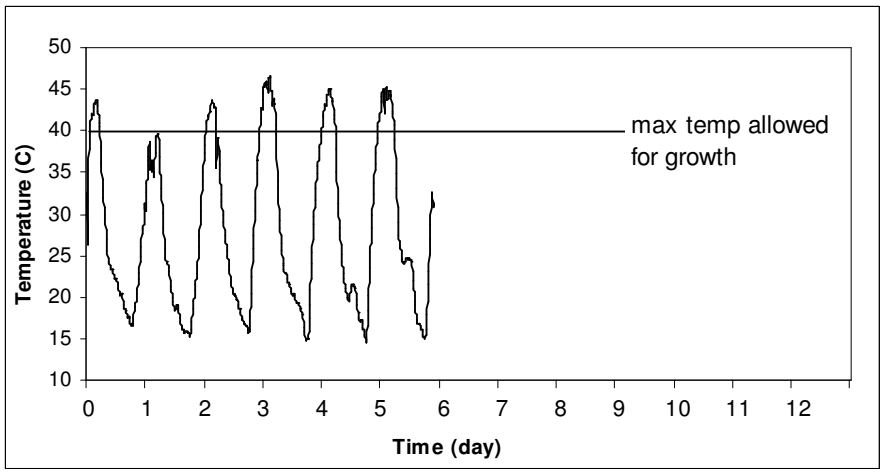


Figure 6.28 Temperature of bioreactor which was not shaded (Run 140807).

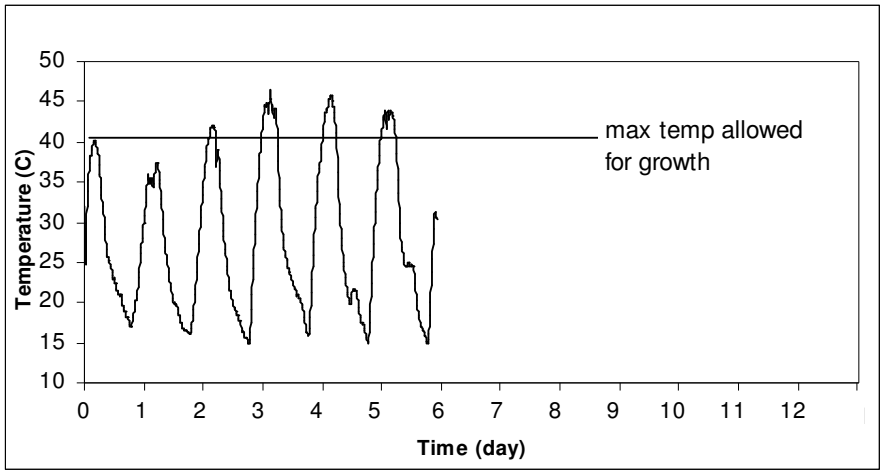


Figure 6.29 Temperature of the bioreactor which was shaded to block 60 % of the incident sunlight intensity (Run 140807).

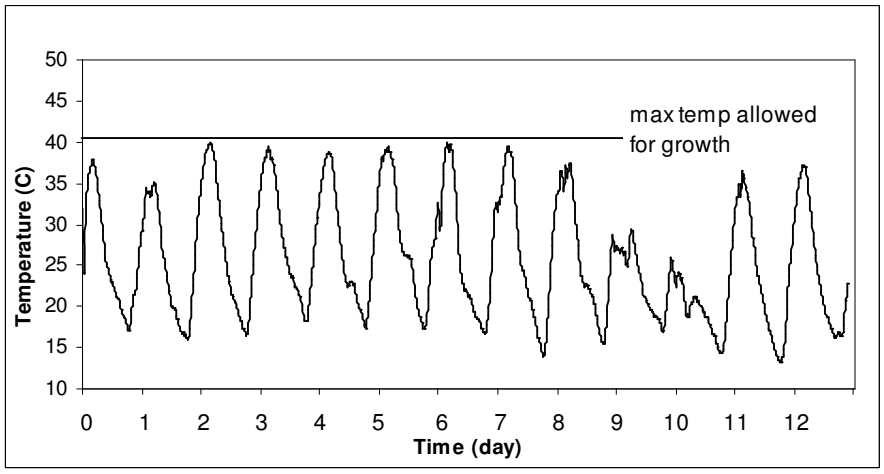


Figure 6.30 Temperature of the bioreactor which was shaded to block 77 % of the incident sunlight intensity (Run 140807).

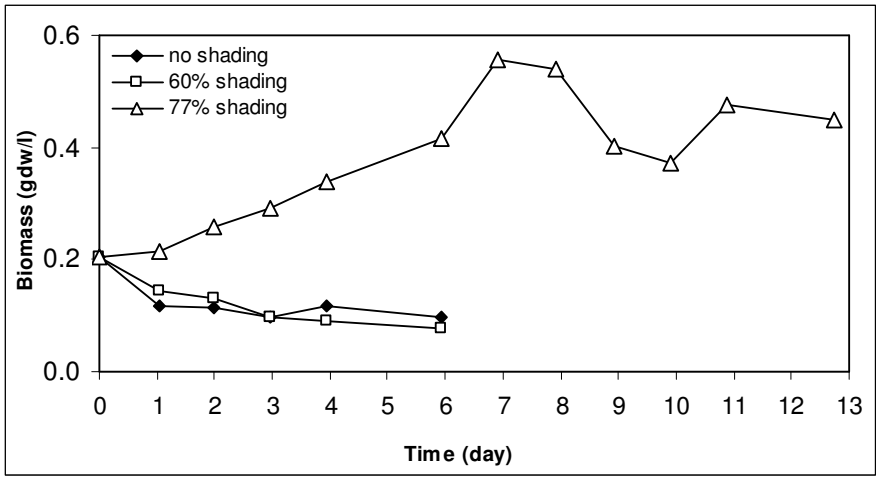


Figure 6.31 Effect of shading on cell growth in the bioreactors (Run 140807).

Table 6.4 summarizes the results of Run 140807. Bacteria did not grow in the bioreactor which was not shaded and which was shaded by a single layer of filter paper, probably due to the high temperature reached. No gas accumulation was observed in these two bioreactors. These two bioreactors stopped after 6 days.

A slow growth was observed in the bioreactor which was shaded by a double-layer filter paper (77% shading). In this bioreactor, temperature was never above 40ºC and bacteria grew slowly, 255 ml of hydrogen was collected after 240 hours. The pH values in all three bioreactors were between 6.8-7.2 during the run.

During this run, it was observed that the shading material used (white filter paper) was not appropriate for outdoor conditions (i.e. is not resistant to water), therefore greenhouse shading material specifically designed and manufactured for this purpose was employed in the later studies.

Table 6.4 Summary of the experimental conditions and results of Run 140807

Bacteria used	*R. capsulatus*		
Experiment period	14.08.2007 – 25.08.2007		
Bioreactor volume (ml)	320		
C source in media	30 mM acetate + 7.5 mM lactate		
N source in media	2 mM glutamate		
Inoculation amount (v/v)	20%		
Shading	77%	60%	none
Cooling	none	none	none
Max bioreactor temp. (°C)	39	45	47
Max biomass (g/l)	0.56	No growth	No growth
Total gas produced ($l/l_{culture}$)	0.77	0	0
Avr. H_2 prod. rate ($ml/l_{reactor}.h$)	3.3	NA	NA

NA: not applicable

6.3.2.2 Effect of Shading and Cooling on the Growth and Hydrogen Production of Mutant and Wild Type Strains

Mutant of *R. capsulatus* (YO3) obtained by Dr. Yavuz Öztürk and wild type bacteria were compared in solar bioreactors. *R. capsulatus* YO3 is a (hup^-) mutant strain lacking uptake hydrogenase enzyme.

First run was carried out between 22.08.2007 – 31.08.2007 (Run 220807). Two flat-panel bioreactors made of glass were used (320ml volume). 20% inoculation was made, the bacteria used were *R. capsulatus YO3* mutant and the wild type. The media contained 30 mM of acetate and 7.5 mM of lactate as the carbon source. The photobioreactors were placed outdoor under sunlight in south-facing orientation (40º inclined from the ground). Both of the bioreactors were shaded by greenhouse shading material (blocking ~60 % of light intensity). The bioreactors were cooled by water spraying between 10:00-16:00 (cooling could not be performed due to the tap water absence in Ankara between 3rd and 5th days). The growth, bioreactor temperatures and sunlight intensity curves obtained were given in Figures below:

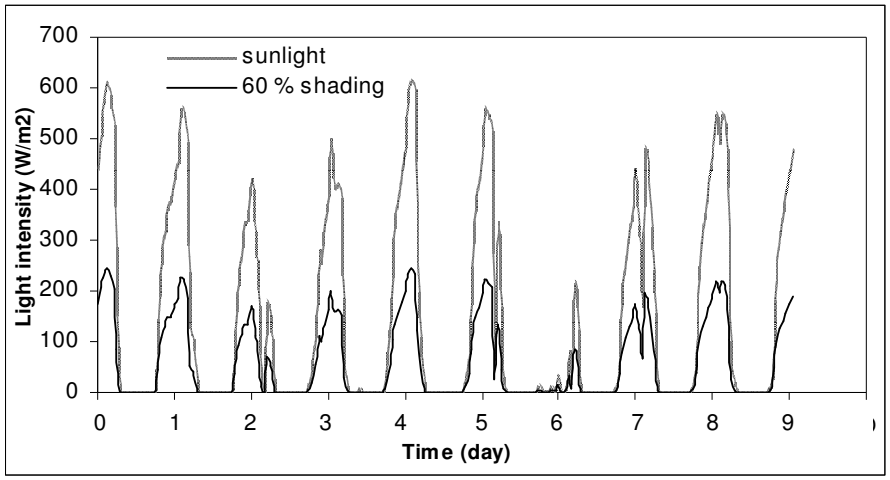


Figure 6.32 Direct sunlight intensity and the sunlight received by bioreactors during the run (Run 220807).

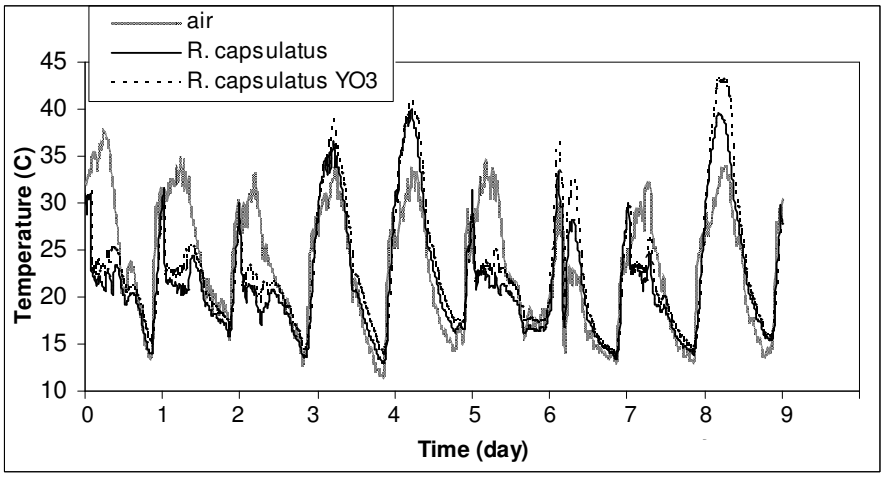


Figure 6.33 Air and bioreactor temperatures during the run (Run 220807).

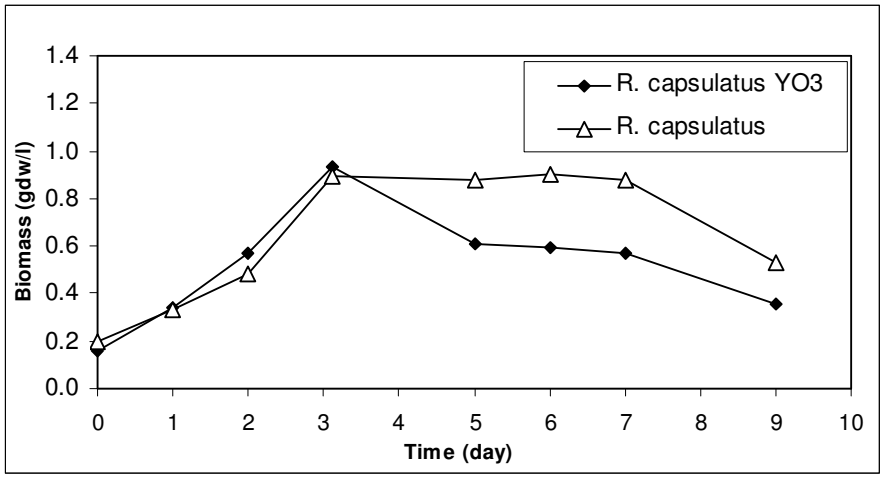


Figure 6.34 Cell growth in bioreactors during the run (Run 220807).

The pH values in both bioreactors were between 6.6 - 7.4 during the run.

Unfortunately there was a leakage out of the gas collection system and although it was observed that hydrogen gas was produced in both of the bioreactors most of the produced gas could not be collected. Therefore hydrogen productivities could not be determined quantitatively for comparison.

This Run shows the growth patterns of both strains in outdoor conditions. It can be seen that both type of bacteria started to grow in a similar pattern but after reaching to the end of the exponential growth phase, the mutant strain started to die unlike the wild type strain.

On the other hand, due to water shortage in Ankara, cooling water could not be provided during the third and the fourth days, resulting in high bioreactor temperatures. It can be seen that bacteria died on days that high temperature (>40ºC) was reached (day 4 and especially day 8). Otherwise, the shading and water spraying was shown to be very effective for keeping the temperature under control.

Table 6.5 summarizes the experimental conditions and the results of Run 220807.

Table 6.5 Summary of the experimental conditions and results of Run 220807

Experiment period	22.08.2007 – 31.08.2007	
Bioreactor volume (ml)	320	
C source in media	30 mM acetate + 7.5 mM lactate	
N source in media	2 mM glutamate	
Inoculation amount (v/v)	20%	
Bacteria used	*R. capsulatus*	*R. capsulatus* YO3
Shading	60%	60%
Cooling	Water spraying	Water spraying
Max bioreactor temp. (°C)	40	40
Max biomass (g/l)	0.90	0.93

Second run was carried out between 27.09.2007 – 05.10.2007 (Run 270907) in order to show the hydrogen productivity of both strains. Six bioreactors made of glass were used (550ml working volume). 20% inoculation was made, the bacteria used were *R. capsulatus* YO3 mutant and the wild type. The media contained 30 mM of acetate and 7.5mM of lactate as the carbon source. The photobioreactors were placed outdoor under sunlight. For each type of the bacteria; one bioreactor was shaded by greenhouse shading material (blocking ~60% of light intensity) and the other two were not shaded.

The bioreactors were cooled by water spraying for 5-10 minutes when the bioreactor temperature exceeded 40ºC, which usually happened at 13:00. One cooling session that lasted 5-10 minutes were enough per day, that cooling period dropped bioreactor temperature down to 25ºC and after cooling stopped the bioreactor temperature did not exceeded 40ºC during the rest of the day as can be seen in the temperature profiles given below.

The wild type *R. capsulatus* performed very poorly compared to the YO3 strain; there was no growth in bioreactors that were not shaded after 8 days, and a slow growth after three days of lag time in the shaded bioreactor. A slight hydrogen production started in the shaded bioreactor after six days but was not measured since the batch was almost ended and it was very small compared to the hydrogen production by mutant strain.

The air temperature and sunlight intensity, growth and bioreactor temperature curves obtained for the mutant strain in shaded and not shaded bioreactors were given in Figures 6.35 - 6.38 (average values were taken for the two not shaded bioreactors):

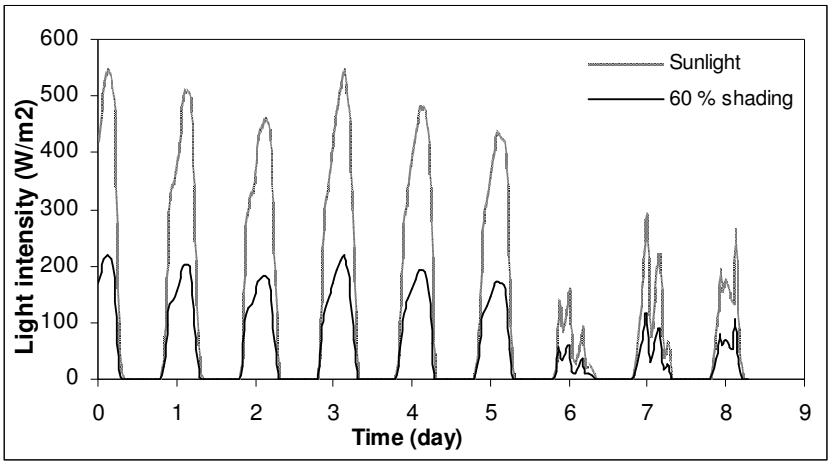


Figure 6.35 Sunlight received by bioreactors during the run (Run 270907).

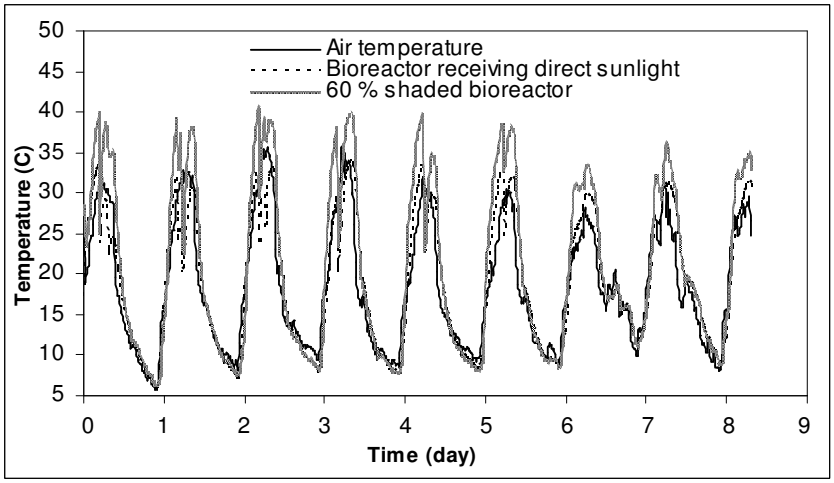


Figure 6.36 Air and bioreactor temperatures during the run (Run 270907).

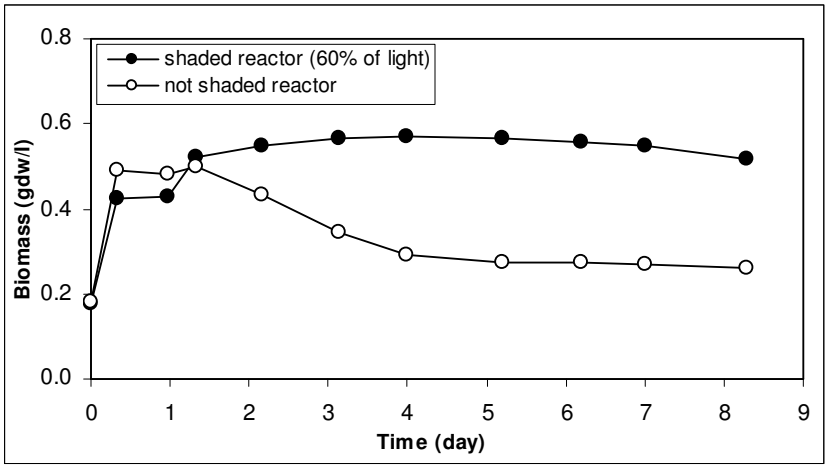


Figure 6.37 Cell growth in bioreactors during the run (Run 270907).

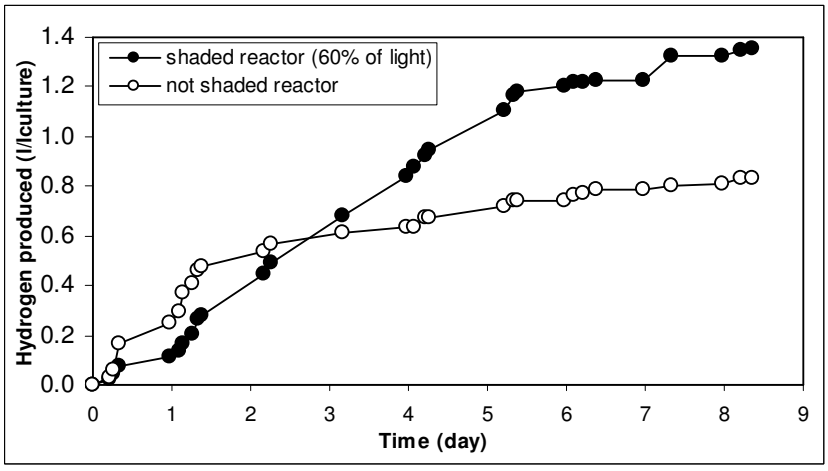


Figure 6.38 Hydrogen production in bioreactors during the run (Run 270907).

The pH values in the bioreactors were between 6.9 - 7.8 during the run.

Bacteria grew in all three bioreactors and produced hydrogen. Significantly more hydrogen gas was produced in the bioreactor which was shaded to reduce incident light compared to not-shaded bioreactors.

There is also a noticeable difference between the growth curves: although the biomass density in all three bioreactors reached to approximately the same concentration, the biomass density of not-shaded bioreactors started to decrease after the maxima has been reached whereas the biomass density in the shaded bioreactor stayed almost the same. This results support the finding of the previous set, where the biomass started to decrease similarly in the not-shaded bioreactor which contained YO3 strain.

Table 6.6 summarizes the experimental conditions and the results of Run 270907.

Table 6.6 Summary of the experimental conditions and results of Run 270907

Bacteria used	*R. capsulatus* YO3	
Experiment period	27.09.2007 – 05.10.2007	
Bioreactor volume (ml)	550	
C source in media	30 mM acetate + 7.5 mM lactate	
N source in media	2 mM glutamate	
Inoculation amount (v/v)	20%	
Shading	60%	No shading
Cooling	Water spraying	Water spraying
Max bioreactor temp. (°C)	34	40
Max biomass (g/l)	0.57	0.50
Total gas produced ($l/l_{culture}$)	1.35	0.83
Avr. H_2 prod. rate ($ml/l_{reactor}.h$)	7.7	5.3

It is evident that the shading of the bioreactor improved the hydrogen productivity considerably. There might be two reasons causing this difference between the shaded and not-shaded cases: The bacteria are susceptible to photoinhibition and the high light intensities reached during the day negatively affect both the biogrowth and the hydrogen production. Second reason might be the temperature, although the temperature stayed below the lethal level, it

increased up to 39-40ºC in the not shaded bioreactors and up to 35ºC in the shaded bioreactor. High temperature may cause the inhibition of the process.

A third run was carried out between 08.10.2007 – 11.10.2007 (Run 081007) in order to clarify the cause of the inhibition of the hydrogen production and to ensure the stability of the process in outdoor conditions. The difference between this and the previous run was the upper temperature limit allowed in the bioreactors: The bioreactor temperatures did not exceeded 35ºC during this run (it was 40ºC in the previous run) to eliminate the effect of high temperature.

The experimental setup was similar to the previous run: Four bioreactors made of glass were used (550ml working volume). 20% inoculation was made, the bacteria used were *R. capsulatus* YO3 mutant and the wild type. The media contained 30 mM of acetate and 7.5mM of lactate as the carbon source. The photobioreactors were placed outdoor under sunlight. For each of the strains, one of the bioreactors was shaded by greenhouse shading material (blocking ~60% of light intensity) and the other one was not shaded.

The temperatures of the bioreactors were monitored during the daytime and they were cooled by water spraying a few minutes when the bioreactor temperature exceeded 35ºC, until the temperature dropped down to 27-28ºC.

The air temperature and sunlight intensity, growth, substrate consumption (for YO3 mutants only) and bioreactor temperature curves obtained were given in Figures 6.39 - 6.44:

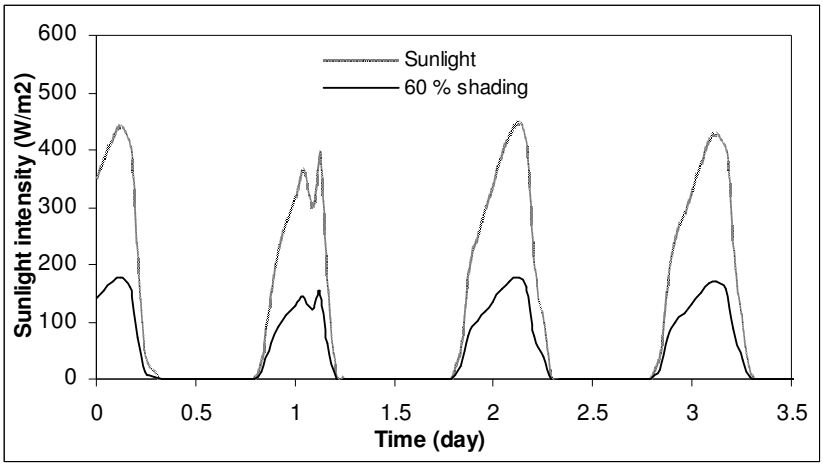


Figure 6.39 Sunlight received by bioreactors during the run (Run 081007).

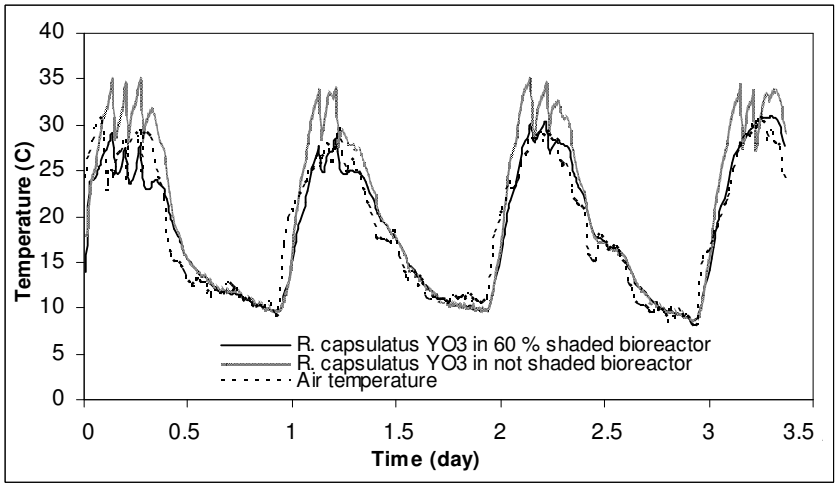


Figure 6.40 Temperature of air and bioreactors that contain *R. capsulatus* YO3 mutant (Run 081007).

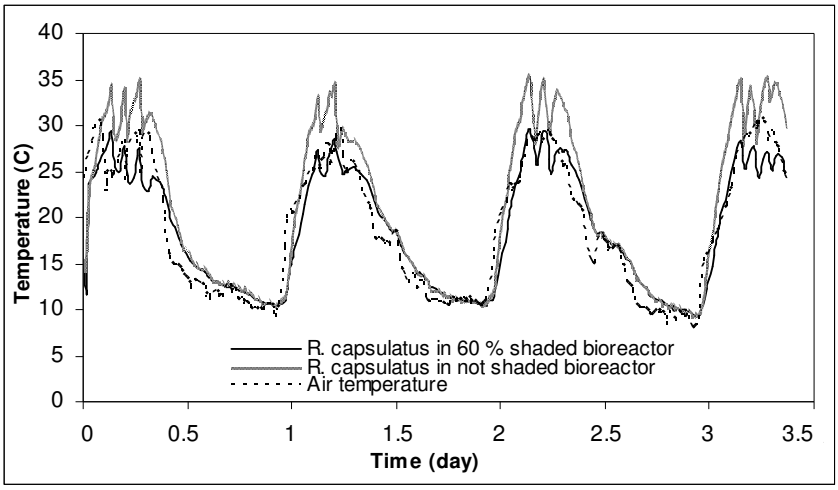


Figure 6.41 Temperature of air and bioreactors that contain wild type *R. capsulatus* (Run 081007).

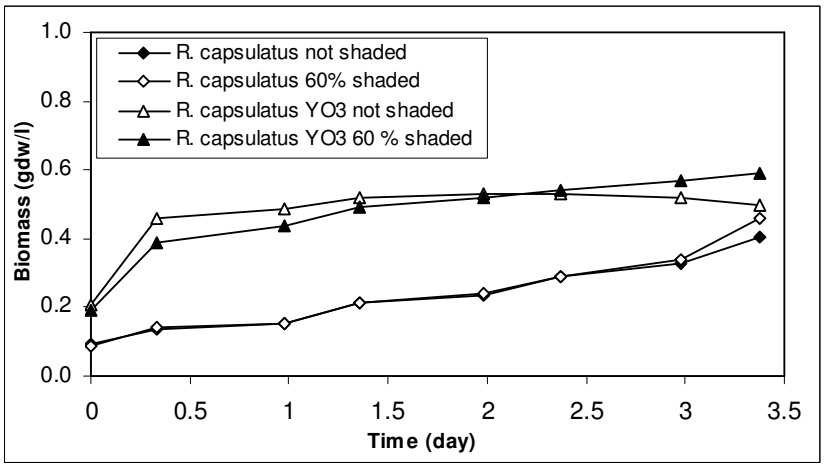


Figure 6.42 Cell growth in bioreactors during the run (Run 081007).

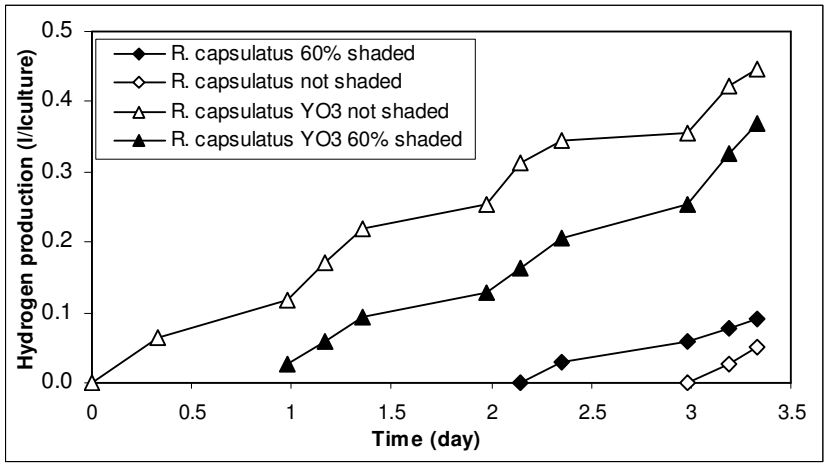


Figure 6.43 Hydrogen production in bioreactors during the run (Run 081007).

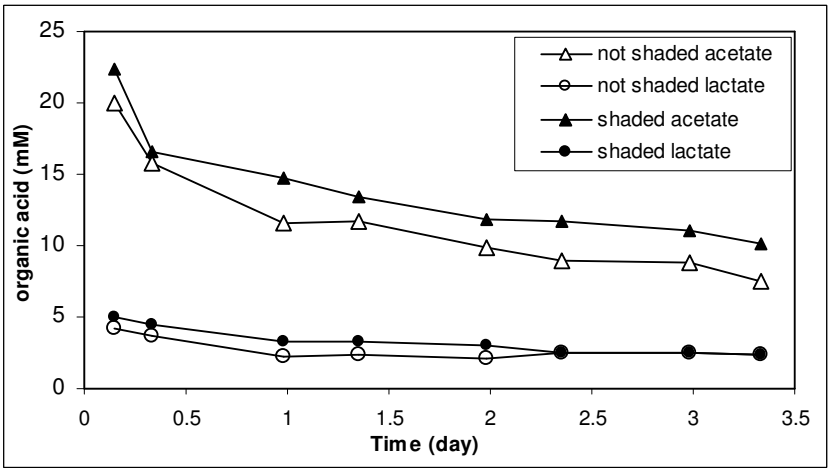


Figure 6.44 Substrate consumption in bioreactors containing *R. capsulatus* YO3 during the run (Run 081007).

The pH values in the bioreactors were between 6.8 - 7.8 during the run.

Bacteria grew in all four bioreactors and produced hydrogen. The growth of the wild type was much slower than YO3 strain, similar to the results obtained in the previous run (Run 270907). In the case of the YO3 strain; the bacteria in not-shaded bioreactor started to die after second day, supporting the results obtained in the first and second runs (Run 270907 and Run 220807).

The gas production started immediately in the not-shaded bioreactor whereas it started one day later in the shaded bioreactor. Apart from this difference in the lag times, the hydrogen production patterns in not shaded and shaded bioreactors were comparable. For the wild type strain the gas production lag times were 2-3 days.

The temperature was carefully controlled and stayed below 35ºC in all of the bioreactors showing that shading and cooling by water spraying were effective and enough to keep the temperature at a desired level in bioreactors.

The importance of inoculum can be observed from Figure 6.42. Although same amount of inoculum were added to all bioreactors (20%), the mutant strain inoculum was apparently denser and initial biomass concentrations in bioreactors which contain the mutant strain were higher. As a result, shorter growth and hydrogen production lag times were observed.

Table 6.7 summarizes the experimental conditions and the results obtained with mutant strain in Run 081007.

Table 6.7 Summary of the experimental conditions and results of Run 081007

Bacteria used	*R. capsulatus* YO3	
Experiment period	27.09.2007 – 05.10.2007	
Bioreactor volume (ml)	550	
C source in media	30 mM acetate + 7.5 mM lactate	
N source in media	2 mM glutamate	
Inoculation amount (v/v)	20%	
Shading	60%	No shading
Cooling	Water spraying	Water spraying
Max bioreactor temp. (°C)	30	35
Max biomass (g/l)	0.59	0.53
Total gas produced ($l/l_{culture}$)	NA	NA
Avr. H_2 prod. rate ($ml/l_{reactor}.h$)	6.3	5.9

NA: not applicable since the run stopped after 4 days

The results obtained were evaluated in the next part in terms of the effect of photoinhibition and the temperature dependence of the hydrogen production process.

6.4 Evaluation of the Outdoor Applications

6.4.1 Analysis of the Efficiencies and Rates

The average hydrogen production rates, light and substrate conversion efficiencies were calculated for the *R. capsulatus* wild type and YO3 mutant strains for the outdoor batch runs and given in Table 6.8.

Table 6.8 Summary of the results of outdoor studies with *R .capsulatus*

R. capsulatus strain used	Wild type	YO3 mutant	YO3 mutant	YO3 mutant	YO3 mutant
Experiment period (2007)	14-25 Aug	27Sep-5Oct	27Sep-5Oct	8-11Oct	8–11Oct
Shading	77%	60%	none	60%	none
Cooling	none	Water spraying	Water spraying	Water spraying	Water spraying
Max bioreactor temp. (°C)	39	33	40	30	35
Avr. H_2 prod. rate ($ml/l_{reactor}.h$)	3.3	7.7	5.3	6.3	5.9
H_2 productivity ($mgH_2/ l_{reactor}.h$)	0.27	0.63	0.44	0.52	0.49
H_2 yield ($gH_2/g_{substrate}$)	0.027	0.045	0.028	0.027	0.028
Light conv. Eff.[1] (%)	0.18	0.96	0.59	0.59	0.72
Light conv. Eff.[2] (%)	0.79	2.41	0.59	1.48	0.72
Substrate conv. Eff. [3] (%)	19	33	20	22	26

[1]Incident sunlight intensity (before being filtered by the shade material) was used

[2]Sunlight intensity after passing through shade material was used

[3]HPLC data were used if available, otherwise it is assumed that all the substrate was used during the batch.

The wild type *R. capsulatus* performed poorly compared to the YO3 mutant strain showing the effect of the uptake hydrogenase enzyme absence: The rate of hydrogen production, substrate and light conversion efficiencies were lower. This result was in accordance with the indoor performance of the strains: Ozturk et al (2005) compared the indoor hydrogen production of wild type and YO3 mutant of *R. capsulatus* and reported that the total hydrogen production, substrate conversion efficiency and hydrogen production rate of the mutant was significantly higher compared to the wild type parents.

The light conversion efficiency in the shaded bioreactors was higher than the not-shaded bioreactors, showing that the light energy is in excess in case of the sunlight illumination. Sunlight has very high intensity and the bacteria can not completely utilize all of this light energy.

The substrate conversion efficiencies of the batch runs vary between 19-33%. This is comparable to the values reported in indoor studies.

The average hydrogen production rates were higher in the shaded bioreactors compared to the not shaded bioreactors but the rates reached were low compared to the rates obtained in indoor runs which were illuminated continuously. The reason is the stop of the hydrogen production during dark periods (nighttime), which decreases the overall rate (Uyar et al, 2007).

Average hydrogen production rates varied between 3.3-7.7 ml $H_2/l_{reactor}$.h for the runs (Table 6.8). Eroglu et al (2007) reported comparable results in a 8L outdoor bioreactor with *R. sphaeroides*: 8 ml $H_2/l_{reactor}$.h in 30 mM acetate medium and 5-10 ml $H_2/l_{reactor}$.h in 15 mM malate medium.

Hydrogen yields varied between 0.027-0.045 $gH_2/g_{substrate}$ for the runs (Table 6.8). Eroglu et al (2007) reported similar yields in the 8L outdoor bioreactor with *R. sphaeroides*: 0.033 $gH_2/g_{substrate}$ in 30 mM acetate medium and 0.037-0.067 $gH_2/g_{substrate}$ in 15 mM malate medium.

6.4.2 Comparison of Indoor and Outdoor Hydrogen Production Performances

A parallel bioreactor was run (inoculated by the same culture in the same medium) with the Run 270907 which was placed indoor as a control. The difference between the two bioreactors were that the indoor bioreactor received constant light intensity in a continuous manner and was kept at a constant temperature (30ºC) whereas the outdoor bioreactor was illuminated by diurnal cycle and the temperature was fluctuated depending on daytime and cooling.

The comparison of the hydrogen production of this indoor reactor with the outdoor shaded bioreactor was given in Figure 6.45.

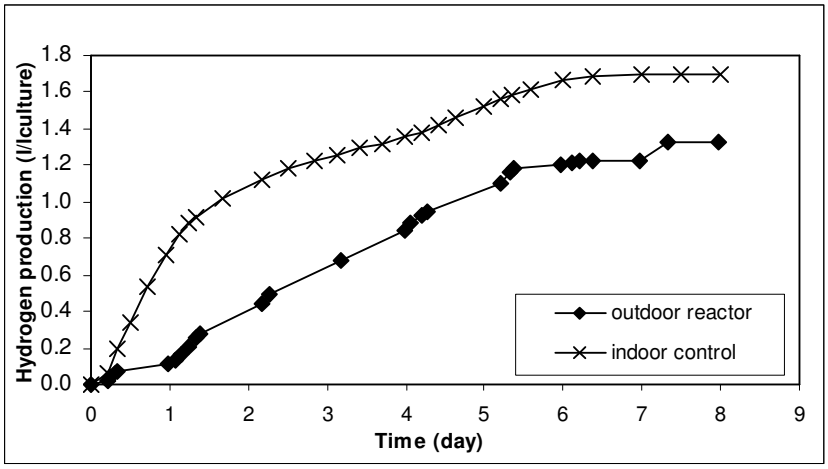


Figure 6.45 Comparison of the hydrogen production by *R. capsulatus* YO3 in indoor and outdoor bioreactors.

From Figure 6.45, it can be seen that the average hydrogen production rate and the total hydrogen produced is more in a continuously illuminated photobioreactor compared to a bioreactor which is illuminated by day-night cycle. The decrease in the hydrogen production rate is due to the stopping of hydrogen production during nights and the decrease in the total hydrogen amount is possibly due to the consumption of available substrates by the bacteria during night.

This result was strongly supported by the findings of the light-dark cycles study (Section 4.4.5), which compared the hydrogen productions in a continuously illuminated bioreactor to a bioreactor which was illuminated by 14 hours light – 10 hours dark cycle (simulation of a summer day). The results were previously given in Figure 4.20b, a reformatted version of this Figure to include only hydrogen production data is given below for comparison with the Figure 6.45:

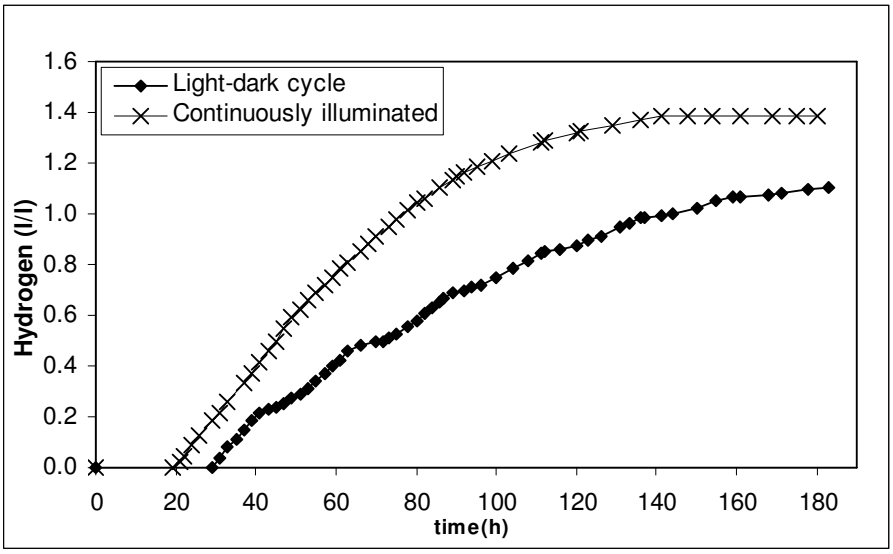


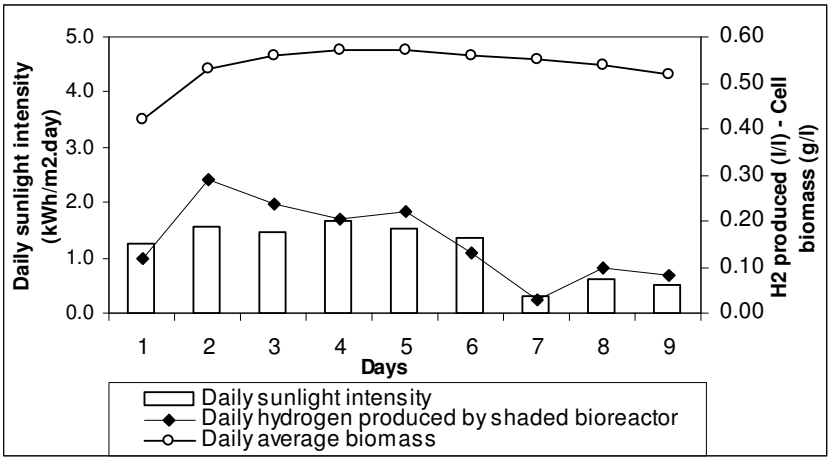
Figure 6.46 Hydrogen production in bioreactors illuminated continuously and with 14 h light - 10 h dark cycles.

It can be clearly seen that the indoor light-dark cycle study was successfully simulated the actual day-night cycle and although the bacteria strains were different, the hydrogen production patterns were quite comparable.

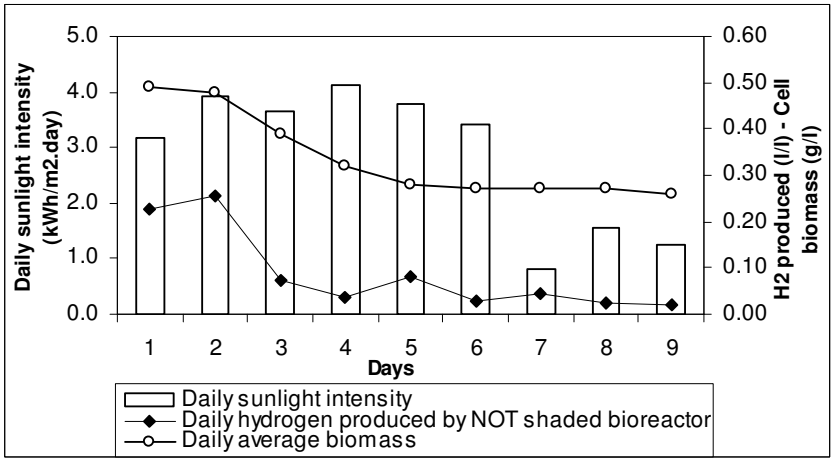
To sum up, artificial illumination during night might be considered based on the gain reported and the cost of such an installment.

6.4.3 Effect of Sunlight Intensity on Hydrogen Production

In order to show the possible relation between the daily hydrogen production, cell concentration and the daily light received by a bioreactor, the light received versus hydrogen production and cell concentration was plotted on a daily basis for the Run 270907 and given in Figure 6.47.



(a)



(b)

Figure 6.47 Daily light intensity versus daily H_2 production and average biomass concentration a) in a 60 % shaded bioreactor, b) in not-shaded bioreactor.

In case of the shaded bioreactor (Figure 6.47a), the average cell concentration was approximately same after the first day. It can be seen that the hydrogen production depends on the light received, especially the decrease in day 7 and restoration in the next day is obvious. The temperature profile of this run shows that the temperature during this batch never exceeded 35°C (Figure 6.36)

therefore the inhibition of the hydrogen production due to the temperature should not be important for this case.

In case of the not-shaded bioreactor (Figure 6.47b), it can be seen that starting from the third day, average cell concentration and hydrogen production decreased significantly. The daily received light intensity for this bioreactor was two times more compared to the shaded bioreactor during the first 6 days of the batch. Therefore this decrease may be attributed to the photoinhibition of the process after the second day. However, the temperature profile of this run shows that the temperature during this batch reached up to 40°C (Figure 6.36) which may also affect the hydrogen production negatively compared to the shaded bioreactor case. As a result, high sunlight intensity or high temperature decreased the cell concentration and hydrogen production until day 5. Yet, there is a more specific region to be considered; the average cell concentration was approximately the same after day 5, and it can be seen that the significant decrease in light energy on day 7 did not decrease hydrogen production, on the contrary, increased it. This may be an indication of photoinhibition.

To sum up, it was shown that photobiological hydrogen process is applicable in outdoor conditions. However, growth and hydrogen productivity of photosynthetic bacteria in an outdoor reactor system is strongly affected by the outdoor parameters such as temperature and sunlight intensity.

The temperature over 40ºC inhibits bacterial growth and hydrogen production, it is suggested that the maximum bioreactor temperature should not exceed 35ºC for optimum hydrogen productivity. Shading and cooling by water spraying were shown to be effective and suggested for temperature control of bioreactors.

It was shown that hup$^-$ mutant of *R. capsulatus* grows faster and have better hydrogen productivity than wild type parent in outdoor conditions. This result confirms the study of Ozturk et al (2005) who compared the indoor hydrogen production of wild type to YO3 mutant and reported that YO3 mutant was superior.

Some indication of photoinhibition of cells was observed in Figure 6.47 on the mutant YO3 strain, indoor studies under controlled light intensities may be performed to confirm these findings. Shading the bioreactors not only helps controlling the temperature but also works against photoinhibiton of the cells.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

7.1. Conclusions

R. sphaeroides grew and produced hydrogen on media containing different carbon sources; malate, acetate, propionate, lactate, pyruvate and butyrate. Highest hydrogen productivity was obtained in malate medium. Butyrate was the least efficient carbon source. Light conversion efficiency changed between 0.27 - 0.87 % and substrate conversion efficiency changed between 14 – 50 % for different organic acids tested.

Acetate did not always ended in hydrogen production by *R. sphaeroides* but biomass instead. If other carbon source(s) were added to the acetate media, stable hydrogen production was observed.

The malate and butyrate consumption rates were found to be first order whereas the first and second order consumption rate equations did not fit to acetate consumption data. In the case of substrate mixtures, it is found that the consumption rate of the main substrate significantly increased when the minor substrates were depleted.

The growth kinetics of *R. sphaeroides* in media with different organic acids were shown to fit into logistic model.

It was shown that *R. sphaeroides* grew and produced hydrogen on media containing different nitrogen sources (malate was the carbon source); glutamate, ammonium chloride, urea, ammonium dihydrogen phosphate. Glutamate was found to be the best nitrogen source for hydrogen production however urea was also suggested as a substitute for glutamate in large scale applications.

Increasing the molybdenum or nickel ion concentration in the media did not affect the hydrogen production or growth of *R. sphaeroides.* The maximum hydrogen was produced in 204µM iron containing bioreactor (10x than original medium). The hydrogen production was severely inhibited in non-iron and 100x iron containing bioreactors.

In the light effects studies, it was shown that the infrared light (750-950nm) played an essential role for the photoproduction of hydrogen and the photobioreactors should receive infrared light. It was also shown that the hydrogen production by photosynthetic bacteria stopped at dark. The outdoor photobioreactor may be illuminated during night to decrease the batch duration, increase the overall hydrogen production rate and total amount of hydrogen produced. It was concluded that the outdoor batch should be started in the morning to let bacteria grow to a threshold concentration during daytime for hydrogen production. Otherwise artificial illumination should be used for an efficient process.

The studies with *R. capsulatus* showed that the growth was severely repressed in media containing only buffer, C and N sources compared to the defined control media. Hydrogen was not produced in such a limited media.

On the other hand, *R. capsulatus* grew and produced hydrogen in any photobioreactor scale (0.1 - 0.5 - 5 - 25 L) on 40/7.5 [acetate]/[lactate] media in a more stable manner compared to *R. sphaeroides*, therefore it was chosen as a candidate bacteria for fermenter effluent studies. Lag time for *R. capsulatus* (both growth and hydrogen production) depended on acetate concentration. Best concentration for growth was 10-20 mM, but 30-50 mM was also acceptable, in case of [acetate]>60mM there was no growth.

R. capsulatus grew on fermenter effluent and produced hydrogen. Addition of vitamins enhanced hydrogen production slightly, addition of iron enhanced hydrogen production drastically.

Several improvements were made on bioreactor design: it was shown that flushing argon gas from the headspace of the bioreactors was not a must, instead the bioreactors could be filled completely to provide anaerobic conditions. On the

other hand, the hydrogen uptake by the bacteria was shown, thus, the hydrogen collection tubes should be equipped by a water trap or one-way valves to prevent hydrogen to go back into the bioreactor.

It was concluded that the photobioreactor depth should be limited depending on the light intensity received by the photobioreactor; 270 W/m^2 should be attained at the darkest point of the photobioreactor for obtaining highest hydrogen production rate.

It was also found that the hottest part of the panel photobioreactor is the top part. Therefore, in order to control the maximum temperature, temperature should be monitored from the top.

Scale up of the process did not introduce any significant problem in the range of 0.1L - 25L photobioreactor size. Lag times, total amount of produced hydrogen, pH and growth patterns were comparable at different scales.

In outdoor applications part of the study, it was found that the sunlight spectra changed during day and shifts to red during sunrise and sunset. The decrease in the light intensity caused by the clouds was between 72 - 92% compared to the sunny days, depending on the cloud density. In case of rain, the decrease in the sunlight intensity could be more than 96%.

It was shown that the bacteria did not grow in the bioreactors if the temperature exceeded 40ºC. The heat generation by bacteria caused bioreactor temperature to rise up to 55ºC (18-20ºC more compared to the air temperature) during the day, requiring the adaptation of a cooling system for the bioreactor during summer.

Shading which decreases the cooling requirement and water spraying which is an effective method for cooling were suggested for controlling temperature of the bioreactors. On the other hand, the orientation of the bioreactor affected the inner temperature of the bioreactor too. The south facing bioreactor stayed over 40ºC for a shorter period of time than the east-west facing and sun-tracking bioreactors, decreasing the cooling requirement.

In outdoor conditions, it was observed that the bacteria can withstand low temperature and light intensity and when the temperature-illumination conditions restored they started to produce hydrogen.

The outdoor studies showed that the growth and hydrogen production of the wild type *R. capsulatus* was inferior compared to the mutant YO3 strain.

Another important conclusion was that the hydrogen production depended on the light intensity received by the bioreactor, moreover some indication of photoinhibition was observed in case of *R. capsulatus* YO3 strain.

Produced gas for all the runs on defined media composed of 91-97% H_2 and 3-9% CO_2. Produced gas on fermenter effluent media contained more CO_2 compared to the gas obtained in defined media (~86% H_2 and 14% CO_2).

7.2. Recommendations

The minimal nutrient requirements of the PNS bacteria may be determined more accurately; this will decrease the cost of defined media at large scales and provide feedback for the requirement of nutrient addition in case of dark fermenter effluent/wastewater usage as substrate.

Larger scale photobioreactors may be operated in the future to prove industrial-scale operation is possible.

Continuous operation may be researched instead of batch processes, this will enable to control some parameters like cell density and pH, and to analyze of the response of the system to the changes in these parameters in real time.

The uptake hydrogenase deficient (hup^-) mutants showed a better performance than wild type strain in this study. This and other mutant strains need to be investigated further in indoor and outdoor conditions for even better performances.

Further studies need to be carried out on finding thermotolerant bacterial strains and exploring their hydrogen productivities since this will decrease the cooling requirement of the photobioreactors in outdoor applications.

REFERENCES

Akkerman, I., Janssen, M., Rocha, J., Wijffels, R.H., 2002, "Photobiological hydrogen production: photochemical efficiency and bioreactor design", Int. J. Hydrogen Energy, 27: 1195 – 1208.

Arai, T., Wakayama, T., Okana, S., Kitamura, H., 1998, "Open air hydrogen production by photosynthetic bacteria used solar energy during winter seasons in central Japan", presented in Int. Conference on Biological Hydrogen Production, Hawaii, USA.

Arik, T., 1995, "Production of hydrogen by *Rhodobacter sphaeroides* O.U. 001", MSc. Thesis in Biological Sciences, Middle East Technical University, Ankara.

Barbosa, M.J., Rocha, J.M.S., Tramper, J., Wijffels, R.H., 2001, "Acetate as a carbon source for hydrogen production by photosynthetic bacteria", Journal of Biotechnology, 85: 25–33.

Biebl, H., Pfennig, N., 1981, "Isolation of members of the family Rhodosprillaceae.", In: The prokaryotes. Editors: Starr, M.P., Stolp, H., Trüper, H.G., Balows, A., Schlegel, H.G., New York: Springer-Verlag, 267-273.

Biel, A.J., 1986, "Control of Bacteriochlorophyll Accumulation by Light in *Rhodobacter capsulatus"*, Journal of Bacteriology, 168: 655-659.

Carlozzi, P., Sacchi, A., 2001, "Biomass production and studies on *Rhodopseudomonas palustris* grown in an outdoor, temperature controlled, underwater tubular photobioreactor", J. Biotechnol., 88: 239-249.

Chen, C.Y., Lee, C.M., Chang, J.S., 2006, "Hydrogen production by indigenous photosynthetic bacterium *Rhodopseudomonas palustris* WP3–5 using optical fiber - illuminating photobioreactors", Biochemical Engineering Journal, 32: 33–42.

Clayton, R.K., 1966, "Spectroscopic analysis of bacteriochlorophylls in vitro and in vivo", Photochemistry and photobiology, 5: 669-677.

Das, D., Veziroğlu, T.N., 2001, "Hydrogen Production by Biological Processes: A Survey of Literature", Int. J. Hydrogen Energy, 26: 13-28.

El-Kahlout, K., 2002, "Effect of bacteriorhodopsin on hydrogen gas production by *Rhodobacter sphaeroides* O.U.001 in a photobioreactor", MSc. Thesis in Biotechnology, Middle East Technical University, Ankara.

Eroglu, E., 2002, "Hydrogen production from olive mill wastewater by *Rhodobacter sphaeroides* O.U. 001", MSc. Thesis in Chemical Engineering, Middle East Technical University, Ankara.

Eroglu, E., 2006, "Biological hydrogen production from olive mill wastewater and its applications to bioremediation", Ph. D. Thesis in Chemical Engineering, Middle East Technical University, Ankara.

Eroglu, E., Gündüz, U., Yücel, M., Türker, L., Eroglu, I., 2004, "Photobiological hydrogen production by using olive mill wastewater as a sole substrate source", Int. J. Hydrogen Energy, 29: 163-171.

Eroglu, I., Kadir, A., Gunduz, U., Yucel, M., Turker, L., 1999, "Substrate consumption rates for hydrogen production by *Rhodobacter sphaeroides* in a column photobioreactor", Journal of Biotechnology, 70: 103–113.

Eroglu, I., Tabanoglu, A., Gündüz, U., Eroglu, E., Yücel, M., 2007, "Hydrogen Production by *Rhodobacter sphaeroides* O.U.001 in a Flat Plate Solar Bioreactor", Int. J. Hydrogen Energy, in press.

Fang, H.H.P., Li, C., Zhang, T., 2006, "Acidophilic biohydrogen production from rice slurry", Int. J. Hydrogen Energy, 31: 683-692.

Fascetti, E., and Todini, O., 1995, "*Rhodobacter sphaeroides* RV Cultivation and Hydrogen Production in One and Two-stage Chemostat", Appl. Microbioal Biotechnology, 44: 300-305.

Fascetti, E., D'addario, E., Todini, O., Robertiello, A., 1998, "Photosynthetic hydrogen evolution with volatile organic acids derived from the fermentation of source selected municipal solid wastes", Int. J. Hydrogen Energy, 23: 753-760.

Favinger, J., Stadtwald, R., Gest, H., 1989, "*Rhodospirillum centenum*, sp. nov., a thermotolerant cyst-forming anoxygenic photosynthetic bacterium", Antonie Van Leeuwenhoek, 55: 291-296.

Fuller, R.C., 1959, "Bacterial photosynthesis subsequent to the photochemical act.", Abstracts Proc., IX Int. Botanical Congress, Montreal, Vol. II: 125.

Fuller, R.C., Smillie, R.M., Sisler, E.C., Kornberg, H.L., 1961, "Carbon metabolism in *Chromatium*", J. Biol. Chem., 236: 2140.

Gurgun, V., Kirschner, G., Pfennig, N., 1976, "Fermentation of pyruvate by seven species of phototrophic purple bacteria". Z Allg Mikrobiol, 16: 573-576.

Hallenbeck, P.C., Benemann, J.R., 2002, "Biological hydrogen production; fundamentals and limiting processes", Int. J. of Hydrogen Energy, 27: 1185 – 1193.

Hawkes, F.R., Dinsdale, R., Hawkes, D.L., Hussy, I., 2002, "Sustainable fermentative hydrogen production: challenges for process optimization", Int. J. Hydrogen Energy, 27: 1339 -1347.

Hillmer, P., Gest, H., 1977, "H_2 metabolism in the photosynthetic bacterium Rhodopseudomonas capsulata: H_2 production by growing cultures", J. Bacteriol., 129: 724-731.

Hirabayashi, H., Amakawa, M., Kamimura, Y., Shino Y., Satoh, H., Itoh, S., Tamiaki, H., 2006, "Analysis of photooxidized pigments in water-soluble chlorophyll protein complex isolated from Chenopodium album", Journal of Photochemistry and Photobiology, 183: 121–125.

He, D., Bultel, Y., Magnin, J.P., Willison, J.C., 2006, "Kinetic analysis of photosynthetic growth and photohydrogen production of two strains of *Rhodobacter Capsulatus"*, Enzyme and Microbial Technology, 38: 253–259.

Hoekema, S., Bijmans, M., Janssen, M., Tramper, J., Wijffels, R.H., 2002, "A pneumatically agitated flat-panel photobioreactor with gas re-circulation: anaerobic photoheterotrophic cultivation of a purple non-sulfur bacterium", Int. J. Hydrogen Energy, 27: 1331– 1338.

Holt, J.G., Stanley, J.T., Bryant, M.P., and Pfennig, M.P., 1984, "Bergey's Manual of Systematic Bacteriology", Williams and Wilkins, Baltimore.

Horiuchi, J.I., Shimizu, T., Tada, K., Kanno, T., Kobayashi, M., 2002, "Selective production of organic acids in anaerobic acid reactor by pH control", Bioresource Technology, 82: 209–213.

Hu, Q., Guterman, H., Richmond, A., 1996, "A flat inclined modular photobioreactor for outdoor mass cultivation of photoautotrophs", Biotechnol. Bioeng., 51: 51-60.

Hussy, I., Hawkes, F.R., Dinsdale, R., Hawkes, D.L., 2005, "Continuous fermentative hydrogen production from sucrose and sugarbeet", Int. J. Hydrogen Energy, 30: 471– 483.

Kars, G., Gündüz, U., Yücel, M.,Türker, L., Eroglu, I., 2006, "Hydrogen production and transcriptional analysis of nifD, nifKand hupS genes in *Rhodobacter sphaeroides* O.U.001 grown in media with different concentrations of molybdenum and iron" Int. J. Hydrogen Energy, 31: 1536 – 1544.

Khanal, S.K., Chen, W.H., Li, L., Sung, S., 2004, "Biological hydrogen production: effects of pH and intermediate products", Int. J. Hydrogen Energy, 29: 1123– 1131.

Khatipov, E., Miyake, M., Miyake, J., Asada, Y., 1998, "Accumulation of Poly-β-hydroxybutyrate by *Rhodobacter sphaeroides* on Various Carbon and Nitrogen Substrates", FEMS Microbiology Letters, 162: 39-45.

Kikuchi, G., Tsuiki, S., Muto A., Yamada, H., 1963, "Metabolism of carboxylic acids in non-sulfur purple bacteria under light anaerobic conditions", in "Studies on Microalgae and Photosyntetic Bacteria", special issue of Plant and Cell Physiology, Tokyo, 547-565.

Kim, I.S., Hwang, M.H., Jang, N.J., Hyun H.S., Lee S.T., 2004, "Effect of low pH on the activity of hydrogen utilizing methanogen in biohydrogen process", Int. J. Hydrogen Energy, 29: 1133 – 1140.

Kim, J.S., Ito, K., Izaki, K., Takahashi, H., 1987, "Production of molecular hydrogen by a semi-continuous outdoor culture of *Rhodopseudomonas sphaeroides*", Agric. Biol. Chem., 51: 1173-1174.

Kim, J.S., Ito, K., Takahashi, H., 1980, "The relationship between nitrogenase activity and hydrogen evolution in *Rhodopseudomonas palustris*", Agric. Biol. Chem, 44: 827–833.

Kitajima, Y., El-Shistawy, R.M.A., Ueno, Y., Otsuki, S., Miyake, J., Morimoto, M., 1998, "Analysis of compensation point of light using plane-type photosynthetic bioreactor", In: Biohydrogen, O. Zaborsky (Editor), Plenum Press, New York, 359-368.

Kojima, E., Lin, B., 2004, "Effect of Partial Shading on Photoproduction of Hydrogen by Chlorella", Journal of Bioscience and Bioengineering, 97: 317-321.

Koku, H., Eroglu, I., Gündüz, U., Yücel, M., Türker, L., 2003, "Kinetics of biological hydrogen production by the photosynthetic bacterium *Rhodobacter sphaeroides* O.U. 001". Int. Journal of Hydrogen Energy, 28: 381-388.

Koku, H., Eroglu, I., Gündüz, U., Yücel, M., Türker, L., 2002, "Aspects of the metabolism of hydrogen production by *Rhodobacter sphaeroides*", Int. J. Hydrogen Energy, 27: 1315-1329.

Koku, H., 2001, "Hydrogen metabolism and factors affecting hydrogen production in *Rhodobacter sphaeroides"*, MSc. Thesis in Chemical Engineering, Middle East Technical University, Ankara.

Kornberg, H.L., Lascelles. J., 1960, "The formation of isocitratase by the *Athiorhodaceae*", J. Gen. Microb., 23: 511.

Krahn, E., Schneider, K., Muller, A., 1996, “Comparative Characterization of H_2 Production by the Conventional Mo Nitrogenase and the Alternative "Iron-Only" Nitrogenase of *Rhodobacter capsulatus* hup- Mutants”, Appl. Biochem. Biotechnol., 70: 301-310.

Lay, J.J., Lee, Y.J., Noike, T., 1999, “Feasibility of biological hydrogen production from organic fraction of municipal solid waste”, Water Res., 33: 2579–2586.

Li, D., Chen, H., 2007, “Biological hydrogen production from steam-exploded straw by simultaneous saccharification and fermentation”, Int. J. Hydrogen Energy, 32: 1742 – 1748.

Lin, C.Y., Lay, C.H., 2005, “A nutrient formulation for fermentative hydrogen production using anaerobic sewage sludge microflora”, Int. J. Hydrogen Energy, 30: 285– 292.

Losada, M., Trebst, A.V., Ogata, S., Arnon, D.I., 1960, “Equivalence of light and adenosine triphosphate in bacterial photosynthesis”, Nature, 186: 753.

Mahakhan, P., Chobvijuk, C., Ngmjarearnwong, M., Trakulnalermsai, S., Bucke, C., Svasti, J., Kanlayakrit, W., Chitradon L., 2005, “Molecular hydrogen production by a thermotolerant Rubrivivax gelatinosus using raw cassava starch as an electron donor”, ScienceAsia, 31: 415-424.

Markov, S.A., Weaver P.F., Seibert M., 1997, “Spiral tubular bioreactors for hydrogen production by photosynthetic microorganisms: design and operation”, Appl. Biochemical Biotechnol., 63: 577-584.

Melis, M., 2002,”Green alga hydrogen production: progress, challenges and prospects”, Int. J. Hydrogen Energy, 27: 1217 – 1228.

Miyake, J., Kawamura, S., 1987, “Efficiency of light energy conversion to hydrogen by photosynthetic bacteria Rhodobacter sphaeroides”, Int. J. Hydrogen Energy, 12: 147-149.

Miyake, J., Mao, X.Y., Kawamura, S., 1984, "Photoproduction of hydrogen from glucose by a co-culture of a photosynthetic bacterium and *Clostridium butyricum*", J. Ferment. Technol, 62: 531–535.

Miyake, J., Wakayama, T., Schnackenberg, J., Arai, T., Asada Y., 1999, "Simulation of the daily sunlight illumination patternt for bacterial photo-hydrogen production", J. Biosci. Bioeng., 88: 659-663.

Modigell, M., Holle, N., 1998, "Reactor development for a biosolar hydrogen production process", Renewable Energy, 14: 421-426.

Nakada, E., Nishikata, S., Asada, Y., Miyake, J., 1998, "Light penetration and wavelength effect on photosynthetic bacteria culture for hydrogen production." In: Biohydrogen, Zaborsky OR, editor. New York: Plenum Press.

Ogbonna, J.C., Toshihiko, S., Tanaka, H., 1998, "Development of efficient large-scale photobioreactors: a key factor for practical production of biohydrogen", In: Biohydrogen, O. Zaborsky (Editor), Plenum Press, New York, 329-343.

Ogbonna, J.C., Toshihiko, S., Tanaka, H., 1999, "An integrated solar and artificial light system for internal illumination of photobioreactors", Journal of Biotechnology, 70: 289–297.

Ozturk, Y., 2005, "Characterisation of the Genetically Modified Cytochrome Systems and Their Application to Biohydrogen Production in *Rhodobacter Capsulatus*", Ph. D. Thesis in Biotechnology, Middle East Technical University, Ankara.

Payne, J., Morris, J.G., 1969, "Acetate utilisation by *R. sphaeroides*", FEBS Lett., 4: 52-54

Pellerin, N.B., and Gest, H., 1983, "Diagnostic Features of the Photosynthetic Bacterium *Rhodopseudomonas sphaeroides*", Current Microbiology, 9: 339-344.

Ramachandran, R., Menon, R.K., 1998, "An overview of industrial uses of hydrogen", Int. J. Hydrogen Energy; 23: 593-598.

Rechenberg, I., 1998, “Artificial bacterial algal symbiosis (prohect ArBAS): Sahara experiments” In: O.R. Zaborsky (Editor), Biohydrogen, Plenum Press, London, 281-294.

Ren, N., Li, J., Li, B., Wang, Y., Liu, S., 2006, “Biohydrogen production from molasses by anaerobic fermentation with a pilot-scale bioreactor system”, Int. J. Hydrogen Energy, 31: 2147 – 2157.

Richmond, A., Cheng-Wu, Z., 2001, “Optimization of a flat plate glass reactor for mass production of *Nannochloropsis* sp. Outdoors", J. Biotechnology, 85: 259-269.

Rocha, J.S., Barbosa, M.J., Wijffels, R.H., 2001, “Hydrogen Production by Photosynthetic Bacteria: Culture Media, Yields and Efficiencies”, In: J. Miyake, T. Matsunaga, A. San Pietro (Editors), Biohydrogen II - An Approach to Environmentally Acceptable Technology, Elsevier Science Ltd., UK, 3-32.

Sarı, S., 2007, Development of helical tubular reactor for hydrogen producing photosynthetic bacteria, MSc. Thesis in Biotechnology, Middle East Technical University, Ankara.

Sasaki, K., 1998, “Hydrogen and 5-aminolevulinic acid production by photosynthetic bacteria”. In: Zaborsky, O.R. (Ed.), Biohydrogen. Plenum Press, London, 133–142.

Sasikala, C. H., Ramana, C. H. V., Rao, P. R., 1995, “Regulation of simultaneous hydrogen photoproduction during growth by pH and glutamate in *Rhodobacter sphaeroides* O.U.001”, Int. J. Hydrogen Energy, 20: 123-126.

Sasikala, K., Ramana, C.V., Rao, P.R., Kovacs, K.L., 1993, "Anoxygenic Phototropic Bacteria: Physiology and Advances in Hydrogen Production Technology", Adv. Appl. Microbiol., 38: 211- 295.

Sasikala, K., Ramana, C.V., Rao, P.R., Subrahmanyam, M., 1990, “Effect of Gas Phase on the Photoproduction of Hydrogen and Substrate Conversion Efficiency in the Photosynthetic Bacterium *Rhodobacter sphaeroides* O.U.001”, Int. J. Hydrogen Energy, 15: 795-797.

Sasikala, K., Ramana, CV., Rao, PR., 1991, "Environmental regulation for optimal biomass yield and photoproduction of hydrogen by *Rhodobacter sphaeroides* O.U.001", Int. J. Hydrogen Energy, 16: 597-601.

Segers, L., Verstraete, W., 1983, "Conversion of organic acids to H_2 by Rhodospirillaceae grown with glutamate or dinitrogen as nitrogen source", Biotechnol. Bioeng., 25: 2843–2853.

Shin, H.S., Youn, J.H., Kim, S.H., 2004, "Hydrogen production from food waste in anaerobic mesophilic and thermophilic acidogenesis" Int. J. Hydrogen Energy, 29: 1355-1363.

Sierra, E., Acien, F.G., Fernandez, J.M., Garcia, J.L., Gonzalez, C., Molina, E., 2007, "Characterization of a flat plate photobioreactor for the production of microalgae", Chemical Engineering Journal, in press.

Sistrom, W.R., Griffiths, M., Stainer, R.Y., 1956, "The biology of a photosynthetic bacterium which lacks colored carotenoids", J. Cell. Comp. Physiol., 48: 61.

Speece, R.E., 1996, "Anaerobic Biotechnology for Industrial Wastewaters", Archae Press, Nashville, Tennessee.

Suludere, D., 2001, "Investigation of Polyhydroxybutyrate (PHB) Production by Photosynthetic Bacterium: *Rhodobacter sphaeroides* O.U.001", M.Sc. Thesis in Biology, Middle East Technical University, Ankara.

Tabanoglu, A., 2002, "Hydrogen production by *Rhodobacter sphaeroides* O.U.001 in a solar bioreactor", MSc. Thesis in Biotechnology, Middle East Technical University, Ankara.

Tabita, F.R., 1995, "Anoxygenic Photosynthetic Bacteria (Chapter 41)", edited by Blankenship, R.E., Madigan, M.T., Bauer, C.E., Kluwer Academic Publishers, Netherlands, 885-914.

Tao, Y., Chen, Y., Wu, Y., He, Y., Zhou, Z., 2007, "High hydrogen yield from a two-step process of dark- and photo-fermentation of sucrose", Int. J. Hydrogen Energy, 32: 200-206.

Tredici, M.R., 2004, “Mass Production of Microalgae: Photobioreactors”. In ‘Handbook of Microalgal Culture, Biotechnology and Applied Phycology”, A. Richmond (Ed.), Blackwell Scientific, 178-214.

Türkarslan, S., 1999, “Identification of hydrogenase from *Rhodobacter* species and hydrogen gas production in photobioreactors”, MSc. Thesis in Biological Sciences, Middle East Technical University, Ankara.

Ueno, Y., Kawai, T., Sato, S., Otsuka, S., Morimoto, M., 1995, “Biological production of hydrogen from cellulose by mixed anaerobic microflora”, J. Ferment Bioeng., 79: 395-397.

Ueno, Y., Sato, S., Otsuka, S., Morimoto, M, 1996, “Hydrogen production from industrial wastewater by anaerobic microflora in chemostat culture”, J. Ferment Bioeng., 82: 194-197.

Uffen, R.L., Wolfe, R.S., 1970, “Anaerobic Growth of Purple non-sulfur bacteria under Anaerobic, Dark Conditions”, J Bacteriol, 104: 462-472.

Uyar, B., Gündüz, U., Yücel, M., Türker, L., Eroğlu, I., 2007, “Effect of light intensity, wavelength and illumination protocol on hydrogen production in photobioreactors”, Int. J. Hydrogen Energy, 32: 4670– 4677.

Vignais, P.M., Colbeau, A., Willison, J.C., Jouanneau, Y., 1985, “Hydrogenase, Nitrogenase, and Hydrogen Metabolism in Photosynthetic Bacteria”, Adv. Microbial Phys., 26: 154-234.

Vignais, P.M., Billoud, B., 2007, “Occurrence, Classification, and Biological Function of Hydrogenases: An Overview”, Chem. Rev., 107: 4206-4272.

Wakayama, T., Miyake, J., 2001, “Hydrogen from biomass”, In: Miyake, J., Matsunaga, T., San Pietro A., (Editors), Biohydrogen II – An Approach to Environmentally Acceptable Technology, Elsevier Science Ltd., UK, 41-51.

Wiessner, W., 1970, “Photobiology of Microorganisms (Chapter 4)”, edited by Halldal P., John Wiley and Sons, Great Britain, 96-132.

Willison, J.C., 1988, "Pyruvate and acetate metabolism in the photosynthetic bacterium *R. capsulatus*", J. Gen. Microbiology, 134: 2429-2439.

Yetiş, M., 1999, "Photoproduction of hydrogen from wastewater of a sugar refinery by *Rhodobacter sphaeroides* O.U. 001", MSc. Thesis in Biological Sciences, Middle East Technical University, Ankara.

Yiğit, D.Ö., 1999, "Identification of by-products in hydrogen producing bacteria; *Rhodobacter sphaeroides* O.U. 001 grown in waste water of a sugar refinery, MSc. Thesis in Biotechnology, Middle East Technical University, Ankara.

Yiğit, D.Ö., Gündüz, U., Türker, L., Yücel, M., Eroğlu I., 1999, "Identification of by-products in hydrogen producing bacteria: *Rhodobacter sphaeroides* O.U. 001 grown in the wastewater of a sugar refinery", J Biotechnol., 70: 125-131.

Younesi, H., Najafpour, G., Syahidah, K., Ismail, K., Mohamed, A., Kamaruddin, A.H., 2007, "Biohydrogen production in a continuous stirred tank bioreactor from synthesis gas by anaerobic photosynthetic bacterium: *Rhodopirillum rubrum*", Bioresource Technology, in press.

Zhang, K., Kurano, N., Miyachi, S., 1999, "Outdoor culture of a cyanobacterium with a vertical flat-plate photobioreactor: effects on productivity of the reactor orientation, distance setting between the plates and culture temperature", Appl. Microbiol. Biotechnol., 52: 781-786.

Zhang, M.L., Fan, Y.T., Xing, Y., Pan, C.M., Zhang, G.S., Lay, J.J., 2007, "Enhanced biohydrogen production from cornstalk wastes with acidification pretreatment by mixed anaerobic cultures", Biomass and Bioenergy, 31: 250-254.

Zinder, S.H., 1984, "Microbiology of anaerobic conversion of organic wastes to methane: recent development", ASM New, 50: 294.

APPENDIX A

PNSB UNDER MICROSCOPE

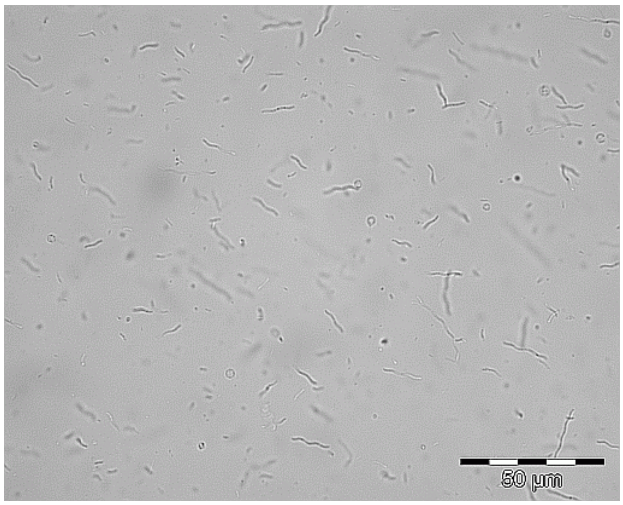


Figure A.1 *R. rubrum* under microscope

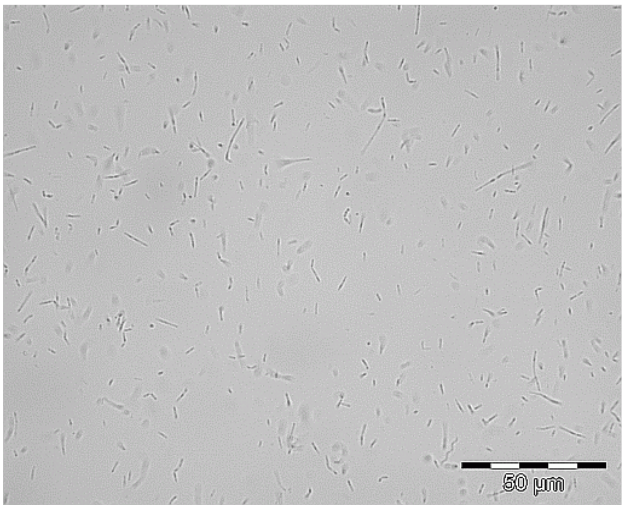


Figure A.2. *R. capsulatus* under microscope

APPENDIX B

PREPARATION AND COMPOSITION OF MEDIUM

The components of the medium given below were dissolved in 1L distilled water, pH was set to 6.5-6.8 by adding NaOH, sterilized by autoclave.

Table B.1. The composition of medium

Composition	pre-activation medium	malate medium	acetate/lactate medium
KH_2PO_4	1.0 g	1.0 g	3.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g	0.5 g	0.5 g
NaCl	0.4 g	0.4 g	-
Na-glutamate	1.8 g	0.36 g	0.36 g
$CaCl_2 \cdot 2H_2O$	0.05 g	0.05 g	0.05 g
Vitamin Solution	1 ml	1 ml	1 ml
Trace Element Solution	1 ml	1 ml	1 ml
Fe-citrate Solution	1 ml	1 ml	1 ml
Acetic acid	-	-	2.29 ml
Malic Acid	1.0 g	2.0 g	-
Lactic Acid	-	-	0.56 ml

Table B.2. The composition of trace element solution

Composition	Amount
HCl (25% v/v)	1 mL
$ZnCl_2$	70 mg
$MnCl_2 \cdot 4H_2O$	100 mg
H_3BO_3	60 mg
$CoCl_2 \cdot 6H_2O$	200 mg
$CuCl_2 \cdot 2H_2O$	20 mg
$NiCl_2 \cdot 6H_2O$	20 mg
$NaMoO_4 \cdot 2H_2O$	40 mg
H_2O	complete to 1L

Table B.3. The composition of vitamin solution

Composition	Amount
Thiamine	500 mg
Niacin (Nicotinate)	500 mg
Biotin	15 mg
H_2O	complete to 1L

Fe-citrate Solution:

Within 100 ml distilled water, 0.5 g Fe-citrate was dissolved and sterilized by autoclaving.

APPENDIX C

THE MINIMAL NUTRIENT REQUIREMENTS FOR BACTERIA

Determining the essential nutrient requirements for bacteria to grow and produce hydrogen is important for cost reduction and dark-photofermentation integration process.

In an attempt to determine the essential nutrients for the bacteria, a test was planned and conducted in which *R. capsulatus* (DSM 155) was employed as the photosynthetic bacteria.

Two glass bioreactors of 100 ml culture volume were prepared. One contained the defined medium (control) and the other one contained tap water which has some elements already (the analysis of the tap water was given in Appendix), carbon source, nitrogen source and buffer to keep pH stable. The C source was 40 mM of acetate and 7.5 mM of lactate, N source was 2 mM of glutamate.

The tap water composition was given in Table C.1. The results obtained was given in Table C.2.

Table C.1 Tap water analysis of Aachen, Germany

	Unit	**Sample 1**	**Sample 2**
pH	—	7.8 - 9.3	7.4 - 8.2
Electric conductivity	µS/cm	150 - 300	300 - 550
Ammonium (NH_4^+)	mg/l	<0.04	<0.04
Nitrite (NO_2^-)	mg/l	—	—
Nitrate (NO_3^-)	mg/l	5 - 15	5 - 15
Total iron (Fe)	mg/l	<0.02	<0.02
Mangane (Mn)	mg/l	<0.01	<0.01
Sulphate (SO_4^{2-})	mg/l	20 - 35	20 - 70
Chloride (Cl^-)	mg/l	10 - 25	10 - 40
Calcium (Ca^{2+})	mg/l	10 - 30	30 - 80
Magnesium (Mg^{2+})	mg/l	3 - 10	30 - 20
Sodium (Na^+)	mg/l	5 - 20	5 - 20
Potassium (K^+)	mg/l	1 - 2	1 - 2
Aluminium (Al)	mg/l	<0.1	<0.1
Fluoride (F^-)	mg/l	<0.2	<0.2
Arsene (As)	mg/l	—	—
Cyanide (CN^-)	mg/l	—	—

Table C.2 The results of the defined medium and minimal medium run

Results	**Bioreactor 1**	**Bioreactor 2**
final pH	6.88	7.81
growth lag time (days)	3	7
H_2 prod. lag time (days)	4	NA
final hydrogen amount (ml)	92	0

Bioreactor 1: defined media
Bioreactor 2: C source + N source + buffer in tap water
(105 ml bottles were used)

It can be clearly seen from the picture that the bacterial growth in tap water is much weaker compared to the growth in the control bioreactor.

R.capsulatus did not produce hydrogen in the tap water and the growth was very slow. The batch was stopped after 26 days, the control bioreactor produced 92 ml gas by then. GC analysis of bioreactor 1 gas at the end was made giving 94%H_2 and 6%CO_2

It can be concluded from this study that C source + N source + buffer is not enough for the bacteria, probably vitamins + iron were also needed.

APPENDIX D

THE COMMERCIALIZED GAS MONITORING PRODUCT

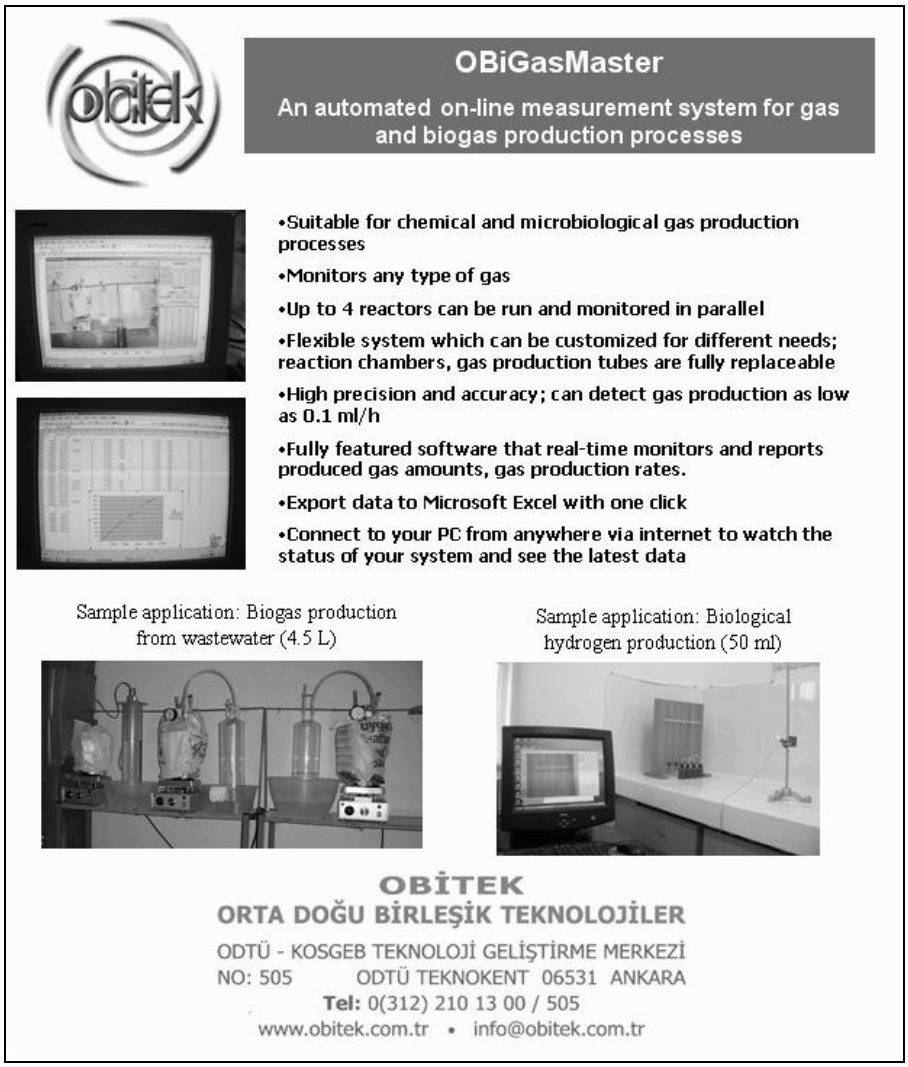


Figure D.1. Commercialized gas monitoring product

APPENDIX E

SAMPLE GAS CHROMATOGRAM

A sample gas chromatogram for a typical hydrogen production experiment is given in Figure E.1. If the bioreactors are filled completely with medium; the first largest peak is hydrogen and the smaller peak at the end belongs to the carbon dioxide gas.

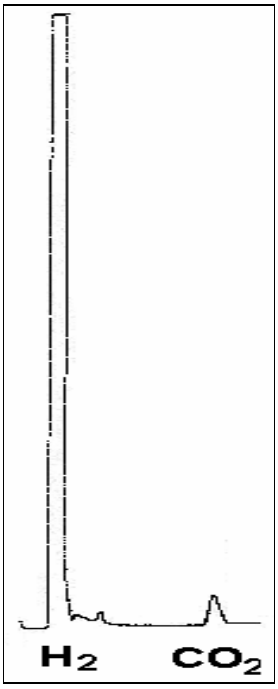


Figure E.1 Gas chromatogram of a hydrogen production experiment (taken by Hewlett-Packard 5890 Series II Gas Chromatography).

APPENDIX F

OD-DRY CELL WEIGHT CALIBRATION CURVES

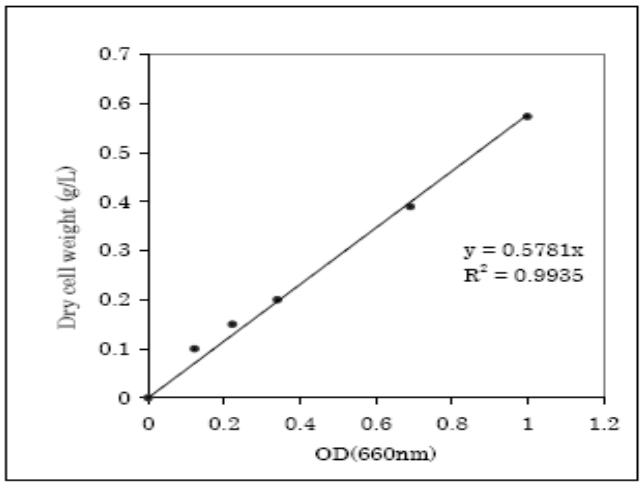


Figure F.1 Calibration curve and the regression trend line for *R. sphaeroides* OU001 dry weight versus OD_{660} (Eroglu, 2006)

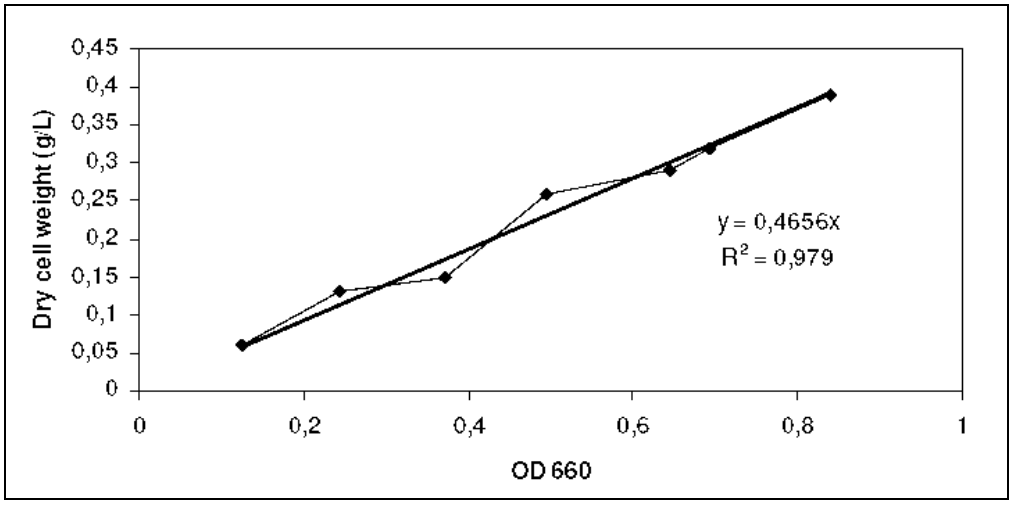


Figure F.2 Calibration curve and the regression trend line for *R. capsulatus* YO3 dry weight versus OD_{660} (Ozturk, 2005)

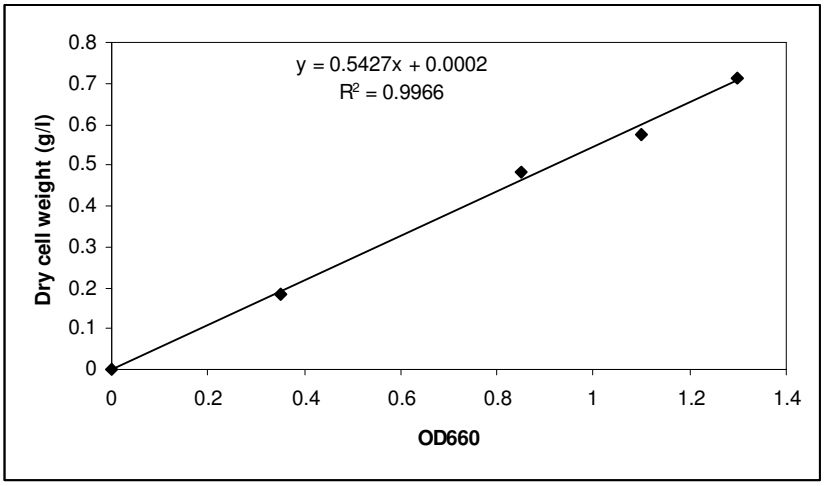


Figure F.3 Calibration curve and the regression trend line for *R. capsulatus* (DSM1710) dry weight versus OD_{660}

APPENDIX G

SAMPLE CALCULATION OF SUBSTRATE CONVERSION EFFICIENCY

This section describes the calculation procedure of the substrate conversion efficiency. For illustration, the calculations will be shown for the malate run given in Section 4.1. In this run, *R. sphaeroides* was used in a 55 ml photobioreactor. The media initially contained 15 mM malate as C source and 2 mM of Na-glutamate as N source.

The raw data required for the calculations are the total gas production and the malate consumption values. In this run, all of the malate was consumed by bacteria and the final amount of hydrogen gas obtained was 55 ml (Table 4.1).

It is assumed that hydrogen is an ideal gas and the temperature and atmospheric pressure are constant throughout the run.

First, the number of moles of hydrogen produced within the period was calculated. Using the ideal gas law, the volume of gas is converted to moles of gas. The conversion factor (P/RT) is 0.0446 mmol/ml. As a result it is found that 2.45 mmol hydrogen was produced.

Next, number of moles of malate consumed within the period was calculated. The consumed substrate amount is calculated by subtracting the final amount from the initial amount. Here, the consumed malate amount is 14.2 mM (Figure 4.1.a), which corresponds to 0.781 mmol malate in a 55ml bioreactor.

Lastly, the substrate conversion efficiency was calculated. Theoretically, 6 moles of hydrogen are produced per mole of malate (Equation 4.2). Therefore the theoretical hydrogen production is 6 times the mmol malate consumed. Using the definition given in Section 4.1, substrate conversion efficiency is found as (2.45/(0.781x6)) x100 = 52 %.

APPENDIX H

SAMPLE CALCULATIONS OF HYDROGEN PRODUCTION RATES AND YIELD

This section describes the calculation procedure of the average hydrogen production rate, hydrogen productivity and hydrogen yields. For illustration, the calculations will be shown for the malate run given in Section 4.1. In this run, *R. sphaeroides* was used in a 55 ml photobioreactor. The media initially contained 15 mM malate as C source and 2 mM of Na-glutamate as N source.

Average hydrogen production rate:

Average gas production rate is useful to determine the efficiency of the process based on the bioreactor culture volume. It is formulated as:

average gas production rate = $(V_{final}-V_{initial})/(t_{final}-t_{initial})$

where;

V is the volume of hydrogen produced ($l/l_{culture}$) and t is the time (hour)

The initial and final values are the values when hydrogen production started and when hydrogen production practically stopped, respectively. Since the gas production continues at a very slow rate towards the end of the batch Koku (2001) suggested using the values when 95% of the hydrogen was produced as final values for the calculation of average gas production rate.

In this run, the final amount of hydrogen gas obtained was 1.00 $l/l_{culture}$ as given in Table 4.1. 95% of this amount corresponds to 0.95 $l/l_{culture}$. This amount was produced at 81^{th} hour of the batch (Figure 4.1.a). On the other hand, hydrogen production started in 20^{th} hour.

Inserting the values in the definition gives:

The average gas production rate = $(0.95-0)/(81-20)=0.016$ $l/l_{culture}.h$

Hydrogen productivity:

Hydrogen productivity is formulated as:

Hydrogen productivity = $(H_{final}-H_{initial})/(t_{final}-t_{initial})$

where; H is the weight of hydrogen produced (g) and t is the time (hour).

The initial and final values are the values when hydrogen production started and when 95% of hydrogen was produced, respectively.

In this run, 95% of the final amount of hydrogen gas obtained was 0.95 $l/l_{culture}$. Multiplying with hydrogen density gives 0.95 $l/l_{culture}$ x $0.082g/l=0.078gH_2/l_{culture}$. This amount was produced at 81^{th} hour of the batch (Figure 4.1.a). On the other hand, hydrogen production started in 20^{th} hour.

Inserting the values in the definition gives:

Hydrogen productivity = $(0.078-0)/(81-20)=0.0013\ g_{hydrogen}/l_{culture}.h$

Hydrogen yield:

Hydrogen yield is useful to determine the efficiency of the process based on substrate used. It is formulated as:

Hydrogen yield = $(H_{final}-H_{initial})/(S_{final}-S_{initial})$

where; H is the weight of H_2 produced (g) and S is the weight of substrate used (g).

The initial and final values are the values when hydrogen production started and when hydrogen production stopped, respectively.

In this run, the final amount of hydrogen gas obtained was 1.00 $l/l_{culture}$ as given in Table 4.1. Multiplying with hydrogen density gives 1.00 $l/l_{culture}$ x $0.082g/l=0.082gH_2/l_{culture}$. Multiplying with culture volume (55ml) gives 0.055 $l_{culture}$ x $0.082gH_2/l_{culture}$ $=0.0045gH_2$.

The initial substrate (malate) amount was 15 mM. Multiplying with culture volume (55ml) gives (0.055 $l_{culture}$ x 0.015 mol malate/$l_{culture}$)=0.000825 mol malate. Multiplying by molar weight of malate (134.1 g/mol) gives (0.000825 mol malate x 134.1 g malate/mol malate) = 0.111 g malate. All of this malate was used during the batch (Figure 4.1.a). Inserting the values in the definition gives:

Hydrogen yield = $(0.0045-0)/(0.111-0)=0.041\ g_{hydrogen}/g_{substrate}$

APPENDIX I

CALCULATION OF IONIC STRENGTH OF THE CULTURE MEDIA

This section shows the calculation of ionic strength of the culture media which contains 40 mM of acetate as C source, 2 mM of glutamate as N source and 22 mM of buffer. pH was taken as 7.2.

Table I.1 Ionic strength in the culture media

Composition	Ion	Molarity (M)	Net Charge (Z)	Z square	M*Z2	% share in total Ionic Strength (for ion)	% share in total Ionic Strength (for compound)
KH_2PO_4	K^+	0.01100	1	1	0.0110	6	34
(pKa=7.2)	$H_2PO_4^-$	0.00550	1	1	0.0055	3	
	HPO_4^{2-}	0.00550	2	4	0.0220	13	
$MgSO_47H_2O$	Mg^{+2}	0.00203	2	4	0.0081	5	14
	SO_4^{-2}	0.00203	2	4	0.0081	5	
$CaCl_2.2H_2O$	Ca^{+2}	0.00034	2	4	0.0014	1	1
	Cl^{-1}	0.00034	1	1	0.0003	0	
NaCl	Na^{+1}	0.00689	1	1	0.0069	4	12
	Cl^{-1}	0.00689	1	1	0.0069	4	
Na-glu	Na^{+1}	0.00200	1	1	0.0020	1	4
	glu^{-1}	0.00200	1	1	0.0020	1	
Acetic acid	$C2H2O2^-$	0.03986	1	1	0.0399	23	35
(pKa=4.75)	$C2H2O2H$	0.00014	1	1	0.0001	0	
NaOH (for pH)	Na^{+1}	0.03000	1	1	0.0300	17	
	OH^{-1}	0.03000	1	1	0.0300	17	34
Fe-citrate	Fe^{+3}	0.00002	3	9	0.0002	0	0
	$C_6H_5O_7^{-3}$	0.00002	3	9	0.0002	0	
Niacin-thiamin solution	*negligible*						0
Trace element	*negligible*						0
Biotin	*negligible*						0
				Total=	**0.1746**		
				Ionic Strength (Total/2) =	**0.0873**		

APPENDIX J

SAMPLE CALCULATION OF LIGHT CONVERSION EFFICIENCY

This section describes the calculation procedure of the light conversion efficiency.

For illustration, the calculations will be shown for the malate run given in Section 4.1. In this run, *R. sphaeroides* was used in a 55 ml photobioreactor. The media initially contained 15 mM malate as C source and 2 mM of Na-glutamate as N source.

The raw data required for the calculations are the total gas production, irradiated area of the photobioreactor, hydrogen density and duration of hydrogen production values.

In this run, the total gas production was 55ml. The light intensity on the bioreactor was adjusted to be 3500 lux (200 W/m^2) and kept constant throughout the run. The hydrogen density was taken as 0.089 g/L. Duration of hydrogen production was 63 hours. Irradiated area of the bioreactor was 0.002 m^2.

Using Equation 4.8 ($\eta=[(33.61\cdot\rho_{H2}\cdot V_{H2})/(I\cdot A\cdot t)]100$), the light conversion efficiency is found as ((33.61*0.055*0.089)/(200*0.002*63))*100 = 0.66 %.

APPENDIX K

RATIO OF WEAK ACID/CONJUGATE BASE PAIR IN THE MEDIA

The ratio of weak acid/conjugate base pair in the media was determined by the pH of the media and the pK_a of the weak acid.

In the case of acetic acid, the following equilibrium establishes in the media between the acetic acid and its conjugate base (acetate):

$$CH_3COOH \longleftrightarrow CH_3COO^- + H^+ \qquad \text{(K.1)}$$

Acetic acid acetate

The pK_a of acetic acid is 4.73.

The ratio of both compounds can be found at different pHs by using Henderson-Hasselbalch equation:

$$pH=pK_a+ \log([A^-]/[HA]) \qquad \text{(K.2)}$$

The hydrogen production processes were carried out between pH 6 – 8, the calculation for this range gives the concentration of the compounds (for 40 mM total concentration):

At pH 6.0 : $[C_2H_3O_2^-]$=2.18mM and $[C_2H_4O_2]$=37.82mM

At pH 7.0 : $[C_2H_3O_2^-]$=0.23mM and $[C_2H_4O_2]$=39.77mM

At pH 8.0 : $[C_2H_3O_2^-]$=0.02mM and $[C_2H_4O_2]$=39.98mM

It is clear that most of the compound is in the base form, therefore one should use the word “acetate” when referring to the carbon source in the media instead of “acetic acid”.

Same holds for other carbon sources too, as butyric acid has pK_a=4.81 and propionic acid pK_a=4.88, both close to acetic acid.

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PUBLICATIONS

Article Published in International Journals

Uyar B., Gündüz U, Yücel M, Türker L, and Eroğlu I, "Effect of light intensity, wavelength and illumination protocol on hydrogen production in photobioreactors", International Journal of Hydrogen Energy. 32 (2007) 4670–4677

Articles Published in International Conferences:

Basar Uyar, İnci Eroğlu, Meral Yucel, Ufuk Gündüz, "Biological Hydrogen Production By *Rhodobacter Sphaeroides* O.U. 001 From Organic Acid Mixtures", World Hydrogen Technologies Convention 2007 (WHTC 2007), article published in the conference CD, 4-7 November 2007, Montecatini Terme – Italy

Basar Uyar, İnci Eroğlu, Meral Yucel, Ufuk Gündüz, Lemi Türker, "Effect of Light Intensity and Illumination Protocol on Biological Hydrogen Production by *Rhodobacter sphaeroides* O.U. 001", Proceedings of International Hydrogen Energy Congress & Exhibition (IHEC-2005), article published in the conference CD, 13-15 July, Istanbul, Turkey, 2005.

Articles Published in National Conferences

Başar Uyar, Meral Yücel, Ufuk Gündüz, Lemi Türker, İnci Eroğlu, "Fotofermentatif hidrojen gazı üretiminde farklı azot kaynaklarının etkisi ve optimizasyonu", 15. Biyoteknoloji Kongresi, 28-31 Ekim, Antalya, 2007, 334-337

Başar Uyar, İnci Eroğlu, Meral Yucel, Ufuk Gündüz, Lemi Türker, "Organik asit karışımlarından *Rhodobacter sphaeroides* O.U. 001 ile hidrojen üretimi", 3. Ulusal

Hidrojen Enerjisi Kongresi, article published in the conference CD, 17 Temmuz 2006, İstanbul

Başar Uyar, Hüseyin Avni Öktem, İnci Eroğlu, Meral Yucel, Ufuk Gündüz, Lemi Türker, "Gaz ve Biyogaz Üretim Süreçleri için İnternetten İzlenebilen Otomatik Gaz Ölçüm Sistemi ve Yazılımı (ObiGasMaster)", 3. Ulusal Hidrojen Enerjisi Kongresi, article published in the conference CD, 17 Temmuz 2006, İstanbul

Başar Uyar, Meral Yücel, Ufuk Gündüz, Lemi Türker, İnci Eroğlu, "Rhodobacter sphaeroides O.U.001'in farklı organik asitler kulanarak hidrojen üretimi", 14. Biyoteknoloji Kongresi, Congress, article published in the conference CD, 31 Ağustos – 2 Eylül, Osmangazi Universitesi, Eskişehir, 2005, 178-181

Abstracts Published in International Conferences

Nihal Doğrusöz, Ufuk Gündüz, Meral Yücel, Başar Uyar, Lemi Türker, İnci Eroğlu, "Effect of Nickel (Ni) and Iron (Fe) concentrations on expression level of uptake hydrogenase genes of *Rhodobacter sphaeroides* O.U.001", Book of Abstracts, 7th International Hydrogenases Conference, 24-29 August, The Univ. of Reading-England, 2004

Başar Uyar, İnci Eroğlu, Meral Yücel, Ufuk Gündüz, Lemi Türker, "Utilization of different organic acids by *Rhodobacter sphaeroides* O.U.001 for biological hydrogen production", Book of Abstracts, 7th International Hydrogenases Conference, 24-29 August, The Univ. of Reading-England, 2004