BIOLOGICAL HYDROGEN PRODUCTION FROM OLIVE MILL WASTEWATER AND ITS APPLICATIONS TO BIOREMEDIATION

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ABSTRACT

BIOLOGICAL HYDROGEN PRODUCTION FROM OLIVE MILL WASTEWATER AND ITS APPLICATIONS TO BIOREMEDIATION

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Hydrogen production by photosynthetic bacteria occurs under illumination in the presence of anaerobic atmosphere from the breakdown of organic substrates, which is known as photofermentation. In this study, single-stage and two-stage process development were investigated for photofermentative hydrogen production from olive mill wastewater by *Rhodobacter sphaeroides* O.U.001 within indoor and outdoor photobioreactors.

It was proven that diluted olive mill wastewater (OMW) could be utilized for photobiological hydrogen production as a sole substrate source. However, pretreatment of the system is needed to reduce the

dark color and bacteriostatic effects of OMW.

In this study, several two stage processes including pretreatment of OMW followed by photofermentation were investigated to increase the hydrogen production yields in addition to the significant remediation of OMW. Explored pretreatment methods contain chemical oxidation with ozone or Fenton's reagent, photodegradation by UV radiation, adsorption with clay or zeolite and dark fermentation with acclimated or non-acclimated sewage sludge.

Among these different two-stage processes; clay treatment method resulted the highest hydrogen production capacity. As a result of clay pretreatment, 65% of the initial color and 81% of the phenolic content were decreased. Hydrogen production capacity was 16 L_{H2}/L_{OMW} without pretreatment, while it was enhanced up to 29 L_{H2}/L_{OMW} by two-stage processes. Moreover, clay pretreatment process made it possible to utilize highly concentrated OMW (50% and 100%) media for hydrogen production and for remediation.

On the aspects of environment, treatment of OMW was achieved in the present work. The final composition of the organic pollutants in the effluent of two-stage processes was below the wastewater discharge limits. The overall results obtained throughout this study may open a new opportunity for the olive oil industry and for the biohydrogen area as a result of the effective biotransformation of OMW into hydrogen gas and valuable by-products.

Key Words: Photofermentative Hydrogen Production, Olive Mill Wastewater, Clay Pretreatment, Remediation, Two-stage Processes

ÖZ

ZEYTİN FABRİKASI ATIKSUYUNDAN BİYOLOJİK HİDROJEN ÜRETİMİ VE ATIKSU ARITIMI İÇİN BU YÖNTEMİN UYGULANABİLİRLİĞİ

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Fotofermentatif hidrojen üretimi sırasında, oksijensiz koşullarda aydınlatılan bazı tür fotosentetik bakterilerin ortamdaki organik maddeleri parçalayarak hidrojene dönüştürmeleri sonucunda gerçekleştirilir. Bu araştırmada *Rhodobacter sphaeroides* O.U.001 ile laboratuvar ve açıkhava koşullarına uygun olarak tasarlanan fotobiyoreaktörlerde zeytin atıksuyundan hidrojen üretilmesi için tek ve çift aşamalı süreçler geliştirilmiştir.

Seyreltilmiş zeytin fabrikası atıksuyunun (karasu) biyohidrojen üretimi amacıyla besiyeri olarak kullanılabileceği gösterilmiştir. Ancak

uygun bir önarıtım ile atıksuyun toksik etkilerinin ve renk gideriminin sağlanması gerekmektedir.

Bu araştırmada, hidrojen üretimini arttırmak ve aynı zamanda da karasu arıtımını gerçekleştirebilmek amacıyla; önarıtım yöntemini takip eden fotofermentasyon işlemini içeren iki aşamalı prosesler önerilmiştir. Fizikokimyasal önarıtım işlemleri olarak ozonla veya Fenton reaktifi ile kimyasal yükseltgenme, UV ışıması ile foto-indirgenme, kil veya zeolitlere adsorplanma; biyolojik önarıtım olarak ise aklimasyonlu veya aklimasyonsuz kanalizasyon çamuru ile karanlık fermentasyon süreçleri araştırılmıştır.

İncelenen önarıtım işlemleri içinde, kil üzerine adsorblanma yönteminin iki aşamlı hidrojen üretim proseslerinde en yüksek hidrojen üretim kapasitesini sağladığı saptanmıştır. Kil önarıtımı sayesinde renk %65 ve fenol %81 azaltılmıştır. Önarıtım olmaksızın, ham karasu ile yapılan fotofermentasyon işlemlerinde hidrojen üretim kapasitesi 16 L_{H2}/L_{karasu} iken, bu değer kil önarıtımı sonrasında gerçekleştirilen fotofermentasyon işlemi ile %100 oranında arttırılarak 29 L_{H2}/L_{karasu} olarak bulunmuştur. Ayrıca, kil önarıtımına tabi tutulan derişik (%50 ve %100) karasu ile hidrojen üretilebilmiştir.

İki aşamalı süreçlerin herbir basamağında karasu arıtımının gerçekleşmesi nedeniyle, fotofermentasyon sonunda elde edilen sıvı kompozisyonunun atıksu deşarj kriterlerinin oldukça altında kaldığı görülmüştür. Bu araştırmada incelenen iki aşamalı süreçler, zeytinyağı endüstrisinde atık gidermede ve biyolojik hidrojen üretiminde yeni olanaklar sunmaktadır.

Anahtar kelimeler: Fotofermentatif hidrojen üretimi, Karasu, Kil önarıtımı, Atıksu arıtımı, İki aşamalı yöntemler

To My Family

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LIST OF SYMBOLS AND ABBREVIATIONS

A : Irradiated area (m^2)

Acetyl-CoA: Acetyl Coenzyme A

ADP: Adenosine di-Phosphate

ATP: Adenosine tri-Phosphate

B_1: DO of BOD_5 blank solution before incubation, mg/L

B_2: DO of BOD_5blank solution after 5-day incubation, mg/L

bchl: Bacteriochlorophyll (bchl a: bacteriochlorophyll a)

BOD_5: Biochemical Oxygen Demand (g/L)

COD: Chemical Oxygen Demand (g/L)

D_1: DO of diluted BOD_5 sample immediately after preparation (mg/L)

D_2: DO of diluted BOD_5 sample after 5-day incubation (mg/L)

DO: Dissolved Oxygen (mg/L)

EDTA: Ethylene Diamine Tetra-Aceticacid

f : Dilution Factor

g: Gravitational acceleration constant (N/kg)

GC: Gas Chromatography

HPLC: High Performance Liquid Chromatography

HPP: Hydrogen Production Potential (L_{H2} / L_{OMW})

hup^-: Uptake hydrogenase deficient (mutant)

I: Light Intensity (W/m^2)

NAD: Nicotinamide Adenine Dinucleotide

OD: Optical Density, i.e. the spectrophotometric absorption of the culture at 660 nm

OMW: Olive Mill Wastewater

PHB: Polyhydroxybutyrate

PNS: Purple Non-Sulphur

RubisCO: Ribulose-1,5-bisphosphate-carboxylase/oxygenase

r_g: H_2 production rate based on culture volume, ($L_{H2}/L_c/h$)

r_g^1: H_2 production rate based on bacterial dry cell weight, ($L_{H2}/g/h$)

S: Ratio of seed in diluted sample to seed in blank solution during BOD_5 analysis

t_{lag}: Starting time for hydrogen evolution (h)

Δt: Duration of hydrogen production (h)

TCA: Tri-Carboxylic Acid

TOC: Total Organic Carbon (g/L)

TS: Total Solids (g/L)

UV: Ultraviolet

$V_{culture}$: Volume of culture (L_c)

V_{H2}: Volume of produced H_2 (L_{H2})

X: Cell concentration (dry weight), (g/L_c)

X_{max} : Maximum cell concentration (g/ L_c)

XRD: X-Ray Diffraction

Greek Letters:

η : Light Conversion Efficiency (%)

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

ν: Total volume of acetone for carotenoid analysis (mL)

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. Energy, economy and political crises, as well as the human health, animal and plant life have all become critical concerns. Molecular hydrogen is one of the environmentally acceptable energy carriers. Therefore, the worldwide conversion from fossil fuels to hydrogen would eliminate many environmental problems. There is an urgent need to accelerate the process of hydrogen energy utilization.

The utilization of hydrogen in fuel cells is gaining worldwide interest. Despite the "green" nature of hydrogen as a fuel, it is still primarily produced from nonrenewable sources such as natural gas and petroleum hydrocarbons via steam reforming. In order for hydrogen to become a more sustainable and a clean source of energy, it must be produced through biological processes utilizing waste materials that also offer an opportunity to utilize renewable resources.

In nature, some microorganisms such as algae, cyanobacteria, anaerobic and photosynthetic bacteria can produce hydrogen in accordance with their metabolisms. Photosynthetic bacteria are the most favorable candidates for biological hydrogen production due to their high conversion efficiency and versatility in the substrates they can utilize. Hydrogen production by photosynthetic bacteria (such as *R. sphaeroides*) occurs under illumination in the presence of anaerobic atmosphere from the breakdown of organic substrates, which is known as photofermentation process.

One way to overcome the economic limitations of biological hydrogen production by photosynthetic bacteria is to associate this process with waste treatment. Because, practical applications of photosynthetic bacteria for H_2 production cannot utilize expensive synthetic culture media like used in most of the laboratory experiments. Thus, several studies of the recent literature are focused on the utilization of cheap organic substances such as residual wastes from the food and agricultural industry, or wastewaters with high levels of organic compounds and thereby provide the advantages of both energy production and waste treatment (Yetiş *et al.*, 2002; Türkaslan *et al.*, 1998; Eroğlu *et al.*, 2004).

Olive oil industry has been playing an important role throughout the Mediterranean region, which accounts for approximately 95% of the worldwide olive oil production (Ergüder *et al.*, 2000). Olive oil extraction process from the pulp of olive fruit produce a dark colored wastewater which consists of some juice, waters of washing and cooling processes, and some vegetative solid particles. In a previous study it was proven that olive mill wastewater could be utilized for photobiological hydrogen production as a sole substrate source (Eroğlu

et al., 2004). That waste material was extremely significant because of its high production potential and severe disposal problems.

In Mediterranean countries, annual OMW production is estimated to be over 30 million m^3 (Monteoliva - Sanches *et al.*, 1996), and around 1 million m^3 of this quantity is produced in Turkey (Çengel and Okur, 2000). This dark colored wastewater is a substantial pollutant not only because of its high organic matter content and recalcitrant compounds such as polyphenols, but also its fairly high chemical oxygen demand (COD) and biochemical oxygen demand (BOD_5) values reaching up to 200 g/L and 100 g/L, respectively. As a consequence, the disposal of such a pollutant waste material becomes an important environmental problem that needs to be solved urgently. Although the photobiological H_2 production capability of diluted OMW samples were found to give satisfactory results (highest H_2 yield by 2% (v/v) OMW containing media was 14 L_{H2}/L_{OMW}), enhancement of the photobiological system is needed due to the dark color and bacteriostatic effects of OMW (Eroğlu *et al.*, 2004). OMW samples were diluted with distilled water to overcome these negative effects. Unfortunately, by dilution the quantity of wastewater was increased.

Color is the most obvious indicator of water pollution. In addition, color interferes with the transmission of sunlight into a stream and therefore reduces the photosynthetic action (Kadirvelu *et al.*, 2000). Both treatment and the color removal of water streams can be achieved with one or more of the following methods including the removal of suspended solids, colloidal particles, floating matters, colorful, and toxic compounds by adsorption, chemical degradation, photodegradation or biodegradation processes. Thus, several pretreatment techniques can be applied prior to photofermentation

process in order to get rid of the toxic characteristics of OMW and its dark color which reduces the photosynthetic efficiency. On the aspect of environment, final composition of the photofermentation process effluent might be kept under the water discharge limits, as a result of effective OMW treatment at each stage.

The main objective of the current study is to develop a suitable two stage process including pretreatment process followed by photofermentation; which will yield an efficient hydrogen production in addition to the significant remediation of OMW.

At first, a comparative study of single-stage photofermentation process (without pretreatment) should be investigated with several OMW samples from different olive-oil mills. In order to do that, detailed physico-chemical characterizations have to be done to understand the effect of OMW composition on photofermentation. Then, on the basis of hydrogen production potential, one of these OMW samples should be chosen for utilization throughout the two-stage studies.

Then, further applications were carried out by applying different two-stage processes for both remediation of the chosen OMW and the enhancement of H_2 production. For this purpose, either some physicochemical (adsorption with clay or zeolite, chemical oxidation with ozone or Fenton's reagent, and photodegradation by UV radiation) or biological (dark fermentation with sewage sludge) pretreatment processes were proposed. Those pretreatment techniques were chosen due to their confirmed remediation potentials. Photofermentation experiments were to be carried out in small scale bioreactors (55 mL) at indoor conditions, which give us the opportunity to investigate

comparative experiments by operating several parallel runs at the same time.

Attempts in two-stage processes also include extensive analyses to investigate the effect of pretreatment on photofermentation. Such a study is unique in this research area due to the simultaneous comparison of many parameters. These analyses are; total phenol, total sugar, color, specific organic acids, phenols, amino acids, sugars and alcohols in addition to the gas analysis, the bacterial concentration and pH measurements. The precision of these results were also checked by applying material balance equations on each stage of the process. The overall results obtained throughout this study may open a new opportunity for the olive oil industry and the biohydrogen area as a result of the effective utilization of OMW during these two-stage processes.

Since the primary objective is to obtain efficient hydrogen production from OMW; the other possible ways of improvement should also be investigated such as scale-up. The effect of outdoor conditions on hydrogen and valuable by-product formation (i.e., carotenoid and polyhydroxybutyrate) was also investigated in a large scale solar bioreactor, which would gain us a beneficial knowledge for further studies on the industrial applications.

In the following two chapters, a general knowledge on the olive mill wastewater (Chapter 2) and biological hydrogen production (Chapter 3) is given in details. In Chapter 2; physicochemical properties of olive mill wastewater, explanation of different remediation processes as well as several studies on the biotransformation of this waste material into valuable by-products are given. 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 two-stage biological hydrogen production processes (i.e., dark fermentation followed by photofermentation) are also given.

Chapter 4 is the part for the explaining the experimental methods. Detailed procedures for several analyzing techniques and for the pretreatment processes are given in addition to the experimental setup for the indoor and outdoor hydrogen production processes. Experimental planning is described at the end of Chapter 4.

Results are given and discussed in Chapter 5. This part includes several results such as the photofermentative hydrogen production by OMW coming from different sources; effect of different pretreatment processes on photofermentative hydrogen production; and the outdoor hydrogen production in a solar bioreactor. As a final point, conclusions and further recommendations are explained throughout Chapter 6.

CHAPTER 2

OLIVE MILL WASTEWATER

Olives are one of the main crops in terms of cultivation surfaces across the Mediterranean basin and played an important role in the diet of people as well as their economy and culture. As a result, olive oil mills are located mainly around the Mediterranean area (i.e. Spain, Italy, Greece, Turkey and Tunisia) and account for approximately 95% of the worldwide olive oil production. Annual world production of olive oil is approximately 3 000 000 tons, and Turkey is the 4th largest country in the olive oil production with a potential of nearly 600 000 tons per year (Çengel and Okur, 2000). In Turkey, olive oil processing is generally carried out by various small plants rather than by large edible oil refineries. These plants are principally located around the Mediterranean, Aegean and Marmara coastal regions where olive trees grow. Olive oil production is a seasonal operation, which starts generally in September and ends in February at the latest.

The manufacturing process of olive oil usually produces an oily phase, a solid residue and a dark colored aqueous phase, the latter of which arises from the water content of the fruit (vegetable water). The so called olive mill wastewater (OMW) consist of a mixture of this vegetable water, some soft tissues from olive pulp in a relatively stable

oil emulsion, and the process waters of the machinery cooling and fruit washings steps.

2.1. Physicochemical Properties of Olive Mill Wastewater

OMW is a considerable pollutant because of its extremely high organic matter contents; comparatively high chemical oxygen demand (COD) and 5-day biochemical oxygen demand (BOD_5) values that can reach up to 200 g/L and 100 g/L, respectively (Yeşilada *et al.*, 1992). Its phenolic content ranges between 1.5 – 4 g/L (Mulinacci *et al.*, 2001). It is a dark colored liquid containing many dissolved and suspended substances. The characteristic black-brownish color of this effluent is chemically related to polymers of low-molecular weight phenolic compounds and lignin derivatives. Color mainly depends on the age and type of oil processed and also the type of the technology used. Fresh OMW has a strong specific smell. It is slightly acidic, having pH values within a range of 3-5.

In Table 2.1, the constituents of OMW from Turkey and Spain are compared with each other. As given, OMW has high amounts of COD and BOD_5. pH values are slightly acidic. Solid content mainly comes from olive fruit residues such as olive pulp, husk, and some lignin derivatives that are hardly biodegradable. On elemental basis, OMW contains relatively high amounts of K, Ca, Na, Mg and Fe (Eroğlu, 2002).

OMW is generally composed of water (83-94%), organic matter (4-16%) and mineral salts (0.4-2.5%) (Ramos-Cormenzana *et al.*, 1996). The main organic constituents are oils (1-14%), polysaccharides (13-53%), proteins (8-16%), organic acids (3-10%), polyalcohols (3-10%) and

polyphenols (2-15%) (Cabrera *et al.*, 1996). More than 50 phenolic compounds, many alcohols, aldehydes and other low molecular weight compounds have been reported in the literature (Saiz-Jimenez *et al.*, 1987). Among these, the ones having a phenolic structure deserve a special attention because of their influence on dark color and phytotoxic effect (Gonzalez *et al.*, 1990). Mineral salts of OMW are mainly carbonates (21%), phosphates (14%), potassium (47%) and sodium (7%) (Cabrera *et al.*, 1996). The total suspended solid (TSS) is principally derived from the olive pulp and contains mainly cellulose and pectins (Hamdi, 1992). However, both quality and quantity of OMW are influenced by several factors (Vlyssides *et al.*, 1996) such as:

1. Type of production process
2. Type of olives
3. Area under cultivation
4. Use of pesticides and fertilizers
5. Climatic conditions
6. Harvesting time (i.e. stage of olive maturity)

Table 2.1 Characteristics of different olive mill wastewater samples

Characteristics	Unit	Turkey - Balıkesir (Eroğlu *et al.*, 2004)	Turkey Overall (Işıklı, 1992)	Spain Overall (Işıklı, 1992)
pH	-	4.86	4.93	5.0
Density	g/cm^3	1.02	n.a	n.a
COD	g/L	72.20	108.33	40.00
BOD_5	g/L	17.88	77.85	33.00
Total Solids	g/L	42.24	47.75	30.00
Total Suspended Solids	g/L	3.48	4.82	n.a
Total Dissolved Solids	g/L	27.38	n.a	n.a.
Color	PtCo APHA Unit	73,500	n.a	n.a
K	g/L	7.81	1.87	1.20
Ca	g/L	0.55	0.15	0.12
Na	g/L	0.41	0.07	0.05
Mg	g/L	0.28	0.08	0.05
Fe	mg/L	59.5	18.7	16.0
Zn	mg/L	9.50	0.91	0.90
Mn	mg/L	2.50	2.69	0.80
B	mg/L	2.49	5.19	n.a
Ni	mg/L	0.60	n.a	n.a
Mo	mg/L	0.34	n.a	n.a
Co	mg/L	0	n.a	n.a
Cr	mg/L	0	n.a	n.a
Pb	mg/L	0	n.a	n.a
Cu	mg/L	0	0.37	0.40
[C] / [N]	M/M	42.3	n.a	n.a

n.a: Not Available

2.2. Remediation of Olive Mill Wastewater

Only in Mediterranean countries, annual OMW production is estimated to be over 30 million m^3 (Ergüder *et al.*, 2000), and approximately 1 million m^3 of this value has been generated from

Turkey. Thus, the disposal of such a pollutant waste material becomes an important environmental problem that needs to be solved in a short time.

As a polluting source, OMW has been present for thousands of years but its effects on the environment have become more significant, since olive oil production has been amazingly increased during the last 30 years. In addition to that, olive oil mills were previously small and discharged their effluents directly on the land or underground. However, they are now much larger and the direct discharge of OMW in sewers has been prohibited for many years, because of its damaging properties. Nevertheless, illegal dumping of olive mill effluents has been practiced as a common disposal method for oil millers.

The difficulties in disposing these wastewaters are mainly related to their high organic content, excessive COD (in the range of 50-200g/L, i.e. two orders of magnitude higher than domestic sewage), the nature of some constituents that are hard to biodegrade such as phenols, and to the seasonal production which lasts approximately 3 months per year.

The treatment and disposal of OMW can be carried out directly at the mill or at centralized treatment plants. Centralized ones are generally more economical to run, even though they require more expensive transportation. There are wide ranges of technological OMW treatment studies available especially in Mediterranean countries that are trying to face the negative effects of the treatment and disposal of this waste liquid.

In general, most of the treatment processes used for high strength industrial wastewaters have also been applied to olive mill wastewater. A number of OMW treatment methods have been employed in recent years and these can be divided into physico-chemical and biological ones. The physico-chemical methods are mainly based on OMW treatment with thermal processes (i.e., evaporation in ponds and incineration), f1occulation/coagulation, ultrafiltration, and reverse osmosis. These methods are generally known to be very expensive and unable to completely solve the problem (Cabrera *et al.*, 1996).

The biological methods can be subdivided into anaerobic and aerobic processes. The former method uses microorganisms that do not require oxygen to degrade the pollutants in the effluent, while the latter needs an external source as air or pure gaseous oxygen. These methods have certain clear benefits due to the potential utilization of their byproducts such as proteins, poly-hydroxy-butyrates, poly-hydroxy-alcanoates and exopolysaccharides (Vlyssides *et al.*, 1996).

Even if most of the OMW constituents are biodegradable, some of them such as polyphenols and lipids are decomposed at reaction rates much lower than others such as sugars or short chain volatile acids. Therefore, OMW represent a decisive problem to the researcher who intends to treat them using a biological process, because efficient treatment of wastewaters requires fast biodegradation. As a consequence, many other non-biological processes have been tested on olive mill effluents. But, most physico-chemical methods give only a partial solution to the problem and should be followed by a biological treatment method. However, more sophisticated technologies like reverse osmosis or ultrafiltration have the disadvantages of high cost and low efficiency. In the recent years there has been increasing

interest in the use of electrochemical methods for the treatment of wastewaters (Israilides *et al.*, 1997). The organic and toxic pollutants present in wastewaters such as dyes and phenols are usually destroyed by a direct anodic process or by an indirect anodic oxidation via in the presence of oxidants such as hydroxyl radicals, ozone, etc. In practice, these different kinds of treatment processes are often combined since their effects differ extensively.

Color is the most obvious indicator of water pollution. The discharge of colored waste is not only damaging the aesthetic nature of receiving streams, but also it becomes toxic to the aquatic life. In addition, color interferes with the transmission of sunlight into a stream and therefore reduces photosynthetic action (Kadirvelu *et al.*, 2000). Recently a number of wastewater treatment methods have been used for color removal from wastewater by several investigators. For example, the characteristic black-brownish color of olive mill wastewater effluent is due to the slowly biodegradable compounds such as polyphenols, which are difficult to remove (Yeşilada *et al.*, 1999). An important step in the decolorization of the olive oil wastewater is the breakdown of colored polymeric phenolics to monomers.

Both treatment and the color removal of water streams can be achieved with one or more of the following methods including the removal of suspended solids, colloidal particles, floating matters, color, and toxic compounds by either adsorption, chemical degradation, photodegradation, biodegradation, ultrafiltration and, in some cases, precipitation (Pizzolato *et al.*, 2002; Oukili *et al.*, 2001). Much of the research in color removal from wastewaters has been conducted by adsorption in low-cost materials such as silicates and zeolites (Yeh and Thomas, 1995; Mansi, 1996) and by advanced oxidation processes

(H_2O_2/UV, UV/TiO_2, O_3/UV, Fe^{+2}/H_2O_2) (Huang *et al.*, 1993; Liao *et al.*, 1999).

2.2.1. Adsorption Processes

One of the powerful treatment processes for the removal of color from water at low cost is adsorption. Adsorption techniques have proven successful in removing colored organics. Several adsorbents (such as activated carbon, natural clay, bentonite, silica, cement, charcoal, etc.) are eligible for such a purpose. Activated carbon is the most popular adsorbent and has been used with great success (Erdem *et al.*, 2005). However, due to its difficulty and expense of regeneration, consumption of an alternative low-cost adsorbent is required. Adsorption takes place without significant pollutant release into the surrounding environment. It is only necessary to eliminate the consumed activated carbon. Incineration is the best way, since activated carbon is very well combustible.

Adsorption method is mainly used for the removal of suspended solid and dissolved organic pollutants in waste water. In the field of olive oil wastewater, these are coloring substances (mainly tannic acid), hard to degrade or non-biodegradable pollutants that are bactericidal or inhibiting compounds can be removed. Between 60-80% of the organic constituents of OMW can be adsorbed by geomaterials such as activated carbon (Hamdi *et al.*, 2004). Strong contamination has negative effects on the workability of the plant so that the OMW can be pretreated, for example in an activated sludge tank.

Limited plant reliability and the resulting running costs are the disadvantages of this process. Murthy *et al.* (1991) reported a high

removal (beyond 80%) of color by activated charcoal, fuller's earth, and coal ash. Shawwa *et al.* (2001) reported 90% removal of color and COD from bleached OMW by the adsorption process, using activated coke as an adsorbent.

2.2.1.1. Properties of Clay

The term clay implies a natural, earthy, fine-grained material, which is mainly composed of alumina, silica and water; and with appreciable quantities of iron, alkalies and alkaline earths (Grim, 1968). The most important property of clays is their capacity to change volume by absorbing water molecules or other polar ions into their structure which is called swelling property. Clays are divided into two groups such as *swelling* and *non-swelling* types of minerals (Işık, 2002).

All clays are dominated by silica. Their SiO_2 content cannot be used in their identification, but Al, Mg, Fe, K, Na and Ca are useful indicators of clay type. Another important criterion is *basal spacing*, which is the distance between the sheet layers of the crystal structures. Swelling type clays are smectites; while non-swelling clays are illite, glauconite, chlorite, kaolinite, sepiolite and talc (Velde, 1992).

In all clay structures, fundamental molecular units are involved and arrangements of these molecules are common. The most basic unit of a clay structure is tetrahedron in which four oxygen atoms surround each silicon atom. Another basic unit is octahedron, in which cations are coordinated with six oxygens or hydroxyl units (Işık, 2002).
Polymer layered silicate nanocomposites are hybrid materials between an organic phase (polymer) and an inorganic phase (silicate). Layered silicates (phyllosilicates) are talc, mica, vermiculte, hectorite, saponite

and montmorillonite (Zanetti *et al.*, 2000). Montmorillonite has major applications as a reinforcing agent in polymer nanocomposites.

Montmorillonite's crystalline structure consists of two-dimensional layers where a central octahedral sheet of alumina or magnesia is fused to two external silica tetrahedrons (Işık, 2002). Each layer is separated from other layers by van der Waals gaps, which are called gallery. The layer thickness, which is called d-spacing or basal spacing, is 9.6 Å. These layers form stacks with a regular van der Waals gap in between them, which is called the *interlayer*. Isomorphic substitution within the layers, such as Al^{3+} replaced by Mg^{2+}, generates negative charges. These negative charges are counterbalanced by alkali or alkaline earth cations situated in the interlayer (Alexandre and Dubois, 2002). Crystallographic structure of montmorillonite can be seen in Figure 2.1. The partial positive charge, which is formed for each cation within a gallery, makes it highly hygrophilous. Because of this reason, montmorillonite can hold a large amount of water in its galleries which neutralizes these partial charges by ion-dipole interactions (Zanetti *et al.*, 2000). The crystallographic structure of montmorillonite can be characterized by X-Ray Diffraction (XRD).

The unique characteristic of clays is their capacity to exchange cations between each of their individual layer. Montmorillonites can sorb certain cations and retain them in an exchangeable state as a characteristic feature. These intercalated cations can be exchanged by treatment of other cations in a water solution (Kwolek *et al.*, 2003). If clay is placed in a solution of a given electrolyte, an exchange occurs between the ions of the clay (X^+) and those of the electrolyte (Y^+) (Işık, 2002):

$$X.clay+Y^+ \leftrightarrows Y.clay+X^+ \qquad (2.1)$$

The cation exchange capacity (CEC) is measured as a function of the number of cations which can be measured on the clay surface once it is washed free of exchange salt solution, measured as milliequivalents per 100 g. (meq/100g) (Velde, 1992). The CEC of montmorillonite varies from 80 to 150 meq/100g.

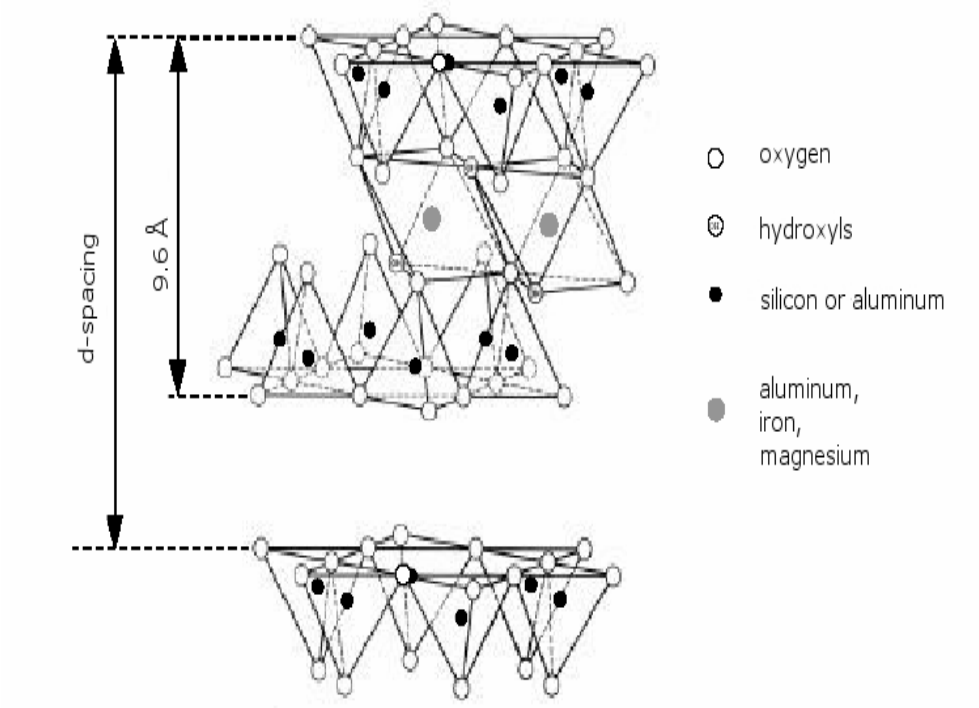


Figure 2.1 Idealized structure of montmorillonite (Kornmann, 2000)

2.2.1.2. Adsorption on Clay

The development of industrial methods for the conversion of raw materials into useful products; completely without generating pollutants such as waste gas, wastewater, and solid waste materials; is expected to be a significant conversion technique based on zero emission. There are many techniques to decrease the by-products of industrial production processes. Clay treatment is widely used in industry for separation, purification, and recovery processes.

Among several removal technologies, adsorption with natural clays has great importance due to the ease of operation, comparable low cost of application, and relatively high specific surface area. However, their sorption capacity for hydrophilic (polar) organic molecules is very low due to the hydrophilic nature of the mineral surfaces. Natural clay has a negative charge that is compensated by exchange cations (i.e., Na^{+} and Ca^{2+}) on their surfaces. These exchangeable cations are strongly hydrated in the presence of water, resulting in a hydrophilic environment at the clay surface (Akçay *et al.*, 2004)

The treatment of clays with inorganic or organic reagents increases the sorption capacity. There has been an increasing interest in the organic treatment of clays, i.e. organo-clays, whereby, the metal cations on the mineral surfaces are released in the case of change observed with the organic cations. The organic cations may also enter into ion-exchange reactions with exchangeable cations between the layers. The surface of clay may be modified and become strongly organophilic. As a result, the organo-clay complex becomes an excellent sorbent for poorly water-soluble organic contaminants.

Oukili *et al.* (2001) proposed the use of the clay as adsorbent and the hydrogen peroxide as oxidizer for the physicochemical treatment of OMW, in order to clarify water from the black-brownish color and to reduce the amounts of both polyphenols and the COD. Within their study, the bleaching led to 87% decrease of polyphenols and 66% decrease of COD, whereas the structure of clay had an effective catalytic and adsorbent effect on the removal of polyphenols. The color was observed to be changed from black-brown to lucid yellow. This remaining yellow colored liquid can be retreated by other processes,

such as biological ones to remove phenolic compounds responsible for that yellow color.

Al-Malah *et al.* (2000) observed the decolorization of OMW with 81% removal efficiency of phenolic compounds and 71% of organic matter, after post-treatment using clay. In that study, a series of treatment steps composed of settling, centrifugation and filtration was consecutively used to condition OMW. Then the filtrate was subjected to a post-treatment process with adsorption on clay molecules. The maximum adsorption capacity for the tested concentrations of clay was reached in less than 4 hours. Additionally, the researchers suggested that the adsorption of phenols and organics was reversible and mainly caused by the hydrophobic interactions.

2.2.1.3. Regeneration

In addition to the significant benefits from industrial waste utilization, the current studies for the re-usage and the disposal of clay waste is classified as follows (Toya *et al.*, 2004):

1. raw material for glass-ceramics
2. additive in the production of mortar
3. soil improvement
4. fuel
5. utilization in brick industry
6. utilization in cement furnaces
7. addition to animal feed
8. burning
9. regeneration

Among these alternatives, only a few studies are found in the literature, dealing with the regeneration of spent clay and then re-used

in environmental applications (Pollard *et al.*, 1992; Low *et al.*, 1996). Kalam and Joshi (1988) indicated that spent clay during the vegetable-oil industry was regenerated with the pretreatment of hexane extraction, and then reclaimed by an autoclave with the methods of wet oxidation or of heating in aqueous medium. Ng *et al.* (1997) indicated that spent clay was regenerated by acid and heat treatments, on the other hand Hou *et al.* (2000) investigated that spent clay was thermally regenerated in a box furnace. Boukerroui and Ouali (2000) regenerated clay by thermal processing followed by washing with a solution of hydrochloric acid.

2.2.2. Separation Processes

2.2.2.1. Flocculation and Coagulation

Precipitation means to transform a water-soluble substance into its insoluble particular form by means of a chemical reaction. Certain chemicals cause precipitation because they react with the dissolved and suspended organic compounds. After adding flocculants or coagulants, the finest suspended compounds or those dissolved in colloidal form are then transformed into a separable form.

Flouri *et al.* (1996) investigated the removal of color from OMW using aluminum sulphate, lime and hydrogen peroxide. In general terms, all these chemical substances exerted a clear decolorizing effect, yet the most effective one was hydrogen peroxide, followed by lime and alumina with the maximum color removal efficiencies of 50%; 25% and 15% respectively.

According to a remarkable study of Aktaş *et al.* (2001), after the lime treatment of different types OMW, the amount of total solids could be

dropped by 63%; polyphenols and phenols by 65% and 28%; reducing sugar, nitrogen and oil by 78%, 87% and 95%, respectively. When the structures of these OMW were considered, it was observed that the aromatics with two phenolic groups in the molecule (o-diphenols), like cathechin, were totally removed. However, molecules that contain both phenol and carboxyl groups, such as vanillic acid were adsorbed partially, and the ones having only one phenol or carboxyl group, such as tyrosol, were not affected by lime.

2.2.2.2. Distillation and Evaporation

These processes concentrate the organic and inorganic contents of OMW by the evaporation of water phase. The energy required to evaporate the water can be provided either by a man-made heat source or by a natural source such as air.

The main drawbacks of these processes are related to the post-treatment and disposal of the produced emissions. A first problem can be the disposal of the concentrated 'paste'. Its usage as an animal feed is limited by the very high concentration of potassium. Alternatively it can be burned to feed the boiler which provides the thermal energy to the distillation plant, but its combustion induces air pollution which has to be dealt with post-treatment of the gases. A second problem is that to the condensate is not made of pure water but carries away an appreciable fraction of volatile compounds found in OMW such as volatile acids and alcohols. These compounds cause the high COD of the condensate, which can reach 3 g COD/L, and necessitates the additional treatment of the distillate prior to discharge or reuse (Rozzi and Malpei, 1996). Due to that, it might be used as a pretreatment method of biological processes.

A novel evaporation process has been developed in Spain, which solves most of the above problems, by exploiting natural evaporation of OMW in ambient air. OMW is sprayed on special perforated panels with very high specific surface area (Rozzi and Malpei, 1996). A fraction of the wastewater is evaporated and carried away with the air that circulates naturally through the panels. The energy requirements of this process are limited to the recalculation pump. This system is fairly efficient, unless the weather in the selected area is very rainy (which is not likely in most Mediterranean locations).

The evaporation ponds cause serious negative environmental impacts on nearby areas due to the bad odors, insect creation, leakages, infiltrations and sludge accumulations. As the smell of olive mill effluents is normally considered quite strong and unpleasant, plants based on this process should be located at some distance from residential areas, especially those downstream to the direction of prevailing winds. In spite of these disadvantages, OMW is most frequently stored in evaporation ponds, for economic reasons.

Vitolo *et al.* (1999) observed that the evaporation of OMW sample gave result to two main products as a limpid liquid obtained by condensation of the vapors and a concentrated residue in the form of a very viscous liquid. Analysis of the condensed vapors showed the absence of polyphenols, which indicates that they remained in the concentrated residues. Both COD reduction in the condensed vapours and the concentration of the organic load in the residues of the wastewater were around 98%.

2.2.2.3. Membrane Processes

In membrane filtration, e.g. reverse osmosis or ultrafiltration; the OMW is separated in two phases. In plants based on these processes, the pressure of the wastewater is increased by a pump and the solution is then 'filtered' through a special membrane which can block the passage of organic compounds with molecular weights of the order of 10,000-100,000 Da or stop even much smaller inorganic salt molecules with molecular weight of the order of 150 Da (Rozzi and Malpei, 1996). Most biological procedures used for OMW treatment, might begin with a filtration and/or ultrafiltration step in order to eliminate the suspended solids.

Reverse osmosis has over 90% efficiency in removing organic matter, but on the other hand results in high operating cost and sludge-disposal problems (Fiestas Ros de Ursinos, 1991).

Despite optimal COD reduction, these processes are hardly suited for the treatment of olive oil wastewater. This is especially due to the very high costs, which would not be accepted in the olive oil producing countries where environmental awareness is not yet strongly developed. Additionally, process control is rather complex and requires highly qualified personnel.

These processes have been proposed several times for the treatment of OMW but their advantages in this particular application have still to be clearly demonstrated. Rozzi and Malpei (1996) claimed that the membrane processes allow one to separate valuable compounds from OMW, for example polyphenols (as an antioxidant and a flavoring agent), but not a single demonstration plant is in operation yet.

2.2.3. Chemical Oxidation Processes

The last decade has witnessed chemical oxidation processes emerging as promising alternatives to tertiary treatment, owing to their high potential to provide partial and ultimate destruction of many refractory compounds including dyestuff, halogenated and aromatic organics (İnce and Apıkyan, 2000). These processes involve the formation of highly reactive free radical species, which are far more powerful as oxidizing agents than commonly known strong oxidants like molecular oxygen and ozone. All chemical oxidation processes are based mainly on hydroxyl radical chemistry. Hydroxyl radical is the major reactive intermediate responsible for organic substrate oxidation (Chiron *et al.*, 2000).

Besides oxygen, oxidizing chemicals are also used so that even hardly degradable constituents of OMW can be destroyed or attacked. Possible oxidizing agents are ozone (0_3), hydrogen peroxide (H_20_2) or Fenton's reagent, etc. Among the oxidation studies, the oxidation by ozone has shown to be effective for the degradation of most phenolic compounds.

In terms of ecological aspects, chemical oxidation has to be regarded as critically considering the strong air emissions and the high-energy demand. Disadvantages of these processes are limited to the higher running costs of the plant. Low space requirements and normally dischargeable - treated water production are the main advantages.

2.2.3.1. Ozone as an Oxidizing Agent

Ozone is the most powerful chemical oxidant available for water treatment. It has been used as a disinfectant for drinking water treatment. Once dissolved in water, it may react with many organic compounds according to the direct reaction as molecular ozone or by

indirect reactions through formation of secondary oxidants like free radical species (Rice, 1997).

Andreozzi *et al.* (1998) investigated the possibility of coupling ozonation process with anaerobic digestion process. Their experiments showed that both total phenols and unsaturated lipids were reduced about 50% after 3 hours of ozonation but the COD was unchanged. Nevertheless, ozonated OMW exhibited in general a longer lag phase and a lower yield in methane than OMW without pretreatment. This was observed, because ozone selectively oxidized phenols and unsaturated lipids in OMW. Also, oleic acid was less inhibitory to methanogenesis than its ozonation products (especially, azelaic acid). However, it is remarkable that the ozonated OMW did not present any additional inhibition effects on acidogenic bacteria.

On the other hand, studies of Benitez *et al.* (1999) were successful when the degredation of pollutant organic materials in OMW was carried out by the combined processes of ozonation followed by an anaerobic degradation, and aerobic degradation followed by ozonation. Overall COD removal efficiency for the former case was 84.6% with 12.2% reduction in the ozonation stage and 82.5% reduction in the aerobic digestion stage. For the combined process of aerobic degradation followed by ozonation, 81.8% COD was removed at the end with 73.6% from biological treatment part and 30.3% from the ozonation. Heredia *et al.* (2000) had implied a similar conclusion that the aerobic pretreatment enhanced the latter ozone oxidation by removing most of the biodegradable organic matter, while the ozonation step degraded some of the non-biodegradable organic matter plus most of the phenolic compounds not removed before.

Beltran *et al.* (1999) investigated an integrated process in which ozonation is followed by biological oxidation. They acclimatized activated sludge units to ozonated olive mill wastewater in order to provide adaptation of microorganisms to different ambient and physical conditions. It was also stated that acclimation of biomass to the wastewater components could improve the efficiency of secondary biological treatment. Also, Narkis and Schneidel-Rotel (1980) claimed that when acclimated seed was used in the biodegradability tests, the lag period disappears and COD degradation was increased.

2.2.3.2. Fenton's Reagent as an Oxidizing Agent

Fenton's reagent is a mixture of ferrous salt and hydrogen peroxide, and produces free radicals, e.g. hydroxy radicals and hydroperoxy radicals, easily even at rather low temperatures (Guedes *et al.*, 2003). This is a very promising oxidation process wherein wastewater reacts with hydrogen peroxide (H_2O_2) in a non-pressurized reactor at low temperatures, in the presence of a low-cost catalyst (e.g. iron sulfate), yielding carbon dioxide and water and/or other oxidation products. The oxidation of many organic substances with H_2O_2 is improved by the addition of a catalyst (Fe(II) or more rarely Cu(II) or other transition metal ions) to activate the H_2O_2 molecule, leading to the formation of hydroxyl radicals (OH^-). These are the true oxidant species, which exhibit a very high oxidation potential (Legrini *et al.*, 1993).

Perez *et al.* (2002) reported that the combination of Fenton and photo-fenton reactions proved to be highly effective for the treatment of olive mill effluent. Hassan and Hawkyard (2002) studied the removal of color by combined oxidation with ozone and Fenton's reagent and stated that around 100% color removal was achieved at a pH of 4–5 in

the case of ferral (derived from natural clay sources, which contains 2% ferric sulfate and 6% aluminum sulfate) and ferric sulfate.

2.2.3.3. Hydrogen Peroxide as an Oxidizing Agent

Hydrogen peroxide is one of the most powerful oxidant readily applied to wastewater treatment in the past. It is stronger than chlorine, chlorine dioxide, and potassium permanganate, and through catalysis, H_2O_2 can be converted into hydroxyl radicals ($OH\cdot$). Hydrogen peroxide has been used in the industrial effluent treatment for detoxification of cyanide, nitrite and hypochorite, for the destruction of phenol aromatics, formaldehyde, removal of sulfite, thiosulfate and sulfide compounds (Gogate *et al.*, 2004).

2.2.4. Photocatalytic Oxidation Processes

The photocatalytic or photochemical degradation processes are gaining importance in the area of wastewater treatment, since these processes result in complete mineralization with operation at mild conditions of temperature and pressure (Gogate *et al.*, 2004). The photo-activated chemical reactions are characterized by a free radical mechanism initiated by the interaction of photons of a proper energy level with the molecules of chemical species present in the solution, with or without the presence of the catalyst. The radicals can be easily produced using UV radiation by the homogenous photochemical degradation of oxidizing compounds like hydrogen peroxide and ozone. A major advantage of the photocatalytic oxidation based processes is the possibility to effectively use sunlight or near UV light for irradiation, which should result in considerable economic savings especially for large-scale operations. Sensitizers or catalysts, e.g. ferrous ions, silver

ions, manganese ions etc., can also be used to improve the treatment efficiency of photocatalytic oxidation process.

Most of these methods require long treatment periods of high-energy photons and rarely achieve a complete degradation of the pollutant. The most common reactions observed when a contaminant is irradiated with irradiation of UV light are dechlorination, substitution of chlorine atoms by hydroxyl groups, and formation of radical species (Chiron *et al.*, 2000).

The degradation efficiency of photochemical oxidation process is greatly enhanced using either homogeneous or heterogeneous photocatalysis (Legrini *et al.*, 1993). Heterogeneous processes employ semiconductor slurries (e.g. TiO_2/UV, ZnO/UV) for catalysis, whereas homogeneous photochemistry (e.g. H_2O_2/UV, Fe^{3+}/UV) is used in a single-phase system. Photochemical oxidation processes are light induced reactions, mainly oxidations that rely on the generation of hydroxyl radicals by the combination with added oxidants or semiconductors.

The photocatalytic degradation of protocatechuic acid, a biorecalcitrant polyphenolic compound typically found in olive processing and wine distillery wastewaters, was investigated in aqueous heterogeneous solutions containing semiconductor powders (TiO_2, ZnO) as photocatalysts, both in the presence of artificial and natural illumination (Poulios *et al.*, 1999). It was observed that ZnO is more efficient as a photocatalyst, both in respect of degradation (100%) as well as mineralization (90%) after 3 h of light exposure. In the presence of TiO_2, 80% of the initial acid concentration and 60% of the initial mineral concentration was removed. The combination of TiO_2 catalysts

with H_2O_2 enhanced the photodecomposition rate and the percentage of mineralization (95%).

2.2.5. Electrolytic Oxidation Processes

This method is based on the electrolytic oxidation of OMW constituents, using especially titanium/platinum for the anode and stainless steel for the cathode.

Previously, graphite was frequently used as an anode during this electrochemical treatment as it was relatively economical and gave satisfactory results (Szpyrkowicz *et al.*, 1995). Titanium electrodes covered with very thin layers of electrodeposited noble metals have recently been used (Vigo *et al.*, 1988). Apart from titanium; ruthenium (Murphy, 1992) or rhodium (Vigo *et al.*, 1988) are also used as electrocatalysts for electro-oxidation of pollutants present in OMW, which are difficult to eliminate biologically such as phenols. Previous investigations have established that to obtain a high degree of efficiency, an anode of titanium covered with platinum should be used (Naumczyk *et al.*, 1996; Szpyrkowicz *et al.*, 1995).

However, this method is still in experimental stage. According to the laboratory results of Israilides *et al.* (1997), after ten hours of electrolysis the organic load was reduced by 93 % in terms of COD (chemical oxygen demand) and by 80.4 % in terms of TOC (total organic carbon).

The greatest disadvantage of this method is its high energy consumption. However, it can be applied as an oxidation stage prior to the biological treatment of OMW.

2.2.6. Combustion and Incineration Processes

In general, incineration process is expensive, and complicated by high-energy demand and emission of toxic air pollutants. The foot cake produced by the traditional system can be treated with hexane after drying. A residual oil is extracted and called olive kernel oil. The rejection of this extraction is named as the exhaust foot cake (EFC). This by-product is used as a combustible material. However the combustion of EFC is generally ineffective, because its incomplete combustion emits a strong smoke rich in carbon and carbonmonoxide (Masghouni *et al.*, 2000).

2.3. Bioremediation of Olive Mill Wastewater

Most of the treatment methods are focused on bioremediation, as a means of reducing the polluting effect of OMW by the help of microorganisms. However, bioremediation of OMW presents severe difficulties because phenolics and certain aromatic compounds are very phytotoxic and are held responsible for the strong antimicrobial properties and the recalcitrant black color of the wastewater. The research on OMW treatment is particularly focused on the degradation of phenolic compounds, since their breakdown is considered as the limiting step in the bioremediation of OMW.

2.3.1. Aerobic Biological Processes

In aerobic biological wastewater treatment plants, aerobic microorganisms degrade a fraction of the pollutants in the effluent by oxidizing them with oxygen that is provided by an external source (either as air or pure oxygen). These microorganisms use most of the remaining fraction of the pollutants to produce new cells (termed biomass or sludge), which have to be removed from the water.

Aerobic processes are usually exploited to remove dissolved or colloidal pollutants from wastewaters at low concentrations, usually in the order of 1 g COD/L. However, the high concentration of OMW makes it unsuitable for direct treatment by aerobic biological treatments. Besides, the aerobic treatment of concentrated wastewaters yields huge volumes of excess secondary sludge. It is also difficult to use aerobic processes to reach the required removal efficiency for pollutants such as polyphenols, which are toxic to many microorganisms. In order to overcome this problem, dilution of the wastewater would be necessary prior to aerobic treatment of the raw waste (Tsonis and Grigoropoulos, 1993). For instance, Balice *et al.* (1988) diluted the OMW samples by 70 times with tap water before aerobic activated sludge treatment.

The oldest method for OMW treatment is the use of aerated-ponds. It has been developed from the discharge of OMW into natural stagnant waters. This method includes the sedimentation process and the biodegradation in which aerobic as well as anaerobic metabolism occur, in addition to photosynthesis. All these four major processes occur simultaneously, evidently in different zones of a pond. Rather low COD reduction rates and high space requirements are the disadvantages of this method, but the costs are very low compared to the other aerobic processes. But, it is not practical due to the creation of ground water pollution, bad odor and flies (Cabrera *et al.*, 1996).

Repetitive addition of OMW to soil under aerobic conditions leads progressively to its enrichment with dinitrogen fixers, the activity of which is beneficial to soil fertility. The microbial consortium that develops in soil is dominated mostly by members of *Azotobacter* (Balis *et al.*, 1996). In a study (Piperidou *et al.*, 2000) where *Azotobacter*

vinelandii was used to remediate the OMW in a biowheel reactor to be further used as a biofertilizer, up to 96% COD removal yields could be achieved after 7 days of treatment.

Fadil *et al.* (2003) studied growth and polyphenol biodegradation of OMW by three microorganisms namely *Geotrichum sp.*, *Aspergillus sp.* and *Candida tropicalis*. These three microorganisms were detected for their tolerance to the polyphenols. Average COD removals were 55%, 53% and 63% in OMW samples fermented with *Geotrichum sp.*, *Aspergillus sp.* and *Candida tropicalis*, respectively. Maximum removal of polyphenol was obtained as 47% (*Geotrichum sp.*), 44% (*Aspergillus sp.*) and 52% (*C.tropicalis*), in addition to the significant removal of color.

In a study of Assas *et al.* (2002), 75% color removal was obtained by *Geotrichum candidum* in an aerobic batch reactor. *G.candidum* growth on fresh OMW decreased pH and reduced COD by 65% removal. On the other hand, *G.candidum* growth was inhibited while using stored-OMW samples and resulted lower COD reduction (25%) with no decolorization since phenol polymerization was amplified by the increased pH and oxygen. It is also known that storage period results the auto-oxidation and subsequent polymerization of phenolic compounds and tannins. This give rise to darker phenolic compounds which are not readily biodegradable. In contrast, simple phenolics and tannins are highly toxic but biodegradable (Hamdi, 1992).

OMW is usually diluted before biological treatments. In order to overcome this requirement, Robles *et al.* (2000) isolated seven strains of *Penicillum* from OMW disposal ponds and tested for biomass production and degradation of undiluted OMW. Best results were

obtained by using strain P4, which formed 21.5 gram dry weight of biomass per liter of undiluted OMW, after 20 days of cultivation. Additionally, COD and phenolic contents of OMW were reduced by 60-75% and 50-70%, respectively.

The aerobic treatment of OMW by fungi was demonstrated to be a promising way to reduce its toxicity and dark color (Hamdi *et al.*, 1991). Unfortunately, aerobic biodegradation is hindered by auto-oxidation and polymerization of tannins and polyphenolics of high molecular weight. Moreover, high molecular weight polyphenols of OMW adsorb strongly to mycelia by hydrogen bounding between polyphenolics and proteins or by coagulation. The absorption of polyphenols on cells and extracellular enzymes, which seems to be the limiting factor in their biodegradation, has not been studied. A system that would reduce the redox potential and then avoid the polymerization and the adsorption of polyphenols on proteins would certainly be of interest (Lamia and Moktar, 2003).

Vinciguerra *et al.* (1995) had observed a highly significant correlation between decolorization, total organic carbon and total phenols. They experimented OMW degradation in agitated liquid cultures of the white-rot fungus *Lentinus edodes*. About 45% of decoloration and 75% of total organic carbon reduction were achieved within 4 days. Over the same period, the content of total phenols was reduced by 66%.

Scioli and Vollardo (1997) examined the growth of the yeast *Yarrowia lipolytica* on OMW. Their results showed that the yeast was capable of reducing the COD level by 80% within 24 h, when grown in a 3.5 L fermentor. This produced a useful biomass of 22.45 g/L and the enzyme lipase.

Yeşilada *et al.* (1999) observed high phenol, COD and color removal efficiencies with various types of white-rot fungi, proportional to the biomass production in OMW. Up to 93% of the phenols, 70% COD and 80% color reduction were detected at the end of this aerobic treatment process. In addition to the bioremediation, high laccase enzyme activity was obtained in the fungi living inside the OMW culture. This suggests the possibility of using this effluent to improve the production of such biotechnologically important enzymes.

2.3.2. Anaerobic Biological Processes

In the last decade, most of the research conducted on OMW treatment has been focused on the usage and the development of anaerobic methods and bioreactors that can remove efficiently the high organic load as well as reduce the toxicity of OMW.

Anaerobic digestion process is carried out in airtight vessels by several microorganisms (anaerobic mixed cultures), which do not require oxygen to decompose organic compounds. The organic content of the mill wastewater with high concentrations of soluble, colloidal and suspended matter make anaerobic digestion the first choice for the treatment of either the raw material or the waste following a solid separation step. Furthermore, production of much less biomass and valuable end product formations may balance the associated treatment costs. In addition, the seasonal production of OMW (3 to 4 months out of the year) is not a disadvantage for anaerobic treatment because the decay rates of anaerobic microorganisms are rather low and a digester can be easily restarted even after several months of the mill shutdown (Ubay and Öztürk, 1997). For such kind of reasons, anaerobic treatment of OMW has been the subject of several studies.

Even though anaerobic treatment techniques are considered to be feasible for the treatments of OMW, several difficulties are also present (Rozzi and Malpei, 1996). It should not be overlooked that the overall character and composition of the wastewater would suggest some inhibitory effects which are possibly imposed by high COD values and the presence of toxic substances. Also growth rates of anaerobic microorganisms are appreciably lower than the rates of aerobic ones and the metabolic degradation pathways require several specific microbial populations, which make process control of anaerobic digestion more delicate than aerobic ones.

Anaerobic lagooning has been used for pollution control and OMW disposal as fertilizer after solar drying (Fiestas Ros de Ursinos and Borja-Padilla, 1996). COD removal efficiencies, ranging from 20-30% to 75-80%, have been obtained after 2-4 months of lagooning (Balice *et al.*, 1988). Nevertheless, there has been no attempt to recover methane gas from OMW treatment ponds, which operate under anaerobic conditions.

The anaerobic contact process, which can also be defined as the anaerobic digester system, has been tested by several researchers. These studies refer to the digestion of more or less concentrated OMW ranging from 15 to 70 g COD/L in laboratory and pilot scale digesters, and COD removal efficiencies up to of 80-85% were obtained (Fiestas Ros de Ursinos and Borja-Padilla, 1996).

Anaerobic filters are vessels filled with natural or synthetic media, which are colonized by bacteria to form a fixed biofilm. They can operate as upflow or downflow reactors, and most experimental tests on OMW have been performed on the former. The main advantages of

anaerobic filters are that they require very little process control and that they can withstand high temporary overloads (Rozzi and Malpei, 1996). Marques (2001) studied the anaerobic digestion of OMW plus piggery effluent in an up-flow anaerobic filter type, and observed a COD removal of 70-80% and produced 1-3 $m^3/m^3/d$ of biogas (65-75% CH_4), and a more stabilized effluent with a neutral pH.

In the study of Borja *et al.* (1994) a quicker steady state was reached with the anaerobic contact process than an anaerobic filter, during the anaerobic treatment of OMW with *Geotrichum candidum*. The daily methane production and COD removal recorded with the anaerobic filter were greater than those obtained in the anaerobic contact reactor. Additionally, the anaerobic filter yielded a biogas with a higher percentage of methane and effluent with a lower volatile fatty acid (such as acetic acid, propionic acid and butyric acid) and volatile solid content than the anaerobic contact reactor.

In most anaerobic treatment processes, alkaline chemicals (i.e., sodium bicarbonate, soda and lime) as well as urea are added to the wastewater in the laboratory scale reactors. Because OMW is found to be deficient in nitrogen, while in some pilot plants (Rozzi and Malpei, 1996) the effluent is diluted with settled sewage that provides the missing nitrogen.

Ergüder *et al.* (2000) treated OMW anaerobically, with additional basal media including various types of micro and macro-nutrients. They observed 85-93% COD removal efficiencies in addition to methane gas formation (58 L CH_4 / 1 L OMW at which 413 mL of methane gas was produced from the degredation of 1 g of COD found in OMW).

Lamia and Moktar (2003) investigated the effects of *Lactobacillus plantarum* growth on the decolorization and biodegradation of phenolic compounds. The growth of *L. plantarum* in 10 times diluted OMW led to the degredation of phenolic compounds (46%), with a resultant decolorization (58%) and COD removal (55%). Removal of phenolic compounds was associated with depolymerization, their partial adsorption on the cells and biodegradation of certain simple phenolic compounds.

Ettayebi *et al.* (2003) used *Candida tropicalis* YMEC14 as an extremophile strain to design an anerobic biotreatment process for the treatment of OMW. The process was enhanced by directing yeast methabolism towards biodegradation pathways using hexadecane as co-metabolite and by immobilizing yeast cells in calcium alginate beads. Under immobilization conditions, *C.tropicalis* YMEC14 grown at 40 °C in OMW supplemented with hexadecane resulted around 70% COD and monophenol reduction, as well as 55% polyphenol depletion after a 24 h fermentation cycle. They also pointed out that the black color of OMW become yellow-brown and brighter as the strains grew.

Eroğlu *et al.* (2004) observed 35% COD and 60% phenol (meta and orto substitutions)and 58% BOD_5 removal as a result of photosynthetic hydrogen production by *Rhodobacter sphaeroides* O.U. 001 with diluted OMW (2% v/v) containing media.

2.4. Valuable Products Obtained from Olive Mill Wastewater

In addition to the treatment purposes, some studies were also focused on the reutilization of this waste material to convert it into some valuable products such as hydrogen gas, methane gas, biodegradable

polymers, activated carbons, liquid fertilizer, and etc. Most of these transformation processes have been accomplished by bioconversion (biotransformation) studies that require microorganisms with enzyme systems capable of metabolizing a particular culture media. Some literature studies, transforming OMW into valuable products are given in Figure 2.2.

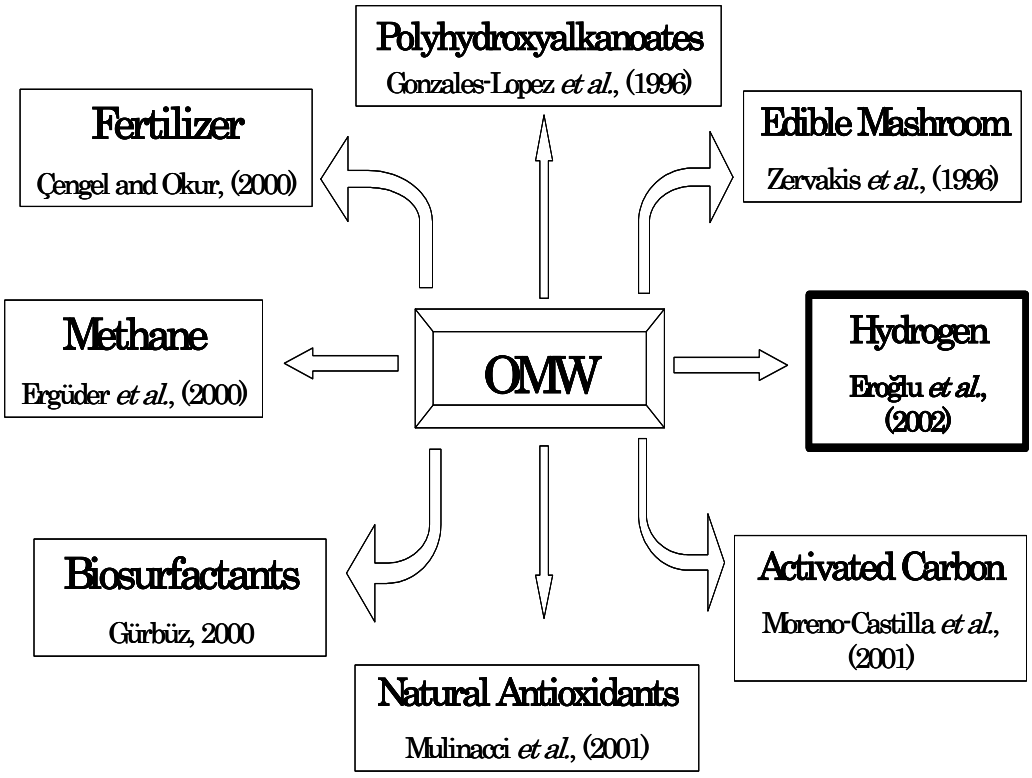


Figure 2.2 From OMW into valuable products

Lopez and Ramos-Cormenzana (1996) used OMW as a sole substrate for the production of the extracellular polysaccharide xanthan, by *Xanthomonas campestris*. Because of its special rheological properties, this biopolymer is widely used in food, cosmetics, pharmaceuticals, paper, paint, textiles, and adhesives. Growth and xanthan production on dilute OMW as a sole source of nutrients were obtained at OMW

concentrations below 60%, yielding a maximal xanthan production of 4.4 g/L at 30-40% OMW concentration. Addition of nitrogen and salts led to the significant increase in xanthan formation with a maximum of 7.7 g/L.

Pollulan, used in food and pharmaceutical industries, is another extracellular polysaccharide produced as a result of OMW fermentation(Ramos-Cormenzana *et al.*, 1996).

Çengel and Okur (2000) used OMW as an organic fertilizer on agricultural lands, since OMW contains significant amounts of organic matter (3.5-15%), K and P; and can easily decompose in soils. In their study, OMW obtained from İzmir (in Aegean Region of Turkey) were applied to three different soils with different textures (sandy, loamy and clay). Results proved that application of wastewater decreased soil pH by 0.42 units in loamy soil, 0.05 units in clay soil, but increased 0.32 units in sandy soil. Total nitrogen content increased between 0.07 to 1.47% in all soil samples after the application. In addition, OMW stimulated the growth of most organisms (especially aerobic cellulose decomposing bacteria) in sandy and clay soils.

In the study of Balis *et al.* (1996), OMW was utilized because of its low content in nitrogenous organic components and richness in carbon sources. These properties offer a highly favorable environment for the growth of free-living dinitrogen fixing bacteria (i.e. *Azotobacter vinelandii*). Repetitive addition of OMW to soil under aerobic conditions resulted an increase in the number of dinitrogen fixers, the activity of which is beneficial to soil fertility.

The investigations of Zervakis *et al.* (1996) were aiming to examine whether OMW could be exploited for the cultivation of edible mushrooms of the genus *Pleurotus*. The optimal concentration of OMW was found to lie within 25-50% range for *Pleurotus* mycelial growth, which was assessed through the measurements of the biomass produced in liquid nutrient media. Furthermore, it was suggested to dilute the spent OMW substrate with water and spread into soil as a natural herbicide for enhancing nutritive properties.

Another important by-product can be classified as polyhydroxyalkanoates (PHA), which are reserve polyesters and accumulated as intracellular inclusions in a variety of bacteria including *R. sphaeroides*. Of these biopolymers, poly-β-hydroxybutyrate (PHB) is the most common type. Thus, Gonzales-Lopez *et al.* (1996) described the production of PHA by *Azotobacter chroococcum* strain H23 when grown in an ammonium supplemented OMW media. As a result; PHA (homo- and copolymers) were formed up to 50% of the cell dry weight after 24 hours. Their production resembles hydrogen production as they usually include an excess of carbon source or limitation of a single nutrient such as nitrogen, oxygen or phosphate.

In the study of Ribera *et al.* (2001), the nutritionally versatile *Pseudomonas putida* were shown to grow in OMW. The transformation with the plasmid pSK2665, harboring *Alcaligenes eutrophus* genes needed for the synthesis of PHB; allowed *Pseudomonas* strain to grow in high concentration of OMW while accumulating biodegradable thermoplastics.

Gürbüz (2000) used OMW as a raw material to obtain biosurfactant through fermentation processes. *Torulopsis bombicola* was used as the

microorganism. Fermentation medium was containing OMW (10%), sesame oil (59%), urea (1%), glucose (10%) and the yeast (10%). In that study, three different types of glycolipids were observed by a thin layer chromatography, Glycolipids are the main group of biosurfactants, and commercially utilized in personal hygiene products such as soaps and detergents.

OMW was also used as a raw material to produce activated carbons by both chemical and physical activation methods (Moreno-Castilla *et al.*, 2001). The physical activation method included the carbonization of the raw material and the subsequent activation at high temperature in a carbondioxide or steam atmosphere. In the first case, KOH and H_3PO_4 were utilized as activating agents, and in the second case CO_2 at 840 ºC was used at different periods of time. Results indicated that the chemical activation with KOH at 800 ºC in an inert atmosphere yielded activated carbons with better quality, which had much lower ash content, higher nitrogen surface area and much better developed porosity.

Olive oil and its extraction by-products (i.e., OMW), exert potential biological activities including antioxidant and free radical scavenging actions. In fact, the possible protective effects of hydroxytyrosol on hydrogen peroxide-induced oxidative alterations were investigated in human erythrocytes throughout the study of Manna *et al.* (1999). They gave experimental supports to the hypothesis of a protective role played by antioxidant components of olive oil on oxidative damage in human systems. Since there is a growing interest in novel sources of natural antioxidants in the onset of several human diseases, oxidative degradation of food, and other goods as such as cosmetics; many researchers have been trying to investigate for a possible recovery of

polyphenols from OMW. For this purpose, Mulinacci *et al.* (2001) had analyzed different types of OMW samples from different Mediterranean countries such as Portugal, France, Spain and Italy. Their results demonstrated that Italian commercial OMW were the richest in total polyphenolic compounds with amounts between 1.5 and 4 mg/mL of wastewater.

Ergüder *et al.* (2000) anaerobically treated OMW, by mixing it with additional basal media including various types of micro and macro-nutrients. They observed that 1 L OMW resulted in the production of approximately 58 L of methane gas (413 mL CH_4 / g COD).

Eroğlu *et al.* (2004) used olive mill wastewater (OMW) as a sole substrate for the production of hydrogen gas by *Rhodobacter sphaeroides* O.U. 001. A maximum hydrogen yield of 13.9 ml H_2/ml OMW was obtained at 2% OMW containing diluted media. In addition to hydrogen production, the maximum yield of polyhydroxybutyrate accumulation (0.12 g/L OMW) and carotenoid pigment formation (0.10 g/L OMW) was also observed at 1% OMW containing media.

CHAPTER 3

BIOLOGICAL HYDROGEN PRODUCTION

Hydrogen is a sustainable energy system in which it is produced from available sources and used in every application where fossil fuels are being used in transportation, residential, commercial and industrial sectors, and for electricity generation. It is the fuel of the future, mainly due to its high conversion efficiency, recyclability and nonpolluting nature, yielding only water after combustion. The ultimate goal for conversion to the "Hydrogen Era" is the substitution of clean hydrogen for the present fossil fuels (Das and Veziroğlu, 2001).

In many respects, hydrogen is the ideal energy carrier. It has the highest energy to mass ratio of any known fuel (Nath and Das, 2003). 1 kg of hydrogen contains the same amount of energy with 2.1 kg of natural gas or 2.8 kg of gasoline. Consumption of hydrogen produces just water.

The future energy economy will have an important role for hydrogen as a clean energy source for use in vehicles and for decentralized electricity generation in stationary fuel cell systems. In fuel cells, hydrogen can be efficiently converted to electricity, producing only water as a waste product, thus drastically reducing CO_x, NO_x, particulate and other emissions that accompany the use of fossil fuels.

A crucial feature of fuel cell technology is that highly efficient electricity generation is feasible at all system scales in contrast to other technologies which show a strong drop in efficiency with diminishing scale. This allows the application of fuel cells in vehicles and in decentralized electricity production for industry, for public distribution at the city region level and even for smaller scaled personal uses such as mobile phones, laptops and etc. Broad implementation requires the development of cost effective fuel cell technology, hydrogen storage systems and related infrastructure (Reith *et al.*, 2003).

Hydrogen-powered fuel cells and engines would be as common as the gasoline and diesel engines of the late 20th century. They would power cars, trucks, buses, and other vehicles, as well as homes, offices and factories.

Low-cost hydrogen based fuel cells, which have been expensive or not readily available, are now entering commercial production and are finding applications in residential housing and buses. Several automobile manufacturers such as Ford, Toyota, Daimler-Benz and General Motors, will be introducing cars in the next few years that use fuel cells, and Shell and BP have established core hydrogen divisions in their companies.

Hydrogen is not a primary energy source, but rather serves as a medium through which primary energy sources(such as nuclear and/or solar energy) can be stored, transmitted and utilized to fulfill our energy needs (Das and Veziroğlu, 2001). Certain hydrogen production processes have reached maturity for commercial utilization, such as

steam reforming or catalytic decomposition of natural gas, partial oxidation of heavy oils, coal gasification and electrolysis of 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 (McAuliffe, 1980). 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 previously mentioned 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.

The use of solar energy for H_2 production attracts much interest because sunlight is a renewable and a powerful supply of energy. Among different approaches, photocatalysis has received much attention as a possible method for photoelectrochemical conversion and storage of solar energy.

Biological hydrogen production processes open a new avenue for the utilization of renewable energy resources; because they represent an ecological and a less energy intensive method as a result of operating at ambient conditions and facilitating waste recycling (Das and Veziroğlu, 2001).

Biological processes have the potential to be an important source of hydrogen in the 21st century. Biological H_2 production holds the promise of generating a renewable fuel from nature's most plentiful resources, sunlight, water and biomass. The process would have a positive impact on climate change, environmental pollution and the question of energy supply and demand. These processes are performed under mild operating conditions, and do not require complex equipments.

In nature, only bacteria and algae have the capability of hydrogen production. Amongst these organisms, those currently selected for research are anaerobic bacteria, photosynthetic bacteria, cyanobacteria and green algae. Table 3.1 gives an overview of biological hydrogen production processes, which are being explored in fundamental and applied research. Several processes are currently under development, ranging from dark fermentation of biomass to photobiological processes through which hydrogen can be produced directly from sunlight. Biological hydrogen production processes are mainly categorized into three groups as follows (Das and Veziroğlu, 2001):

a.Biophotolysis of water using algae and cyanobacteria

b.Dark fermentative hydrogen production from organic compounds

c.Photodecomposition (photofermentation) of organic compounds by photosynthetic bacteria

Table 3.1 Overview of currently known biological hydrogen production processes

Process	General Reactions	Microorganisms
Direct Biophotolysis	$2\ H_2O + light \rightarrow 2\ H_2 + O_2$	Algae
Photo-Fermentation	$CH_3COOH + 2\ H_2O + light \rightarrow 4\ H_2 + 2\ CO_2$	Photosynthetic bacteria, Algae
Indirect Biophotolysis	a $6H_2O + 6CO_2 + light \rightarrow C_6H_{12}O_6 + 6\ O_2$ b $C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CH_3COOH + 2CO_2$ c $2\ CH_3COOH + 4H_2O + light \rightarrow 8H_2 + 4CO_2$ General reaction: $12H_2O + light \rightarrow 12H_2 + 6O_2$	Algae, Cyanobacteria
Water Gas Shift Reaction	$CO + H_2O \rightarrow H_2 + CO_2$	Fermentative bacteria, Photosynthetic bacteria
Dark Fermentation	$C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2\ CH_3COOH + 2CO_2$	Fermentative bacteria
Two phase Fermentation (Dark ferm. + Photoferm.)	a $C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2\ CH_3COOH + 2CO_2$ b $2CH_3COOH + 4\ H_2O + light \rightarrow 8\ H_2 + 4\ CO_2$ Overall: $C_6H_{12}O_6 + 6H_2O \rightarrow 12H_2 + 6CO_2$	Fermentative bacteria, Photosynthetic bacteria
Anaerobic Digestion (Methanogenic Fermentation) ($H_2 + CH_4$)	a $C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2\ CH_3COOH + 2CO_2$ b $2\ CH_3COOH \rightarrow 2CH_4 + 2\ CO_2$ Overall: $C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CH_4 + 4CO_2$	Fermentative bacteria, Methanogenic bacteria

Algae and cyanobacteria directly decompose water to hydrogen and oxygen with light energy. This is the process by which the earth obtained oxygen in the distant past. The reaction requires only water

and sunlight and is very attractive from the viewpoint of environmental protection, without the requirement of any organic compounds. However, the evolution of oxygen as an end product inhibits hydrogen production and the production rates are low due to the complicated reaction system which remains to overcome the large free energy (+242 kj / mol hydrogen) (Miyake *et al.*, 1999a).

During dark fermentative hydrogen production, anaerobic bacteria produce hydrogen by decomposing organic substrates. Anaerobic bacteria easily degrade biomass such as macromolecular polysaccharides and produce hydrogen at high velocity. However, they cannot completely degrade organic compounds. As the decomposition is incomplete, lower molecular weight organic compounds are produced together with hydrogen and carbondioxide. Accordingly, the hydrogen production efficiency is low. In contrast, photosynthetic bacteria utilize organic acids more than organic compounds. 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).

3.1. Hydrogen Production by Photofermentation

Photosynthetic bacteria produce hydrogen from organic compounds by an anaerobic light-dependent electron transfer process. These kinds of 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 or without light, and salty waters.
d. Large database is available for both future genetic improvement attempts and the photosynthetic hydrogen production mechanism.
e. They can trap energy at a wide range of the light spectrum (Fig 3.1) and can withstand high light intensities.

The photosynthetic efficiency (PE) is defined as energy stored as biomass per unit of light energy absorbed. In the current literature, the light energy absorbed is usually based on the Photosynthetically Active Radiation (PAR) range (i.e., 400 to 700 nm for green algae and 400-950 nm for purple photosynthetic bacteria). Figure 3.1 shows the i. light absorption spectrum of a purple bacteria and ii. green algae compared to the sunlight (Reith *et al.*, 2003). The X-axis represents the wavelength, with the visible part indicated by the shaded bar, and the relative light intensity shown on the Y-axis. It is important to note that purple bacteria can also absorb light energy at a wide range, including the non-visible part of the spectrum.

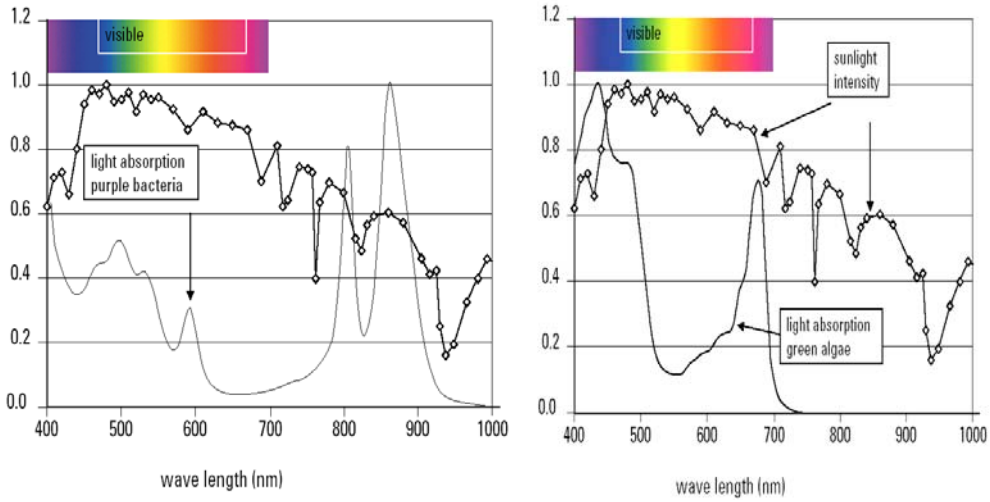


Figure 3.1 Sunlight and light absorption by (i) photosynthetic purple bacteria and (ii) green algae (Reith *et al.*, 2003)

3.1.1. General Characteristics of Purple Non-sulphur Bacteria

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).

Rhodobacter sphaeroides (*R. sphaeroides*) is a member of the purple non-sulphur (PNS) bacteria group, which is among the Gram-negative organisms found in a wide range of environments, including marine and fresh water systems. The classification of *R. sphaeroides* is given in Table 3.2. Cells of *R. sphaeroides* are usually ovoid or spherical in shape with a diameter of 0.5 to 1.2 μm (Pfennig and Truper, 1981).

Table 3.2 The Classification of *Rhodobacter sphaeroides*

Superkingdom	*Prokaryota*
Kingdom	*Monera*
Subkingdom	*Eubacteria*
Division (Phylum)	*Gracilicutes*
Group (Class)	*Photosynthetic Eubacteria*
Order	*Rhodospirillates*
Family	*Rhodospirillaceae*
Genus	*Rhodobacter*
Species	*Sphaeroides*

R. sphaeroides 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).

3.1.2. Overview of Metabolism

Hydrogen production by *R. sphaeroides* and the 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. This kind of metabolism is referred as photofermentation. 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 3.2) 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). An overall scheme for the hydrogen production metabolism is given in Figure 3.2.

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. The transfer of electrons from the TCA cycle to the nitrogenase is accomplished by consecutive oxidation/reduction of electron carriers. These carriers are namely nicotinamide adenine dinucleotide (NAD) and ferredoxin (Fd), and the hypothesized electron path is as follows:

$$\text{Substrate} \rightarrow \text{TCA-cycle} \rightarrow \text{NAD/NADH} \rightarrow (\text{Fd})_{ox}/(\text{Fd})_{red} \rightarrow \text{Nitrogenase} \quad (3.1)$$

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).

The substrate consumption has the alternate pathway of biosynthesis and growth and it is also possible that certain biosynthesis products (such as PHB reserves) can later be degraded by means of the endogenous metabolism. ATP synthase, which can be considered as a part of the photosynthetic apparatus, might function reversibly to generate ATP from a proton gradient, or to create a proton gradient by consuming ATP.

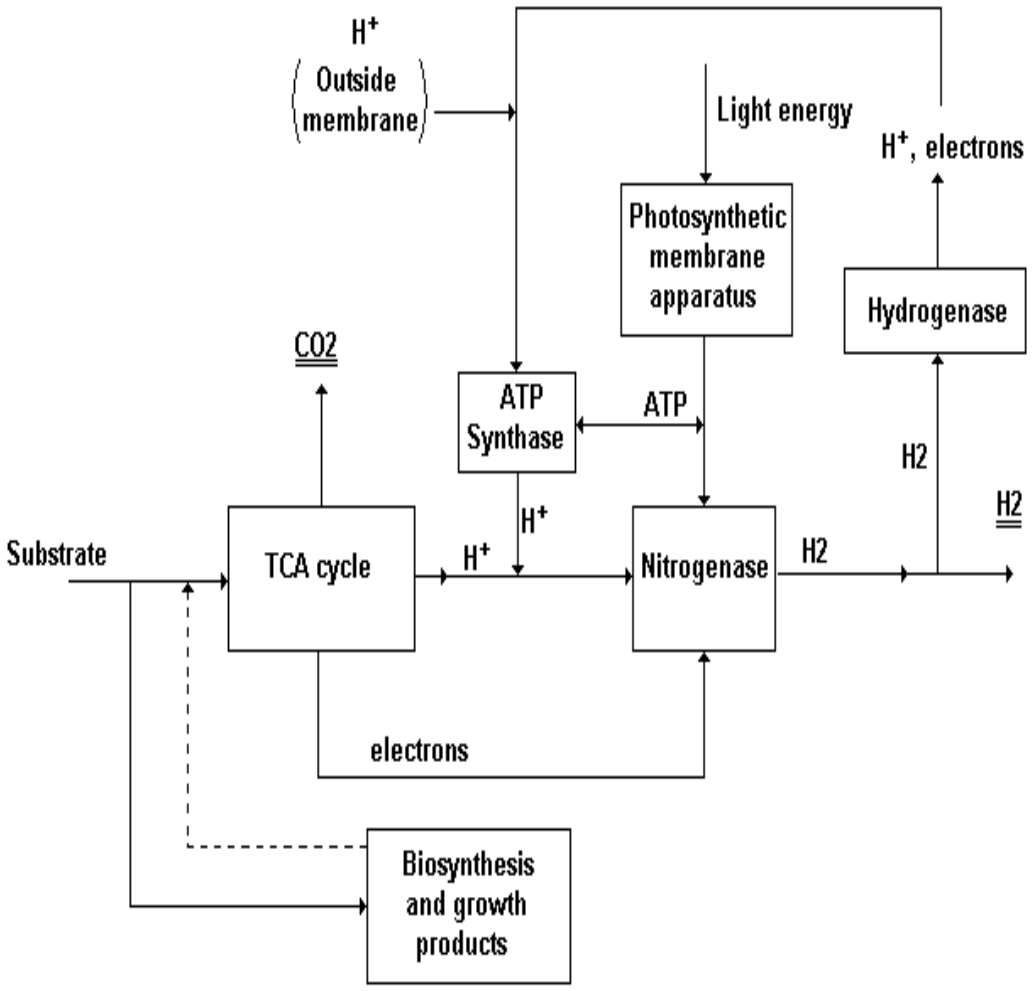


Figure 3.2 The overall scheme of hydrogen production by PNS bacteria (Koku *et al.*, 2002)

Though a wide variety of substrates can be used for growth, only a portion of these is suitable for hydrogen production (Figure 3.3). 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 (Suludere, 2001; Yiğit *et al*, 1999). All these individual components of the hydrogen production interact and are subject to strict regulatory controls.

An overall scheme for the carbon flow is given in Figure 3.3. In this scheme, sugars and structurally similar components like glycerol are utilized through the Embden-Mayerhoff and Entner-Doudoroff pathways. Carbon dioxide is assimilated through Calvin cycle. Lactate, pyruvate, acetate, and butyrate are utilized at the acetyl-CoA and pyruvate junctions. Acids such as malate, succinate and fumarate are utilized as intermediates of the TCA cycle.

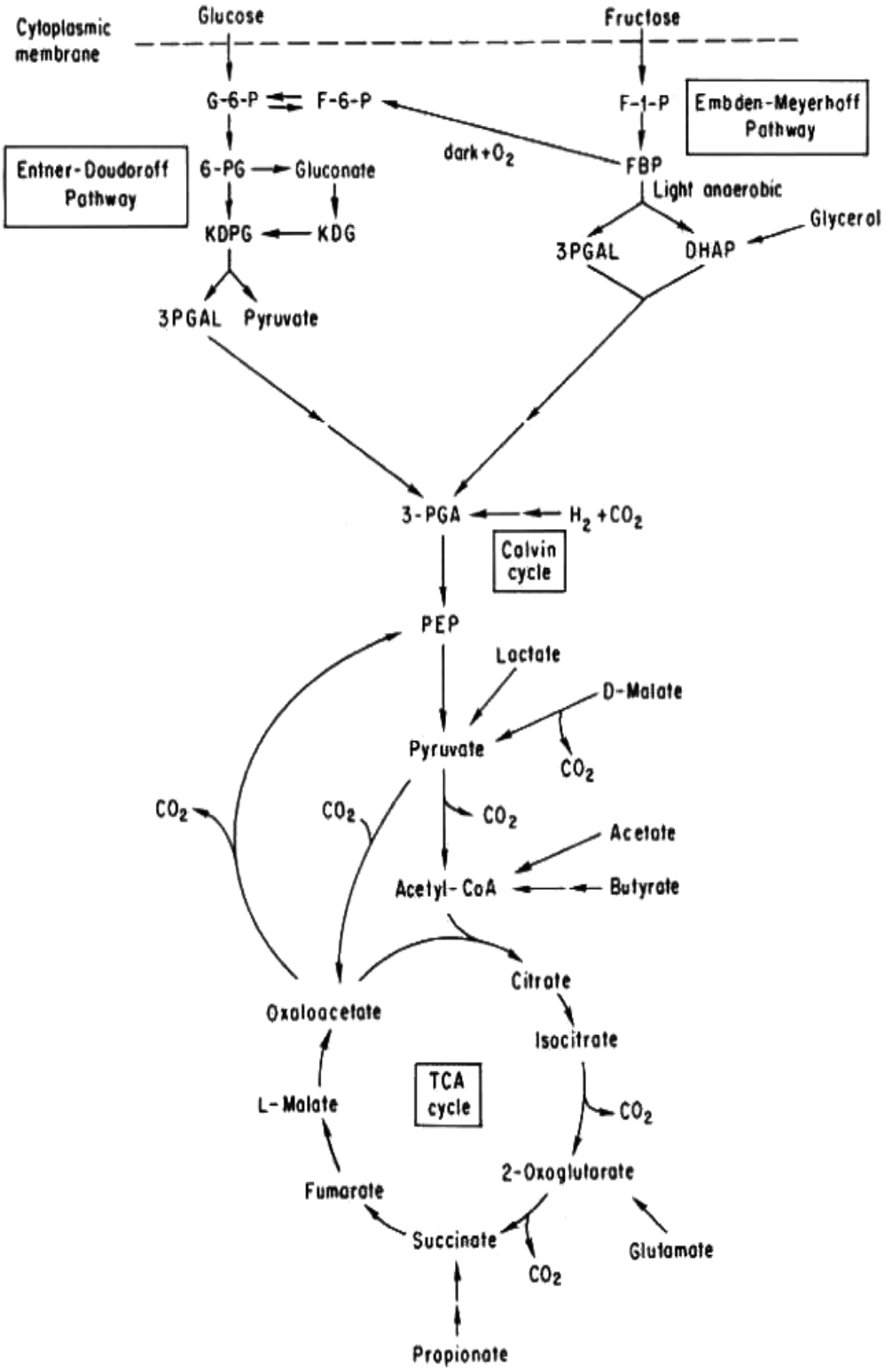


Figure 3.3 Simplified overall scheme of the carbon metabolism in PNS bacteria (Koku *et al*, 2002)

3.1.3. By-products of Photofermentation Processes

One way to overcome the economic restrictions of biological hydrogen production by photosynthetic bacteria is to associate this process with wastewater treatment. Another way 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. An electron micrograph, of PHB granules in *R. sphaeroides* O.U.001 is seen in Figure 3.4 (Suludere, 2001). PHB granules were fixed with glutaraldehyde and uranyl acetate, dehydrated with acetone, and stained with lead citrate.

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. A better understanding of PHB synthesis would allow for controlling the process to increase the production of the desired product.

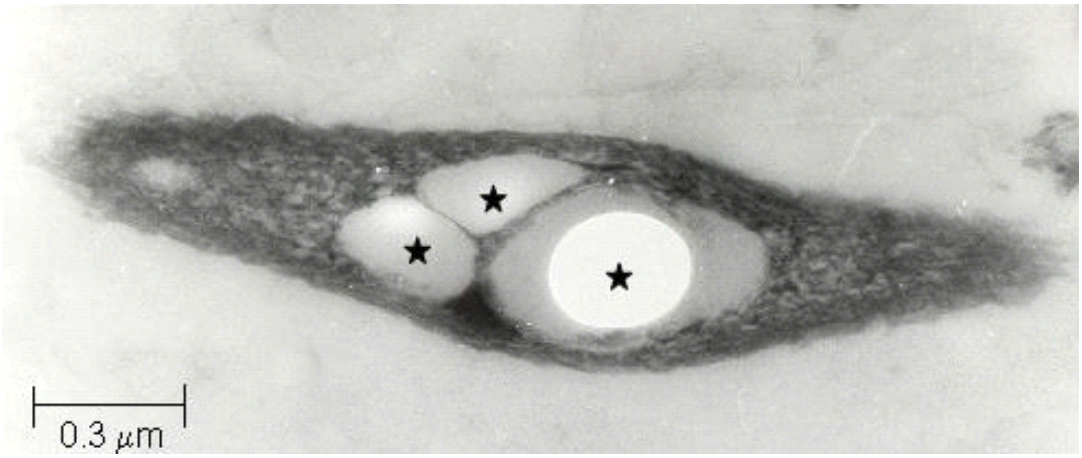


Figure 3.4 Electron micrograph of PHB granules (*) in *Rhodobacter sphaeroides* O.U. 001 (Suludere, 2001)

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. Also, it results the duration of photosynthesis under oxygenic atmosphere.

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). Carotenoid 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.

3.2. 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.

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 the conventional anaerobic treatment of wastewater by anaerobic mixed cultures, organic pollutants are generally converted into methane (Hulshoff Pol and Lettinga, 1986) as given in Figure 3.5. The metabolic stages involved in the production of methane from wastes are hydrolysis, acidogenesis, and methanogenesis. Referring to Figure 3.5, the first step in the process involves the enzyme-mediated transformation (hydrolysis) of higher molecular weight compounds into compounds suitable for use as a source of energy and cell carbon. The second step (acidogenesis) involves the bacterial conversion of the compounds resulting from the first step into lower molecular weight intermediate compounds. Acidification step by the acidogenic bacteria produces hydrogen as a by-product, which in turn is used as an electron donor by many methanogens at the last stage of the methanogenesis process. This stage includes the bacterial conversion of the intermediate compounds into simpler end products, principally methane and carbon dioxide (Tchobanoglous and Burton, 1991).

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 10^8 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).

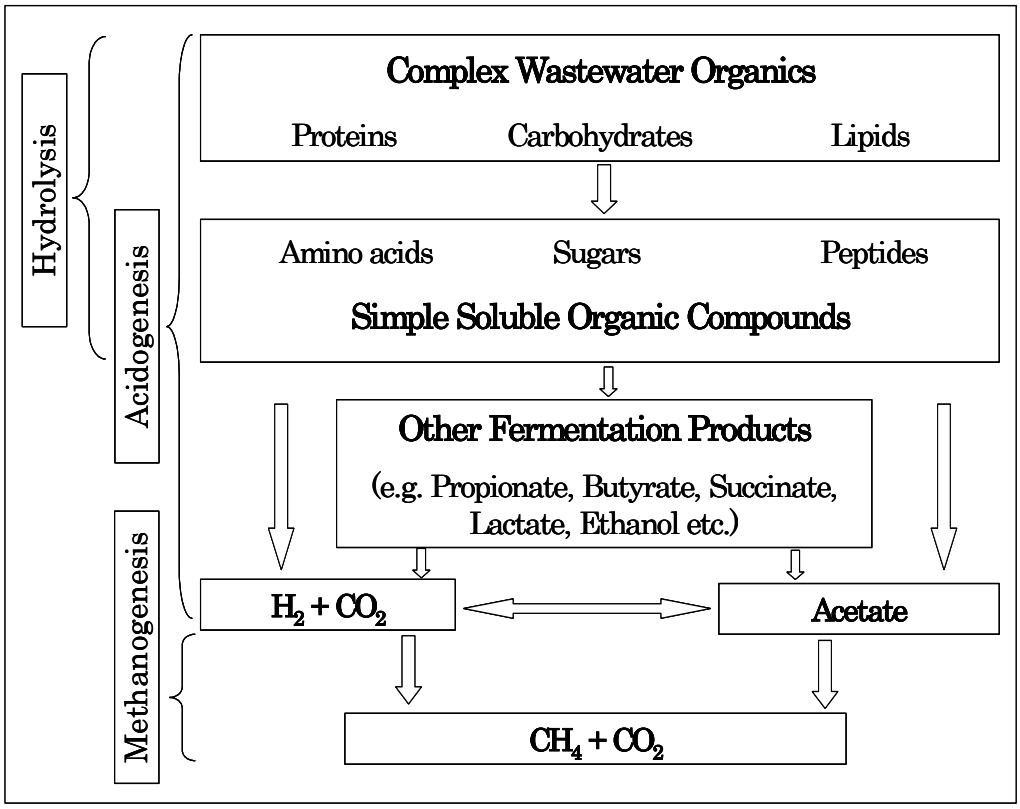


Figure 3.5 Phases of the anaerobic digestion process

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 H_2 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).

3.3. Recent Developments in Two-Step Hydrogen Production Bioprocesses

Dark fermentative and photosynthetic bacteria are known to be coupled with each other in two subsequent stages to enhance the hydrogen production. In the first stage organic waste is heterotrophically fermented to organic acids by the fermentative bacteria. Thereafter, a second stage for phototrophic conversion of organic acids to H_2 is applied using photosynthetic bacteria. During dark fermentative hydrogen production, fermentative bacteria produce

hydrogen by decomposing organic substrates under anaerobic and dark conditions. As the decomposition is incomplete, lower molecular weight organic compounds are produced together with hydrogen and carbondioxide. The organic acids, produced as a result of fermentation, can further be utilized by photofermentative bacteria for hydrogen generation. Higher amounts of H_2 are produced because organic acid enriched media enhance photosynthetic H_2 production (Reith *et al.*, 2003). Thus, coupled systems using photosynthetic and fermentative bacteria become an efficient way for biological hydrogen production.

Hydrogenase enzyme is playing an important role in the dark fermentative H_2 production. Solvent production and hydrogen or acid formation are competing with each other during the pathway of pyruvate decomposition into butyrl-CoA (Lay *et al.*, 1999). Thus, in order to increase photofermentative hydrogen production following a dark fermentation stage; organic acid production must be enhanced (acidogenetic fermentation) or solvent production must be depleted by changing the environmental conditions like T; pH; HRT (hydraulic retention time) during the dark fermentation stage.

Following figure (Figure 3.6) illustrates the energetic-view of H_2 production by dark anaerobic and photosynthetic bacteria. During dark fermentation, anaerobic bacteria decompose carbohydrates to obtain both energy and electron. Complete degradation of glucose to H_2 and CO_2 is impossible by dark fermentation since reactions with negatively charged free energy could be possible. Photosynthetic bacteria could use light energy to overcome the positively charged free energy reaction by utilizing the organic acids for hydrogen production. These combinations of bacteria reduce the light energy demand of

photosynthetic bacteria, but also increase H_2 production (Das and Veziroğlu, 2001).

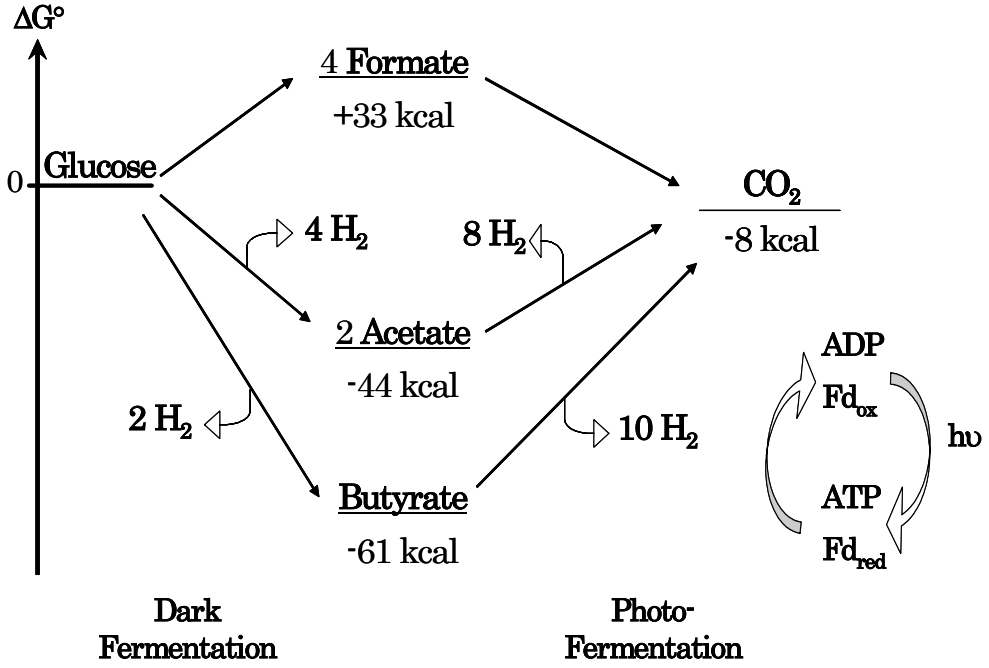


Figure 3.6 Energetic-view of H_2 production by dark fermentation, followed by photofermentation

In a study of Fascetti *et al.* (1998), phototrophic hydrogen production by *Rhodobacter sphaeroides* RV cells was enhanced while the cells were cultivated on the lactate containing solutions derived from the dark fermentation of municipal solid wastes by anaerobic mixed cultures.

Kim *et al.* (2001) studied another two-stage process for hydrogen production from makkoli (raw rice wine) and tofu (soybean product) wastewaters. Their process consist of a dark fermentation step by *Clostridium butyricum* NCIB 9576 obtained from sewage sludge, followed by photofermentation using immobilized *R. sphaeroides* E15-1 cells in hollow fibers. Photofermentative hydrogen production was

increased due to the production of several organic acids such as butyrate, acetate, propionate and ethanol within the dark fermentation broth of both makkoli and tofu wastewaters.

In this context, a new 6th EU Framework integrated project; namely "HYVOLUTION: Non-thermal production of pure hydrogen from biomass"; was started by the 1st of 2006. In HYVOLUTION, 11 EU countries, Turkey (METU-Biohydrogen group) and Russia are represented to assemble the critical mass needed to make a breakthrough in cost-effectiveness. Project focuses on employing thermophilic bacteria which grow at temperatures of 70 ºC or above. These bacteria produce hydrogen together with acetic acid. In this process, the amount of hydrogen produced per unit of biomass is about twice as high in comparison to fermentation at ambient temperatures. Furthermore, 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 (Claassen and de Vrije, 2005).

Nowadays, biophotolysis (green algae) followed by photo-fermentation (photosynthetic bacteria) is also gaining interest among coupled biohydrogen production processes (Melis, 2005). In this study, hydrogen production process was based on unicellular green algae driven by the visible portion of the solar spectrum, coupled with purple photosynthetic bacteria driven by the infrared portion of the solar spectrum. Conditions have been selected for the optimized utilization of solar irradiance and optimized co-cultivation of the two types of unicellular organisms.

3.4. Substrates for Biological Hydrogen Production

The performance of biological hydrogen production by photosynthetic bacteria must be evaluated by several parameters. The first is the hydrogen production rate, which is the rate of gas production (amount/time) on a basis of the culture volume or bacterial dry weight. The second criterion is the substrate conversion efficiency, which is a measure of the amount of substrate utilized for hydrogen production (rather than growth or alternative biosynthesis). It is the ratio of the actual moles of hydrogen produced, to the theoretical amount that would have been obtained if all of the substrate were used for hydrogen (and carbon dioxide) production according to the following hypothetical reaction (Sasikala *et al.*, 1993):

$$C_xH_yO_z + (2x\text{-}z)\ H_2O \rightarrow (y/2 + 2x\text{-}\ z)\ H_2 + x\ CO_2 \qquad (3.2)$$

The expression for percent substrate conversion efficiency is:

$$\%\ \text{Substrate conv. efficiency} = 100 \cdot (\text{actual } H_2 / \text{theoretical } H_2) \qquad (3.3)$$

Table 3.3 displays rates and percent conversion efficiencies from recent hydrogen production studies with different substrates and wild-type strains of various photosynthetic bacteria, generally by *R. sphaeroides*. Similar surveys listing other studies can be found elsewhere (Das and Veziroğlu, 2000; Rocha *et al.*, 2001; Markov *et al.*, 1998; Miyake *et al.*, 1999). In general, it can be suggested from Table 3.3 that the best substrates are anions of organic acids such as lactate and malate while sugars such as glucose and sucrose are not much efficient.

Table 3.3 Hydrogen production studies by photosynthetic bacteria

Org.	Ref.	Carbon source	C/N (mM/ mM)	Gas Production Parameters			
				Conv eff. (%)	Max. Rate (mL/ g_{dwt}/h)	Max. Rate (L/L/h)	Duration (h)
R. capsulatus Z1	Hillmer and Gest, 1977	Glucose Fructose Sucrose Lactate Pyruvate Malate Succinate Propionate Butyrate	30/7	32 27 06 72 68 56 72 - -	88 100 60 130 130 90 100 40 20	n/a	n/a
R. sphaeroides (Berkeley collection)	Macler *et al.*, 1978	Glucose	20/13	24	5.5	0.0126	115
R. sphaeroides B5	Kim *et al.*, 1982	Lactate	50/5	55	138	0.0183	242
R. sphaeroides RV	Miyake *et al.*, 1984	Acetate Lactate Butyrate	16.3/10 52.5/10 46.2/10	40 46 75	37 145 205	117 251 604	285
R. sphaeroides RV	Fascetti *et al.*, 1995	Lactate	50-100/ 4.7	50-80	75	0.0366	n/a
R. sphaeroides O.U.001	Arık *et al.*, 1996	Malate	7.5/10	22	3.3	0.016	46
R. sphaeroides O.U.001	Eroğlu *et al.*, 1999	Malate	15/2	37	3.0	0.017	150
R. sp. HCC 2037	Barbosa *et al.*, 2001	Acetate	22/0.8	73	43	0.025	200

One way to overcome the economic restrictions of biological hydrogen production by photosynthetic bacteria is to associate this process with waste treatment. Because, practical applications of photosynthetic bacteria for H_2 production cannot utilize expensive synthetic culture media like used in most of the laboratory experiments. Thus, several studies of the recent literature are focused on the utilization of cheap organic substances such as residual wastes from the food and agricultural industry, or wastewaters with high levels of organic compounds and thereby provide the advantages of both energy production and waste treatment (Rocha *et al.*, 2001). Table 3.4 lists the different type of waste materials, used for the biological H_2 production in the current literature. The yield and rate results of some of these studies by *R. sphaeroides* are listed in Table 3.5.

Table 3.4 Different type of waste materials used for H_2 production

Study	Microorganism	Waste Material
Salih *et al.*, 1989	*Escherichia coli*	Cheese whey
Sasikala *et al.*, 1991a	*R.sphaeroides* O.U.001	Lactic acid fermentation plant wastewater
Sunita *et al.*, 1993	*R.sphaeroides* SMINSOU	Sewage sludge
Kalia *et al.*, 1994	*Bacillus licheniformis*	Wheat grains
Tanisho *et al.*, 1994	*Enterobacter aerogenes*	Molasses
Fascetti *et al.*, 1998	*R.sphaeroides* RV	Municipal solid wastes such as fruit and vegetable residues
Türkaslan *et al.*, 1998	*R.sphaeroides* O.U.001	Milk industry wastewater
Zhu *et al.*, 1999	*R.sphaeroides* RV	Tofu wastewater
Yetiş *et al.*, 2000	*R.sphaeroides* O.U.001	Sugar refinery wastewater
Eroğlu *et al.*, 2004	*R.sphaeroides* O.U.001	Olive mill wastewater
Yokoi *et al.*, 2002	*Clostridium butyricum* & *Enterobacter aerogenes*	Potato starch residue
Vrije *et al.*, 2002	*Thermotoga elfii*	Lignocellulosic biomass (e.g. Miscantus plant)

Table 3.5 Example studies on the use of wastewater for hydrogen production by *R. sphaeroides*

Waste source + Additives	Strain	H_2 prod. rate ($L_{H2}/L_c/h$)	H_2 prod. rate ($mL_{H2}/ g_{cell}/ h$)	H_2 prod. Yield (L_{gas}/L_{WW})	Waste water (%)	Study
Lactate ferment. plant	O.U. 001	0.0050	1.5	4.5	5-100	(Sasikala *et al.*, 1991)
Dairy plant + Malate	O.U. 001	0.0055	5.9	2	30	(Türkarslan *et al.*, 1998)
Tofu factory	RV	0.0590	91	1.9	100	(Zhu *et al.*, 1999)
Sugar Refinery + Malate	O.U. 001	0.0043	5.0	8.6	20	(Yetiş *et al.*, 2000)
Olive Mill Waste Water	O.U. 001	0.0035 0.0095	7.5 36	13.9 9.8	2 1	(Eroğlu et al., 2004)

Zürrer and Bachofen (1979) investigated the utilization of pure lactate and lactic acid containing whey and yogurt wastes as a carbon source by *Rhodospirillum rubrum* S-1 and calculated the maximum hydrogen production rate as 0.006 $L_{H2}/g_{cell}/ h$ and the composition of evolved gas within a range of 70-75%.

Vatsala and Ramasamy (1987) experimented the photoproduction of hydrogen from distillery waste by *Rhodospirillum rubrum* 11170 in

both indoor and outdoor conditions. The hydrogen production rate in indoor conditions was 0.003 L_{H2}/L_c/ h (H_2 percentage of the final gas was 63%) with %100 distillery waste, and in outdoor conditions the rate was 0.0008 L_{H2}/L_c/ h (54% H_2) with %5 distillery waste.

Sasikala *et al.* (1991a) used the wastewater of a lactic acid fermentation plant as an electron donor for hydrogen production by using *R. sphaeroides* O.U.001 cells. Hydrogen production was observed for different dilutions of wastewater, ranging from 0% to 100 % by tap water.

Sunita and Mitra (1993) studied photoproduction of hydrogen by immobilized cells of *Rhodopseudomonas sp.* SM1 NSOU strain using sewage. They observed a hydrogen production rate of 0.003 L_{H2}/L_c/ h with 50% sewage. But the compositions of the produced gas ranged between 10-90% H_2. Both of these substrates were diluted with modified (nitrogen-free) Biebl and Pfennig medium (Biebl and Pfennig, 1981).

Singh *et al.* (1994) studied hydrogen production with free and immobilized (Ca-alginate) cells of *Rhodopseudomonas sp.* (BHU strains 1-4) using 1% vegetable starch, sugarcane juice and whey in each case. Dilutions were achieved with modified Biebl and Pfennig medium (Biebl and Pfennig, 1981). Among these three substrates, sugarcane juice resulted a maximum amount of hydrogen production, followed by potato starch and whey.

Tanisho and Ishiwata (1994) carried out continuous hydrogen production studies by *Enterobacter aerogenes* E.82005, with the

utilization of 4% molasses as a substrate source. Continuous hydrogen was prolonged for 42 days with a rate of 20 mmol H_2/h/L_c. The H_2 content of the gas was 60%.

Fascetti *et al.* (1998), used acidogenic fermentation driven by anaerobic bacteria as the first step to decompose municipal solid wastes (refuse from vegetable markets) into organic acids. Then the effluent of this step was reutilized during photobiological hydrogen production by photosynthetic bacteria (*Rhodobacter sphaeroides* RV). The retention time of the first step was adjusted such that the effluent was composed of mainly lactic acid and small amounts of acetic acid. Using dilutions of this effluent, hydrogen production at high rates (100 mL_{H2}/g_{cell}/ h) was accomplished from continuous runs using 1 liter chemostats.

Türkarslan *et al.* (1998) studied hydrogen production with the wastewater of a dairy plant, using *R. sphaeroides* O.U. 001. However, the nutrient in waste from the milk factory was not sufficient alone to support the growth of *R. sphaeroides*, probably because it lacked some essential minerals. Thus, when blends of wastewater and malate were used, not only growth and hydrogen production was observed, but also hydrogen production was enhanced through the shortening of the lag time for hydrogen production.

Zhu *et. al.* (1999) worked with immobilized cells of *R. sphaeroides* RV for hydrogen production from the wastewater of a tofu factory (tofu is a kind of soybean product that resembles cheese and mostly utilized in far eastern countries). They obtained relatively higher hydrogen production rates. Apart from the immobilization, two additional factors might have contributed to these higher rates as: The use of a strain of *R. sphaeroides* RV that is capable of utilizing relatively higher

amounts of glucose, and the feeding of waste water after the cells begun to evolve hydrogen from a pre-culture of lactate medium.

Yetiş *et al.* (2000) used sugar refinery wastewater for hydrogen production. No or very little hydrogen generation took place at different dilutions of wastewater alone but when 20 % diluted wastewater was used together with malate, similar rates with those from malate alone were obtained. In addition, less lag times for hydrogen production and slightly increased growth rates were observed. It should be noted that no hydrogen was produced with sucrose alone.

In a repeated batch culture, hydrogen production with high yield of 2.7 mol H_2 / mol glucose was obtained by a mixed culture of *Clostridium butyricum* and *Enterobacter aerogenes* in the starch waste medium consisting of sweet potato starch residue as a carbon source and corn steep liquor as a nitrogen source (Yokoi *et al.*, 2002). Hydrogen yield was increased (4.5 mol H_2 / mol glucose) by culturing *Rhodobacter sp.* M-19 in the supernatant supplemented with 20 μg/l Na2MoO4.2H2O and 10 mg/l EDTA in a repeated batch culture.

Eroğlu *et al.* (2004) used olive mill wastewater (OMW) as a sole substrate for the production of hydrogen gas by *Rhodobacter sphaeroides* O.U. 001. Growth and hydrogen production on diluted OMW were investigated between 20% (v/v) and 1% (v/v) OMW media. Although bacterial growth could be achieved in all inspected OMW concentrations; hydrogen production was observed for the ones below 4%. A maximum hydrogen yield of 13.9 ml H_2/ml OMW was obtained at 2% OMW containing diluted media.

3.5. Scope of the Thesis

The main objective of the current study is to develop a suitable two stage process including pretreatment process followed by photofermentation; which will yield an efficient hydrogen production in addition to the significant remediation of OMW.

Initially, several OMW samples from different olive-oil mills in Western Anatolia were subjected to a single-stage photofermentative hydrogen production process in addition to detailed physico-chemical characterization. As regards to its relatively highest amount of hydrogen production in parallel to its organic acid rich medium, one of these samples was chosen to be utilized during further studies.

Then, different two-stage processes were investigated on the basis of efficient OMW remediation and H_2 production. For this purpose, different physicochemical (adsorption with clay or zeolite, chemical oxidation with ozone or Fenton's reagent, and photodegradation by UV radiation) and biological (dark fermentation with sewage sludge) pretreatment processes were compared with each other. According to its highest hydrogen production potential, ease of operation and comparable low cost of application; clay pretreatment technique was found to be a good alternative for a two stage hydrogen production process. For this reason, a two-stage process at which clay pretreatment step was followed by photofermentation; was explored in detail.

In the following studies, the aim was to gain further insight into the effect of clay pretreatment process on the photofermentative hydrogen production. In order to do that, comparative studies for the

determination of the effect of clay quantity, and the dilution rates of the clay pretreatment effluents were inspected in a sequential manner. Afterwards, the regeneration of spent clay and the possibility of its reutilization for photofermentative hydrogen production process were also investigated in the context of solid waste minimization.

Further challenges in two-stage processes include extensive analysis to investigate the effect of pretreatment on photofermentation. These are; total phenol, total sugar, color, specific organic acids, phenols, amino acids, sugars and alcohols in addition to the gas analysis, the bacterial concentration and pH measurements. For control purposes, untreated (raw) OMW containing medium was examined in parallel to the clay pretreatment effluents. Finally, process evaluation was carried out by applying material balance equations on the clay pretreatment and photobioreactor stages.

For scale-up; single-stage photobiological hydrogen production was carried out in a large scale solar-bioreactor under the illumination of sun. Hydrogen and valuable by-product formation (i.e., carotenoid and polyhydroxybutyrate) were investigated under these conditions.

CHAPTER 4

MATERIALS AND METHODS

4.1. Photofermentation Process

Rhodobacter sphaeroides O.U.001 (DSM 5864) was used in this study.

4.1.1. Anaerobic growth of *Rhodobacter sphaeroides*

In the anaerobic photoheterotrophic growth mode, malic acid as carbon source and sodium glutamate as nitrogen source were shown to be effective for the growth of bacteria (Sasikala *et al.*, 1995). Therefore, these carbon and nitrogen sources were utilized for pre-activation purposes throughout this study. 10% inoculum of the pre-activated bacteria was transferred into the hydrogen producing liquid media containing olive mill wastewater (OMW).

The minimal medium of Biebl and Pfennig (1981), supplied with L-malic acid (7.5 mM) and sodium glutamate (10 mM) without ammonium chloride and yeast extract, was used. Additional components of this medium were vitamins, trace elements and iron-citrate that their compositions are given in Appendix A.

The initial pH of the medium was adjusted to 6.8 by the addition of NaOH. Then, 50 mL medium was injected into a 100 mL glass bottle with a rubber cap. This bottle was sterilized in an autoclave (Prior

Clave) for 15 minutes at 121 ºC. After cooling to room temperature, argon gas (99.995 % purity) was sparged at a flow rate of 100-150 mL/min for about 5 minutes, in order to obtain an anaerobic atmosphere inside. Anaerobic culture preparation bottle is shown in Figure 4.1.

Finally, 10% inoculum of *Rhodobacter sphaeroides* O.U.001 was injected using sterile syringe needles into the prepared anaerobic liquid media, which were then incubated at 36 ºC in an incubator (Gallenkamp PLC). The bottles were illuminated by a 100 W tungsten lamp, placed at a distance of 25-30 cm.

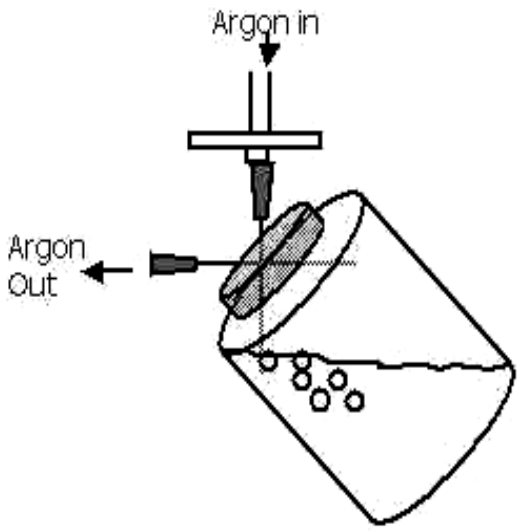


Figure 4.1 Anaerobic culture preparation bottle

For the storage purpose of bacteria, 30 mL growth medium containing penicillin bottles were inoculated with 3 mL active culture of *Rhodobacter sphaeroides* O.U.001. After 48 hours of growth, approximately 3.5 mL of sterile glycerol (10% of total volume) was injected into the bottle with a sterile syringe, in order to protect the

cells from freezing. Then, that bottle was shaken by hand and stored in a freezer (Uğur) at -20 ºC.

When inoculating a new culture, the bacteria were transferred at least two times into a fresh medium to get rid of the effects of glycerol. Renewals of the stocks were carried out every 2 or 3 months.

4.1.2. Hydrogen Production

For hydrogen production experiments by *Rhodobacter sphaeroides* O.U.001, different OMW samples were investigated. The OMW samples, used in this study were obtained from different olive oil mills at different regions of Western Anatolia. Previously, OMW samples (either pretreated or raw) were filtered through Whatmann filter paper (0.45μ). After filtration, OMW was diluted with distilled water (dilution ratios were varying for every run). The initial pH of diluted OMW was adjusted around 6.8-7.0 by adding NaOH and then autoclaved for 15 minutes at 121 ºC (Prior Clave).

4.1.2.1. Experimental Setup for Indoor Hydrogen Production

A schematic diagram of the experimental setup is shown in Figure 4.2. For indoor hydrogen production experiments, rubber-tapered glass bottles with 55 ml of volume were used. The temperature of the photobioreactor was maintained at 30-32ºC. The illumination was provided by 150 W tungsten lamp, adjusted to provide a uniform light intensity of 200 W/m^2 at the surface of the reactor.

The photobioreactors were fully filled with the mixture of pretreated or raw OMW media and the bacterial culture, to generate anaerobic conditions. Argon gas is not used and dissolved oxygen in the medium

is neglected. Initial pH of the media in all bioreactors was adjusted to 6.8 by NaOH addition.

The sterilization of the photobioreactors and media were accomplished by autoclaving at 121 ºC for 15 min. Sterilized medium was added into the reactor near flame. The amount of inoculation to the bioreactors was 10% by volume of the fresh medium. During the experiments, the evolved gas was collected and measured volumetrically by graduated-glass burettes as a result of the reversible replacement of water. The produced gas amounts are continuously recorded by a digital camera.

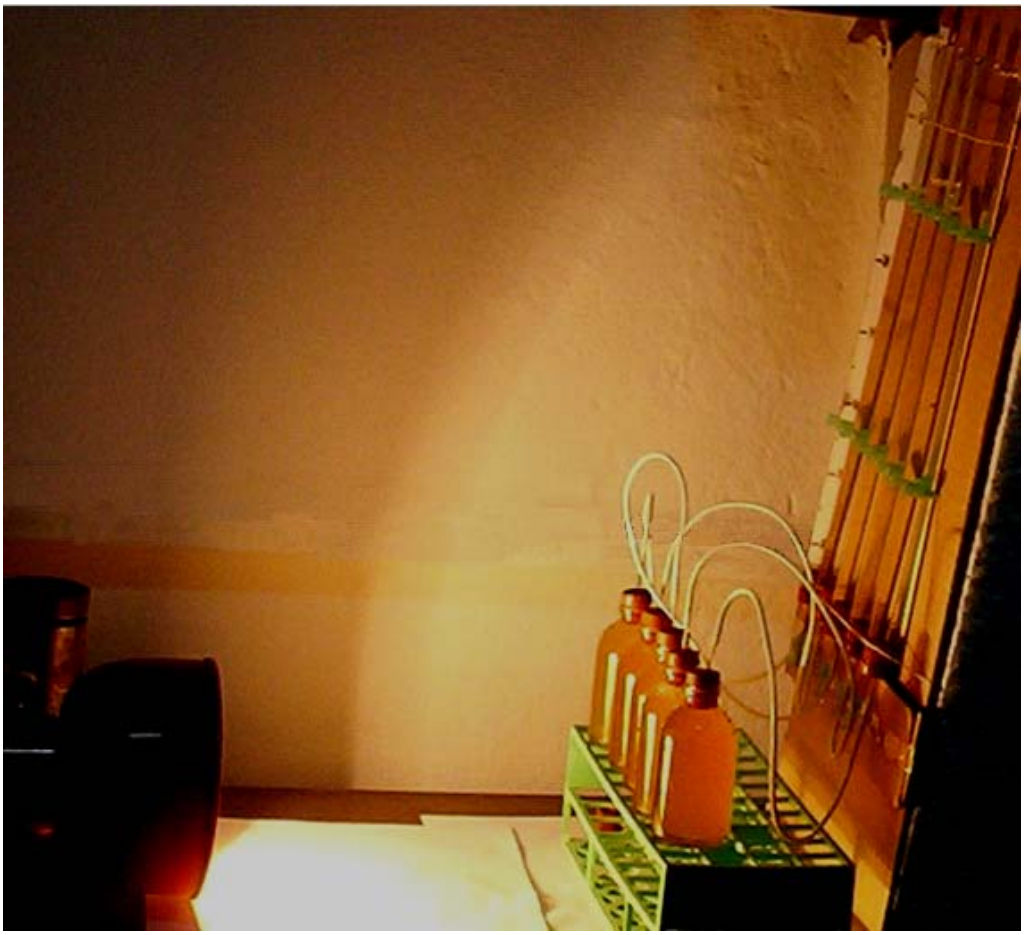


Figure 4.2 Experimental Setup for Indoor Hydrogen Production

4.1.2.2. Experimental Setup for Outdoor Hydrogen Production

For outdoor hydrogen production experiments, 8L volume flat plate solar bioreactor was used which was designed during the study of Tabanoğlu, 2002. It was made of acrylic sheet (Plexiglas) with 5 mm thickness and had an illuminated front area of 0.2 m^2. The schematic diagram of the solar bioreactor and the experimental setup for the outdoor hydrogen production is given in Figures 4.3 and 4.4, respectively. The dimension of the reactor was 50 cm x 40 cm x 4 cm. Total volume of the reactor was 8 L and maximum 6.5 L of it can be filled with culture medium.

In order to obtain light data, Luxmeter (Lutron) was connected to a personal computer (PC). It had a sensor placed at the upper left part of the reactor, through which light density data were collected every after 15 min. A heating blanket was placed on the back face of the reactor. Temperature was controlled with a temperature controller (Elimko-E212), which was connected to a temperature probe (Elimko, Pt-100) inside the reactor. Cooling water was continuously flowed through a coil inside the reactor, made of a glass tube (170 cm long, ID 10 mm).

Gas evolved was collected in graduated glass burettes. The tips of the burettes were inserted into beakers filled with distilled water. Pure argon gas was bubbled through the bioreactor to obtain anaerobic atmosphere. Figure 4.5 shows the views of the solar bioreactor in our sun-laboratory, filled with 4% raw OMW containing media.

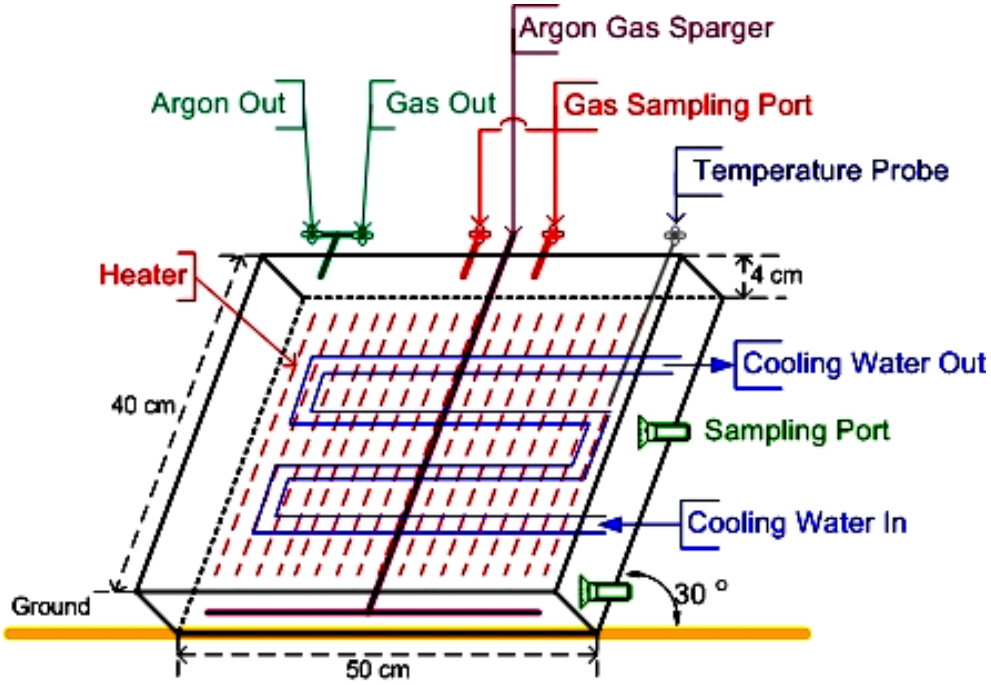


Figure 4.3 Schematic Diagram of the Flat Plate Solar Bioreactor (Tabanoğlu, 2002)

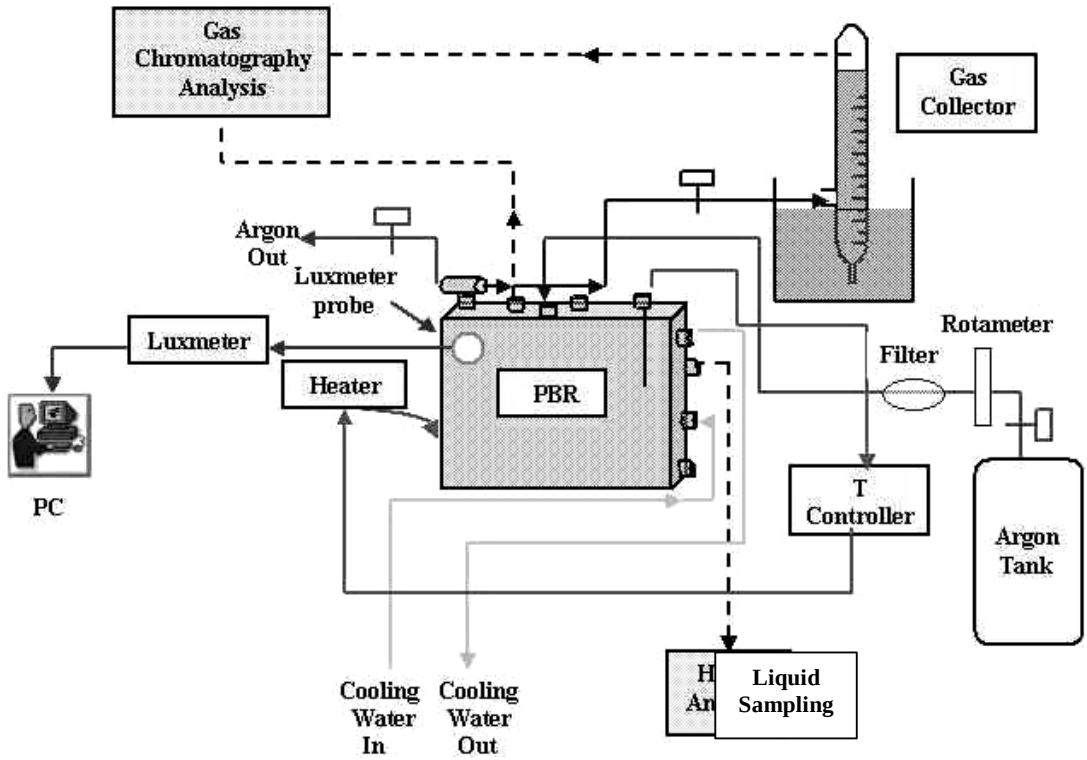


Figure 4.4 Experimental Setup for Outdoor Hydrogen Production (Tabanoğlu, 2002)

Figure 4.5 Views of the solar bioreactor, filled with 4% raw OMW

4.1.2.3. Sampling

Gas samples were taken from the top of the burette by a gas-tight syringe (Alltech), in order to detect the composition of the evolved gas by a gas chromatography (Hewlett-Packard 5890 Series II, thermal conductivity detector).

For indoor photobioreactors; liquid samples were collected after ejecting 1-1.5 mL of the culture media by a sterile syringe from the reactor. Meanwhile, the evacuated volume was simultaneously replaced with the parallel control of the worked media. Control reactor was carried out in the 55 mL vessels under argon atmosphere to obtain anaerobic conditions. For solar bioreactors, around 2 mL of liquid samples were collected from the sampling ports twice a day as in the morning and at night. Liquid samples were taken at certain time intervals, mainly for the pH and the cell concentration analysis. The pH of the culture was measured with a standard combination of a pH electrode (Mettler Toledo 3311) and an electronic transmitter (Nel pHR-1000 Transmitter). Cell concentrations were detected by measuring the optical density of the culture at 660 nm (OD_{660}). The

calibration curve obtained for the cell concentration versus OD_{660} is given in Appendix B.

At the end of each run, the reactor was emptied and the remaining liquid was centrifuged at 10,000 g for 15 minutes at 4 ºC (Sorvall, RC5C) in order to separate solid matter from the liquid media. Then, both phases were collected for further analysis. The solid residue of outdoor experiments was subjected to polyhydroxybutyrate and carotenoid analysis. The supernatant of both indoor and outdoor experiments was tested for some further analysis such as chemical oxygen demand, color, light absorbance, phenols, organic acids, amino acids alcohols, and sugars.

4.2. Pretreatment Processes

4.2.1. Dark Fermentation Process by Sewage Sludge

Sewage sludge culture for the dark fermentation step was obtained from anaerobic digester effluent of Greater Municipality of Ankara Domestic Wastewater Treatment Plant. This process was performed in 55 mL glass reactors under dark conditions in an incubator (Gallenkamp). The reactors are fully filled by mixing the OMW medium and sewage sludge to create anaerobic conditions. The amount of sewage sludge inoculation (either acclimatized or not) to the bioreactors was 10% by volume of the fresh medium.

In order to suppress methanogenic bacteria in the sewage sludge and to enhance the acidogenesis pathway; dark fermentation process was operated at 35°C, and halted after 3 days. Then, the culture media was centrifuged (Sigma) at 3000 g for 10 min in order to separate the

culture from the fermented OMW media. This centrifugation speed and time was chosen to prevent the breaking up of the sludge flocs which might result the extracting of the colorful polymeric material inside its cell (Guibaud *et al.*, 2003). After centrifugation, this fermentation broth was collected and its pH was adjusted around 6.8 by the addition of NaOH prior to sterilization by autoclaving (Prior Clave). This pre-fermented liquid media was then utilized during the photofermentative hydrogen production step.

Dark fermentation was also carried out by acclimatized sewage sludge. 35 mL of fresh seed microorganisms (sewage sludge) obtained from the Greater Municipality of Ankara Domestic Wastewater Treatment Plant (ASKİ) was added into 350 mL glass reactors in an incubator at pH=5, T=35 °C. Acclimation to OMW was carried out with a feeding concentration of 0.5 g COD/L/d, according to the data given in Andreozzi *et al.*, 1998. After 48 hours of incubation, fill and draw procedure is initialized in which as 3.5 mL of mixture was withdrawn from the reactor and the same amount of OMW was added. Acclimation period took about 40 days. The basal medium of Biebl and Pfennig (1981), used for photofermentative hydrogen production, was also utilized as initial substrate source for the sewage sludge. This medium was supplied with basal medium (Appendix-A) containing vitamins, trace elements and iron-citrate which were known to be suitable for anaerobic microbial growth.

4.2.2. Pretreatment with Ozone

The experimental set-up for the ozonation process is given in Figure 4.6 (Erol, 2002). Ozone gas was produced from dry air using a Fischer OZ-502 type ozone generator. The gas flow rate was monitored by a

flowmeter on the generator and two rotameters placed after the generator. The temperature during the experiments was kept at 25 °C using a water bath around the reactor. The gas was diffused into water with a glass sparger at the bottom of the reactor. Gas flow rate and the stirring speed was set to the values 150 L/h and 400 rpm, respectively.

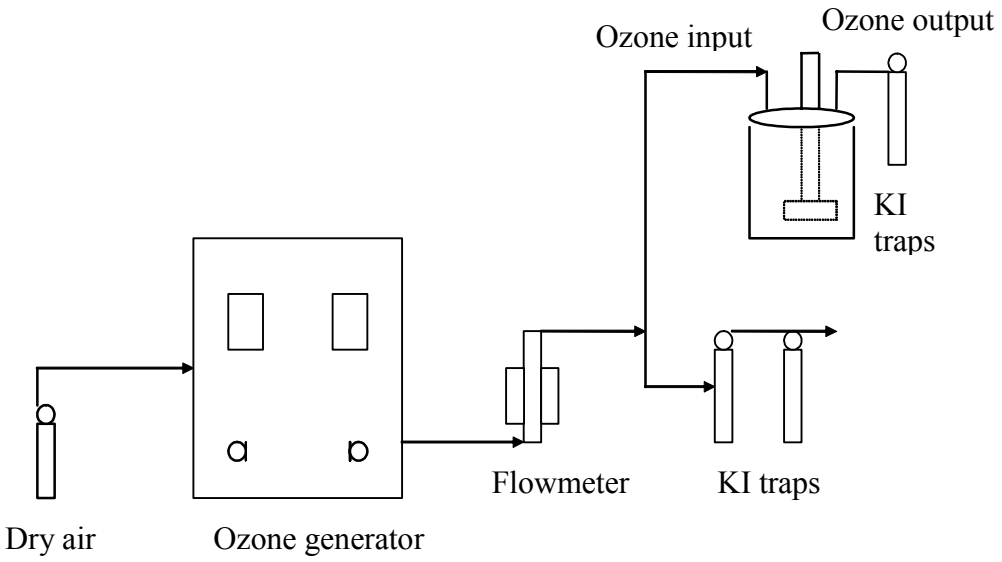


Figure 4.6 Schematic diagram of the ozonation experiment (Erol, 2002)

4.2.3. Pretreatment with Clay

Clay pretreatment process was carried out at pH values around 2 by the addition of HCl to the raw olive mill wastewater to activate clay molecules. Throughout this study, OMW samples were treated with clay samples (Na^{+}- clionite) at several weight fractions (i.e, 0.1, 1, 10, 20 and 50 grams of clay per liters of OMW). Accurately weighed clay material was agitated with raw OMW sample at 250 rpm (5 min) prior to flocculation at 50 rpm (1 hour). At the end, OMW-clay mixture was rested for 2 hours in order to settle down. After settlement, the above liquid was separated from the solids by filtration through a 22 μ

Millipore filter and then subjected to further analysis such as color, COD, organic acid, phenol, sugar and amino acids. Effluent of clay pretreatment process was exposed to further manipulations such as dilution, pH change (6.8), and sterilization before fed into photobioreactors for hydrogen production. Overall flow diagram for hydrogen production with the effluent of clay pretreatment process is shown in Figure 4.7.

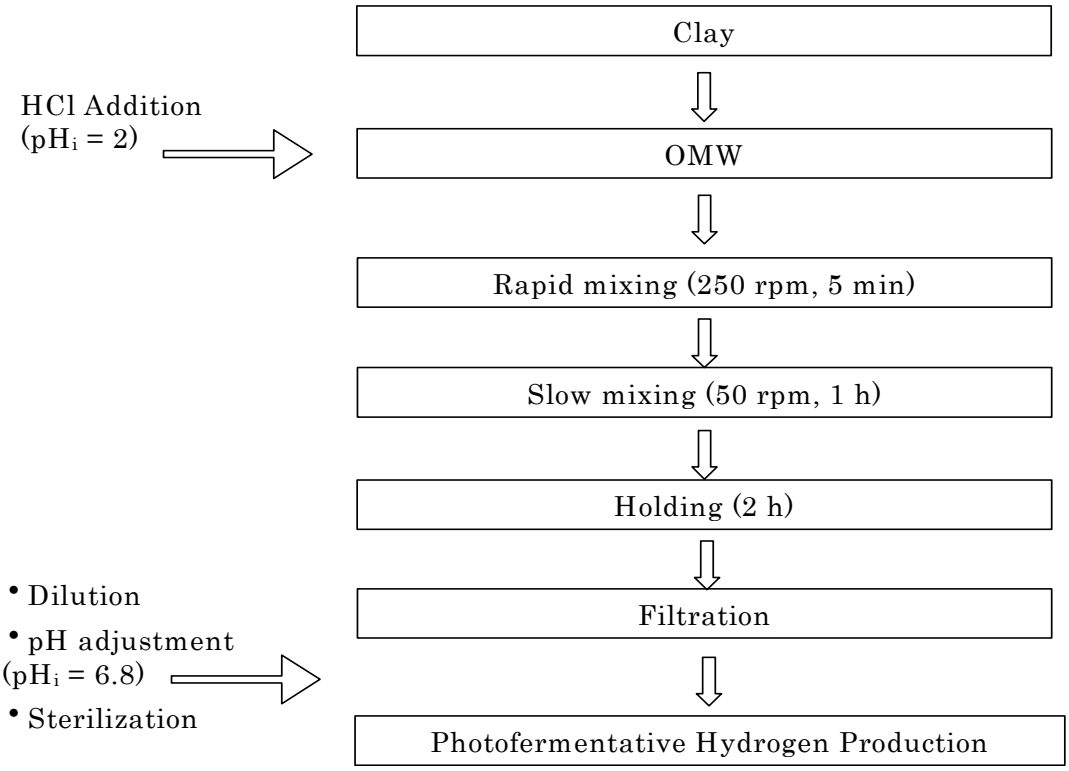


Figure 4.7 Overall flow diagram for clay pretreatment process followed by photofermentative hydrogen production

After the clay pretreatment process, spent clay - OMW complexes were regenerated by washing with water (pH 9) for three times. When the washing process was completed, remaining solid samples were dried at 100 °C for 24 h. Then, regenerated clay was reutilized for the pretreatment of OMW samples.

4.2.4. Pretreatment with Zeolite

OMW was pretreated with Zeolite-4A by following a similar procedure given for the clay material above.

4.2.5. Pretreatment with Fenton's Reagent

The Fenton's reagent was prepared as an aqueous solution of 50 mg/L hydrated ferrous sulfate ($FeSO_4 7H_2O$) and 50 mL/L of hydrogen peroxide (H_2O_2). Then, Fenton's reagent and $KMnO_4$ (1 g/ L) was added to the OMW media in which $KMnO_4$ acts as a catalyst. After precipitation, the above liquid was separated from the solids. Then, its pH was neutralized and rested for 4 hours to remove the significant amounts of H_2O_2 from the media.

4.2.6. OMW Pretreatment with UV Radiation

UV pretreatment method was carried out at room temperature under the radiation of UV_{254nm} lamp (Ultra-Violet Products Inc.) for 1.5 hours, and in the presence of a catalyst $KMnO_4$ (1 g/ L) (Durgakumari *et al.*, 2002).

4.3. Analysis

4.3.1 Evolved Gas Analysis

Composition of the evolved gas was investigated by a gas chromatography (Hewlett-Packard 5890 Series II, 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. All substrates gave hydrogen at high purity. Output gas composition consists of 98.5-

99.5% H_2 and 0.5-1.5% CO_2 for all of the runs. A typical gas analysis chromatogram is given in Appendix C.

4.3.2. Liquid Sample Analysis

4.3.2.1. Cell Concentration Measurements

The cell concentration was obtained by measuring the optical density of culture and also performing the dry cell weight analysis. Measuring the optical density of the culture is one of the simplest and a direct way 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 OMW medium was used as a blank solution.

For the determination of the bacterial dry cell weight, first 1-1.5 mL samples were taken from the reactor and centrifuged (Sigma) at 10,000 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 ^{o}C 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. These reported dry weight values were corrected by subtracting the mass of the pellet, resulting from OMW itself. Thus, fresh OMW media was also subjected to the same drying procedure.

Then, dry cell weight versus OD_{660} calibration was obtained from the samples corresponding to the various points of the growth curve. A sample growth curve and the calibration curve of dry cell weight versus

OD_{660} are given in Appendix B. Calibration shows that OD_{660} value of 1.0 corresponds to 0.58 $g_{dry\ weight}$ / $L_{culture}$.

4.3.2.2. pH Measurements

The pH values of liquid samples were measured with a standard combination of a pH electrode (Mettler Toledo 3311) and an electronic transmitter (Nel pHR-1000 Transmitter).

4.3.2.3. Chemical Oxygen Demand Analysis

Chemical oxygen demand (COD) analysis is widely used as a means of measuring the oxygen equivalent of the organic matter content of the sample, regardless of the biological assimilability. This test includes the oxidation step of organic matter by using a strong chemical oxidizing agent in an acidic medium, and a further colorimetric measurement.

COD values of OMW samples, taken at the beginning and at the end of each hydrogen production experiment, were detected by Micro Digestion Method (Boyles, 1997). Since OMW was filtered before its utilization for biohydrogen experiments, soluble COD values were measured rather than total COD.

COD reagent was prepared by dissolving 9.8 g $K_2Cr_2O_7$, 12 g $AgSO_4$ and 12 g $HgSO_4$ in 1 L concentrated H_2SO_4 solution (95-98%). The solution was mixed about 24-48 hours at room temperature, until its constituents dissolved completely.

The digestion procedure was as follows: OMW sample (2 mL) was mixed with COD reagent (3 mL) within a special COD vial by inverting

it several times, while holding from the cap (the vial becomes very hot during mixing). A blank was also prepared by mixing deionized water (2 mL) with COD reagent (3 mL). Both of the vials were placed in a block-heater (WTW CR-3000 Termoreactor) and were kept at 150 °C for 2 hours. Afterward, the vials were removed and cooled to room temperature. COD value of the OMW sample was detected with a spectrophotometer (Hach, Model DR/2000) at a wavelength 620 nm by selecting the stored program number of 435 (High Range-COD).

4.3.2.4. Biochemical Oxygen Demand Analysis

BOD_5 analysis procedure was taken from Standard Methods for the Examination of Water and Wastewater (Greenberg *et al.*, 1992) – Method 5210, with some modifications. At first, a BOD_5 value was assigned to the OMW sample. Then, dilution ratio was estimated as if the BOD_5 value of the OMW fell within a range assigned in the Standard Methods (Greenberg *et al.*, 1992), with aerated water. Nutrients and bacteria were added to the diluted sample in a BOD_5 bottle (300 mL) and placed in an incubator (Dedeoğlu) working at 20 °C. These were kept for 5 days in the incubator.

A blank solution was also prepared with aerated water and nutrient solutions, without the addition of OMW sample. Then, the DO values of blank and samples were analyzed at the beginning and after 5-day incubation period by titration method. Before titration, 2 mL magnesium sulfate, 2 mL alkali-iodide-azide solution (containing NaOH, NaI and NaN_3), and 2 mL H_2SO_4 were added to the BOD_5 bottles. Then, its content was titrated with sodium thiosulfate solution (0.025 M), till the color of solution becomes light yellow. Afterwards, starch solution (2-3 drops) was added and the color was turned into

dark blue. Titration continued with sodium thiosulfate solution till the color changed from dark blue to colorless.

The amount of consumed sodium thiosulfate solution was related to the DO. The BOD_5 value (mg/L) was calculated from the difference between the final and initial DO values, according to the following equation (Greenberg *et al.*, 1992):

$$BOD_5 = \frac{(D_1 - D_2) - (B_1 - B_2)}{f} . S \qquad (4.1)$$

where,

D_1= DO of diluted sample immediately after preparation, mg/L

D_2= DO of diluted sample after 5-day incubation, mg/L

B_1= DO of blank solution before incubation, mg/L

B_2= DO of blank solution after 5-day incubation, mg/L

f = Volumetric fraction of sample used (Dilution factor)

S= ratio of seed in diluted sample to seed in blank solution. Seed term indicates the amount of bacterial population put into the sample and the control bottle, for oxidizing the biodegradable organic matter. Throughout this study, S was equal to 1.

4.3.2.5. Color Measurements

In order to detect any color change at different stages of the experiments, the color (apparent) of liquid media were analyzed with a Hach spectrophotometer (Model DR/2000) at a wavelength of 455 nm, by selecting the program number of 120. This stored program is calibrated in color units based on the standards of American Public Health Association (APHA). Recommended standard of 1 color unit is equal to 1 mg/L platinum as chloroplatinate ion (PtCo APHA units).

Deionized water was used as a blank solution. Liquid samples were measured without the addition of any reagent, but just diluted with deionized water when the sample was very dark.

4.3.2.6. Light Intensity Measurements

Light intensities of the liquid samples were scanned between wavelengths of 350-1150 nm by a UV-Visible spectrometer (StellarNet EPP2000 VIS 50 Spectrometer). Deionized water was used as a blank solution.

4.3.2.7. Total Phenol Analysis

The Folin-Ciocalteu method was used to assay total phenolic content in this study. The method was originally developed for the analysis of amino acid residues, tyrosine and tryptophane, which was then improved and used by many researchers for the determination total phenolic content (Özcan, 2006). 20 μL liquid sample was mixed with 6.5 mL of deionised water. 0.5 mL of Folin's Reagent (Sigma) and 3 mL of sodium carbonate solution were added to the mixture. Final mixture was kept in shaking water bath at 40°C for 30 minutes. The absorbance was measured at 765 nm by a spectrophotomer (Hitachi-3200 UV-Visible). Results were reported as gram gallic acid equivalents per liter of solvent (g GAE/L). Total phenol content of OMW samples were calculated using the calibration curve obtained by Özcan (2006). That calibration curve for gallic acid equivalent of total phenol concentration is given in Appendix D.

4.3.2.8. Total Sugar Analysis

Total sugar content of olive mill wastewater samples were analyzed by applying Nelson – Somogyi method (Nelson, 1944) cited in Yıldız (2005). According to the experimental procedure given by Yıldız(2005),

liquid OMW samples (2 mL) was mixed with 2 mL of analysis solution(%80 solution A, %20 solution B). Solution A was prepared by dissolving 0.015 g Na-K tartarate, 0.03 g Na_2CO_3, 0.02 g $NaHCO_3$ and 0.18 g Na_2SO_4 in 1 liter of distilled water. On the other hand, 1 L of solution B contains 20 g $CuSO_4$ and 180 g Na_2SO_4.

A blank solution was prepared by distilled water. OMW samples and blank solution were all heated at the water bath up to 100 °C for 20 minutes, then allowed for cooling to a room temperature. After cooling, 1 mL of solution C was added. Solution C was prepared by dissolving 55 g ammonium molybdate and 65 g of $Na_2HAsO_4.7H_2O$ in 1 liter of distilled water. Then, they all mixed with 4.2 mL of H_2SO_4 and 5 mL of distilled water prior to heating at 55 °C for 25 minutes in a water bath. Absorbances of the liquid samples were detected by a spectrophotometer (Hitachi U-3200) at 520 nm. Blank solution was used for the calibration of spectrophotometer. The raw absorbance data obtained from the spectrophotometer was transformed into total sugar content in terms of glucose concentration. In order to obtain glucose concentration, the absorbance values were divided with the calibration constant (0.143) and multiplied with the dilution ratio of the sample. Total sugar content of OMW samples were calculated by using the calibration curve obtained by Yıldız (2005). That calibration curve for glucose concentration is given in Appendix E.

4.3.2.9. Organic Acid Analysis

Liquid samples were initially filtered through a 5μm Nylon filters (R0-000381-55, Varian) for organic acid analysis by HPLC system (Varian ProStar HPLC) in Middle East Technical University's 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. Sample HPLC chromatogram for organic acids is given in Appendix-F.

4.3.2.10. Sugar Analysis

Similar to the organic acid analysis, photobioreactor effluents were exposed to sugar analysis in a HPLC system. All parameters of sugar analysis are same with the ones given for organic acids, except the detector type. ProStar 350 RI was used to detect the column separation. Sample HPLC chromatogram for sugars is given in Appendix-G.

4.3.2.11. Alcohol Analysis

All parameters of alcohol analysis are same with the sugar analysis. Same HPLC column (MetaCarb 87H: 300x 7.8 mm, 5 μm) and the detector (ProStar 350 RI) were sufficient for separating the alcohols present in the OMW samples. Sample HPLC chromatogram for alcohols is also given in Appendix-G.

4.3.2.12. Amino Acid Analysis

Different from organic acid and sugar analysis, liquid samples (20 μL) were analyzed by an Inertsil ODS-3 (250 x 4,6 mm, 5 μm) HPLC column. 0.1 M $NH_4H_2PO_4$ and 1 M of MeCN was used as the mobile phase. The standard analysis of photobioreactor effluent was performed at 40 °C with a mobile phase flow rate of 1 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 (254 nm) was used to detect the column separation. Sample HPLC chromatogram for amino acids is given in Appendix-H.

4.3.2.13. Phenol Analysis

Gas chromatographic analysis of phenols was performed with a Varian CP-3800 GC, equipped with flame ionization detector, split / splitless injector and a 30 m x 0.32 mm x 0.5 μm cross-linked PEG cappilary column (HP-INNOWax). Prior to injection, original solvent extraction technique using n-hegzane was developed. Liquid samples collected from photobioreactor, were initially filtered through a 22 μ Millipore filter paper, and then 0.5 mL of the filtrate was mixed with 0.5 mL n-hegzane. This mixture was centrifuged at 13.000 rpm for 15 minutes (Sigma), and then the supernatant was removed for GC analysis.

The initial temperature of the column was 80 °C for 1 minute followed with a ramp of 15 °C/min and a final temperature of 230 °C for 2 minutes. The temperatures of the injector and detector were 240 °C and 275 °C, respectively. Helium was used as the carrier gas at a flow rate of 25 ml/min. Column head pressure was kept constant for 13 minutes at 12 psi. GC was equipped with a split / splitless injector, an injection volume of 0.5 μL was employed using the autosampler; with

an initial split ratio of 50:1 following a 20:1 ratio after 0.5 minutes. Standard solutions of phenols were prepared by also following the n-hegzane extraction procedure as described above. Sample gas chromatogram for phenols is given in Appendix-I.

4.3.4. By-product Analysis

4.3.4.1. Polyhydroxybutyrate Analysis

Part of the solid residue, obtained after centrifuging the liquid culture at the end of the outdoor hydrogen production experiments, was analyzed for polyhydroxybutyrate (PHB) concentration. These pellets were suspended in 40 mL sodium hypochloride, and incubated at 37 ºC for 1 h. Then, they were centrifuged at 10,000 g (Sorvall RC5C Plus), 4 ºC for 15 min and the supernatant was discarded. 10 mL distilled water was added and centrifuged at 10,000 g for 15 min at 4 ºC, supernatant was discarded again. 5 mL acetone was added and mixed with vortex. It was centrifuged at 10,000 g, 4ºC for 15 min and supernatant was discarded. 5 mL ethanol was added and vortexed. Next, centrifugation was carried out again at 10,000 g, 4ºC for 15 min, supernatant was disposed. Then, 9 mL of chloroform was added. It was kept in boiling water bath for 2 minutes and then waited for cooling. Afterward, it was centrifuged at 10,000 g, 4ºC for 15 min and the supernatant was collected in a graduated cylinder. The volume was completed to 10 mL by the addition of chloroform. Then, that liquid was distributed to 10 test tubes, each containing 1 mL and those were evaporated again. PHB was obtained in the form of white precipitate at the bottom of the tubes. This method was previously studied by Yiğit (1999), Suludere (2001), Tabanoğlu (2002) and Eroğlu (2002); and originated from Bowker (1981).

For the determination of PHB amount, 1 mL sulfuric acid was added to each test tube and these tubes were capped. Then, the tubes were kept in boiling water bath for 10 min and cooled. This provides conversion of PHB to crotonic acid (Bowker, 1981), which absorbs UV light. Then, the average absorbance of these 10 test tubes; by collecting within one tube and making proper dilutions with sulfuric acid for high OD values; was read at 235 nm (Shimadzu UV/Vis Spectrophotometer). PHB concentration was calculated using the calibration curve obtained by Yiğit (1999). That calibration curve is given in Appendix J.

4.3.4.2. Carotenoid Analysis

Total carotenoid amount was analyzed by the previous method (Pietro, 1971) used by Yiğit (1999), Tabanoğlu (2002) and Eroğlu (2002). Remaining part of the solid residue, obtained from the outdoor experiment, was subjected to the carotenoid analysis (some of the solid portion was used for PHB analysis). This pellet was added into the extraction thimbles of Soxilet apparatus. The carotenoid pigments were extracted in the Soxilet apparatus for 12 h with 250 mL acetone. Then, the absorbance of the sample was scanned between wavelengths of 400-550 nm (Unicam UV2-100 UV/Vis Scanning Spectrophotometer).

The total carotenoid amount was then calculated according to the following formula:

$$c = OD_{455} \,.\, v.\, f^{-1}.\, \frac{10}{2500} \qquad (4.2)$$

where, c represents the amount of total carotenoid in mg; OD_{455} is the optical density at 455 nm (there is a maxima at this wavelength); v is the total volume of acetone (250 mL for this study); and f is the

dilution factor. 2500 mL/mg.cm in the formula is an average extinction coefficient for carotenoids (Pietro, 1971). A typical absorption spectrum of the carotenoid extract is given in Appendix K.

4.3.5. Olive Mill Wastewater Analysis

In order to characterize the physical and chemical properties of all OMW samples, the following analyses were performed:

pH, COD, BOD_5, color and total phenol determinations were carried out according to methods described in the previous sections. The density of OMW was detected by weighing the known volume of OMW, with a hydrometer. Elements such as Ca, Mg, Fe, Zn, Mn, Ni, Mo, Co, Cr, Pb and Cu were analyzed by an atomic absorption spectrophotometer (Philips, PU9200X), whereas Na and K were examined by using a flame photometer (Jenway PF P7). Before atomic absorption spectrophotometer and flame photometer analysis, 10 mL of OMW sample was pretreated by filtration through a 22 μ Millipore filter; pH was adjusted below 2 by nitric acid addition; and then sample was diluted to 100 mL by deionized water. Analyzing techniques for these elements are explained properly in Standard Methods (Greenberg *et al.*, 1992). Boron content was detected by the Carmine method (Gamsız and Ağacık, 1976) with a spectrophotometer (Shimadzu UV-1201) at a wavelength of 585 nm.

Total solid (TS) content was detected by weighing the 10 mL OMW sample after drying at 103-105 ºC (Nüve) until weight changes became negligible, as given in Standard Methods (Greenberg *et al.*, 1992) with the number of 2540.B.

After drying the OMW sample for total solid content measurements, the same dried sample was subjected to elemental analyzes for the determination of the elemental carbon, nitrogen and hydrogen contents by an Elemental Analyzer (C-H-N 600, Leco). Then, the molarities of carbon and nitrogen were calculated according to the TS and the elemental analyze results.

4.3.6. Clay Analysis

Cloisite® Na^+ is a natural montmorillonite, and used as a natural clay during our experiments. It was obtained from Southern Clay Products Co[@], which give the possibility to obtain some of its properties (such as particle size and density) from their official web page (20th of April 2006; http://www.nanoclay.com/data/Na.htm). On the other hand, the whole rock analysis of this clay sample was investigated in our laboratories to obtain its elemental contents such as SiO_2, Al_2O_3, Fe_2O_3, Na_2O, Cr_2O_3, P_2O_5, TiO_2, K_2O, MgO, CaO, NiO, ZnO, PbO, CuO, MnO. Atomic absorption spectrophotometer (Philips, PU9200X) was used for the standard rock analysis according to the procedure given by Jackson *et al.*, 1987. In order to obtain its crystalline structure, the layer thickness (basal spacing) was analyzed by a X-Ray Diffractor (XRD). X-ray results of the Cloisite® Na^+ is given in Appendix-L.

4.4. Experimental Planning

a. Four OMW samples (OMW A, OMW B, OMW C, OMW D) were characterized by analysis of the total solid content, biological and chemical oxygen demand, elemental constituents, total phenols, total sugars, and individual organic content (i.e., organic acids, amino acids, simple sugars, alcohols and phenols).

b. Photofermentative hydrogen production capacities of four OMW samples (OMW A, OMW B, OMW C, OMW D) were determined by following the procedure given below.
    1) OMW samples were diluted only with distilled water to 4% by volume (without addition of any other nutrients or trace elements).
    2) Initial pH of all media was around 6.8 - 7.0.
    3) After dilution and pH adjustment, samples were sterilized at 121 ºC.
    4) Rubber-tapered glass bottles with 55 ml of volume were used.
    5) 10% (v/v) of the pre-activated *Rhodobacter sphaeroides* O.U.001 culture was inoculated to the reactor.
    6) Temperature was kept constant at 30-32ºC.
    7) Illumination was achieved with 150 W tungsten lamp, in order to obtain a light intensity of approximately 200 W/m^2 at the front of the reactor.
    8) Anaerobic atmosphere was achieved by fulfilling of the photobioreactors.
c. OMW-D was further investigated in two stage processes. Raw OMW-D sample was pretreated for decolorization by several physicochemical methods such as physical adsorption with clay or Zeolite-4A, chemical oxidation with strong oxidants like Fenton's reagent or ozone, and photooxidation by UV radiation. On the other hand, dark fermentation with sewage sludge (either acclimated or not) was carried out as a biological pretreatment technique. As second stage, photofermentative hydrogen production capacities of the effluents of pretreatment were determined by following the procedure given above.
d. For two stage processes, the significance of clay quantity utilized during clay pretreatment process on the photofermentative

hydrogen production capacity was determined by using different concentrations of clay (0.1, 1, 10, 20 and 50 grams of clay per liters of raw OMW-D).

e. Clay used in pretreatment process was regenerated by washing with water and drying. The photofermentative hydrogen production capacity of the samples pretreated with regenerated clay was investigated.

f. Consumption of organic acids, phenols, amino acids, sugars and alcohols were investigated by detailed analysis of the samples taken at different time intervals during the photofermentation process. Four parallel runs were carried out. Two of them was with untreated OMW-D (single stage operation), and the other two was with clay pretreated OMW-D (two-stage operation).

g. For two stage processes, the significance of dilution ratio of clay pretreatment effluent (4%, 50% and 100%) was investigated. Clay pretreatment processes were done with the addition of 20 g/L clay into raw OMW-D.

h. The photofermentative hydrogen production capacity was determined in an 8 L solar bioreactor in order to investigate the effect of scale-up and outdoor conditions with single stage process utilizing diluted OMW-A (4%) as a sole substrate. The yields of valuable by-products (i.e., carotenoid and polyhydroxybutyrate) were also determined.

CHAPTER 5

RESULTS AND DISCUSSION

5.1. Comparison of Photofermentative Hydrogen Production by OMW Coming from Different Sources

Olive mill wastewater samples utilized throughout this study were obtained from four different olive-oil mills in Western Anatolia. Since their physico-chemical properties depend on local and seasonal factors (i.e., type of processed olive fruit; oil extraction technique; harvesting time; cultivation area and etc.), the detailed analysis of each sample is extremely essential for such a comparative study. Main characteristic properties of the OMW samples are given in Table 5.1.

As can be seen from Table 5.1, all OMW samples are slightly acidic with pH values around 4. They have significant amounts of COD, BOD_5, solid matter, phenol and sugar content, in which their color is proportional with the amount of their solid matter and phenolic content. The sample (OMW-A) having the darkest color (79250 PtCo APHA) also contains the highest total phenol (18.9 g/L) and total solid content (35.9 g/L). Likewise, the lightest color of OMW-C (47500 PtCo APHA) is attributed to its lowest phenol (7.9 g/L) and solid (12.2) content. Solid particles such as the olive fruit residues (i.e. olive pulp, husk, and some lignin derivatives) are known to be consisted of hardly biodegradable and dark colored aromatic compounds (Hamdi, 1992).

On elemental basis, all samples contain relatively higher amounts of K, Ca, Na, Mg and Fe. According to the C/H/N elemental analysis on dry basis, the molar C/N ratio followed a decreasing order as: OMW-D (73.8) > OMW-A (59.5) > OMW-B (48.4) > OMW-C (42.3). Calculation of this parameter is essential for photosynthetic hydrogen production studies, since hydrogen production is known to be favored for the substrates with higher C/N ratio (Eroğlu *et al.*, 1999).

According to the detailed HPLC and GC analysis, it becomes possible to determine the main organic constituents of each OMW samples. On the basis of organic acids, they all contain relatively higher amount of acetic acid; upon which aspartic and glutamic acids are the main amino acids. Besides, ethanol and either normal-phenol or meta-cresol are present at higher levels with respect to the other alcohols and phenols, correspondingly.

Rhodobacter sphaeroides OU001 was grown in these different OMW samples for photofermentative hydrogen production. Volumetric ratio of raw OMW in the photofermentor inlets was 4%, owing to the results of a previous study examining the optimum OMW dilution rates for an efficient hydrogen production (Eroğlu *et al.*, 2004). The experimental data are given in Appendix M1.

Figures 5.1 and 5.2 illustrate the changes in the total volume of hydrogen gas production and bacterial growth at different OMW samples, respectively. The volumetric ratio of raw OMW in the photofermentor inlet was 4%, owing to a previous study examining the optimum OMW dilution rates for an efficient hydrogen production (Eroğlu *et al.*, 2004). Another parameter that gained an importance in biohydrogen studies is the ratio of hydrogen gas production per bacterial weight, which is given in Figure 5.3.

Table 5.1 Characteristics of different olive mill wastewater samples

Property	Unit	OMW(A)	OMW (B)	OMW (C)	OMW(D)
Region	-	Balıkesir - Burhaniye	Balıkesir - Edremit	İzmir- Bornova	İzmir - Bornova
Process Type	-	Centrifugal	Centrifugal	Centrifugal	Traditional
pH	-	4.01	4.23	4.56	4.14
Density (24°C)	g/cm^3	1.02	1.01	1.01	1.01
COD	g/L	66.8	55.4	52.2	52.1
BOD_5	g/L	20.2	19.5	17.9	23.8
Total Solid	g/L	35.9	15.5	12.2	17.8
Total phenol	g/L	18.9	9.6	7.9	12.2
Total sugar	gL	16.2	8.98	6.72	15.1
Color	PtCo APHA	79,250	52,000	47,500	61,500
[C] / [N]	M / M	59.5	48.4	42.3	73.8
ELEMENTS					
K	g/L	4.20	6.72	7.81	2.68
Ca	g/L	0.06	0.15	0.55	0.13
Na	g/L	0.98	1.12	0.41	0.57
Mg	g/L	0.12	0.18	0.28	0.09
Fe	mg/L	51.3	38.8	29.5	13.5
Zn	mg/L	1.90	4.34	9.50	2.01
Mn	mg/L	1.91	1.78	2.50	1.09
B	mg/L	5.22	5.03	2.49	4.93
Ni	mg/L	1.00	0.80	0.60	0.10
Mo	mg/L	2.5	2.8	0.34	1.78
Co	mg/L	0.4	0	0	0
Cr	mg/L	2.5	1.2	0	0
Pb	mg/L	2.4	1.1	0	0
Cu	mg/L	0.20	0.31	0	0.34
ORGANICS					
Glucose	g/L	0.85	0.88	0.42	0.91
Xylose	g/L	0.51	0.78	0.24	0.67
Arabinose	g/L	0.08	0	0.04	0.13
Mannitol	g/L	0.09	0	0	0.07
Methanol	g/L	0.36	0.21	0.11	0.21
Ethanol	g/L	1.87	1.23	0.92	2.32
Acetic Acid	g/L	6.99	5.09	3.95	9.71
Formic acid	g/L	0.45	0	0	0.27
Propionic acid	g/L	1.23	1.17	0.67	1.80
Butyric Acid	g/L	1.41	1.09	0.82	1.95
Lactic Acid	g/L	0.79	0.46	0.54	1.14
Phenol	g/L	1.98	1.44	0.78	2.34
p-cresol	g/L	1.51	0.88	0.56	1.38
m-cresol	g/L	1.88	1.92	1.02	2.56
o-cresol	g/L	1.45	0.98	0.23	0.97
Aspartic acid	g/L	9.45	5.12	2.08	7.32
Glutamic acid	g/L	8.99	7.56	1.92	7.65
Tyrosine	g/L	3.87	1.04	0.63	2.45

Total amount of hydrogen gas production was within the range of 40 – 24 mL (Figure 5.1). The highest amount of hydrogen was obtained from the OMW sample (OMW-D), having the highest organic content (especially acetic, aspartic acid, and glutamic acids) in parallel to its highest carbon to nitrogen molar ratio (73.8) as tabulated in Table-1. Higher light penetration capacity of a medium is known to enhance photofermentation (Eroğlu *et al.*, 2004) nonetheless it is not an adequate parameter for an efficient hydrogen production. Accordingly, the medium (OMW-C) with the brightest color (47500 PtCO APHA) resulted relatively lowest hydrogen production due to its lowest organic content. On the contrary, darkest medium (OMW-A) resulted significantly higher bacterial growth (Figure5.2) due to the probable shift into different metabolic pathways (i.e., dark fermentation) in addition to photoheterotrophic growth. Also, higher nitrogen content coming from amino acids might force the bacteria for nitrogen fixation rather than producing hydrogen (Sasikala *et al.*, 1990). During the photobiological H_2 production processes with PNS bacteria, hydrogen formation is completely related with the action of nitrogenase enzyme. In the presence of molecular nitrogen, this enzyme catalyzes nitrogen fixation whereas it channels the hydrogen production in the absence of molecular nitrogen (Miyake *et al.*, 1989).

OMW-D has significantly higher ratio of hydrogen gas production per bacterial weight (Figure 5.3), which enhances its eligibility for being utilized as a substrate source during photofermentative hydrogen production studies. Besides, the principal motto is to produce hydrogen rather than producing the highest amount of bacteria. No significant differences were observed between pH values of these OMW samples (Figure 5.4). The larger increment in the pH was observed for the media (OMW-D) resulting the highest bacterial growth. This might be

resulted due to the probable accumulation of carbonate ions in parallel to the dark fermentative carbondioxide production and its fixation during bacterial growth. In order to fix CO_2; the membrane bound hydrogenase enzyme (uptake hydrogenase) is known to produce the reducing power required for CO_2 fixation, by splitting the outside hydrogen into protons and electrons (Tabita, 1995).

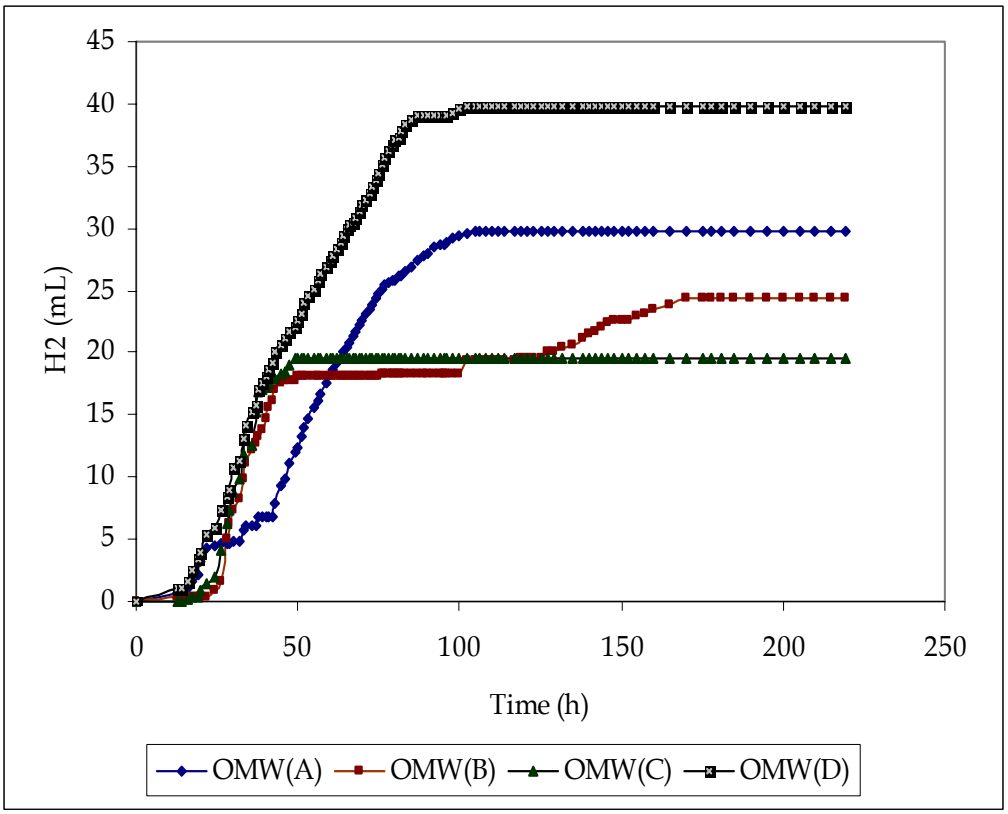


Figure 5.1 Total volume of hydrogen gas production versus time for different OMW samples. [The volumetric ratio of raw OMW, fed to the photobioreactors was 4% (v/v)]

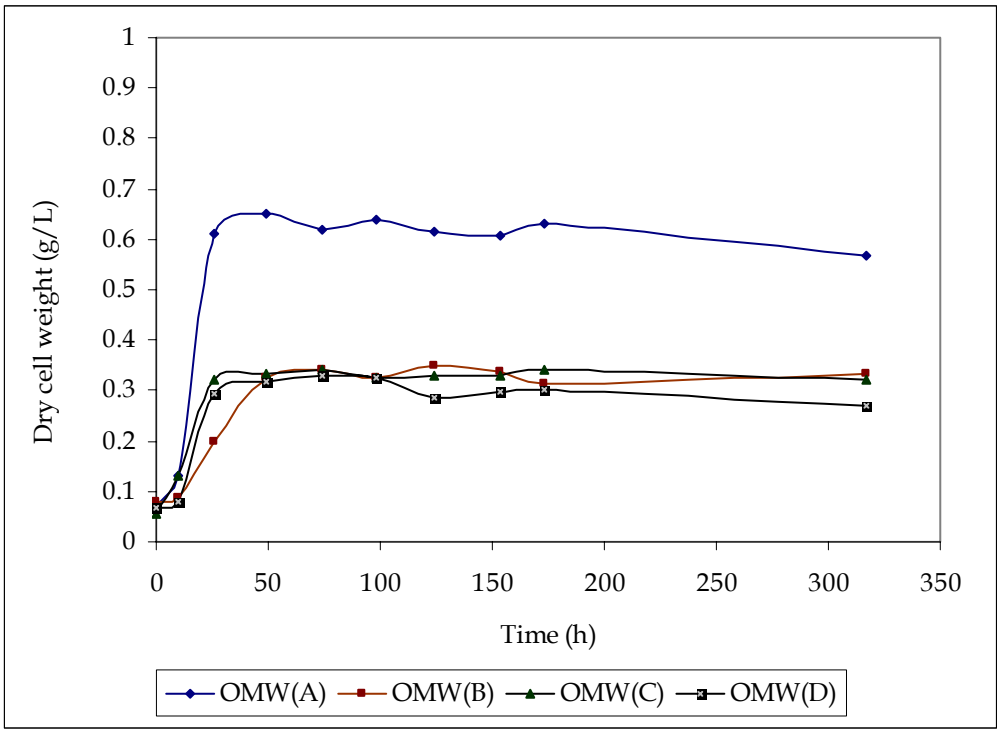


Figure 5.2 Bacterial growth curves for different OMW samples. [The volumetric ratio of raw OMW, fed to the photobioreactors was 4% (v/v)]

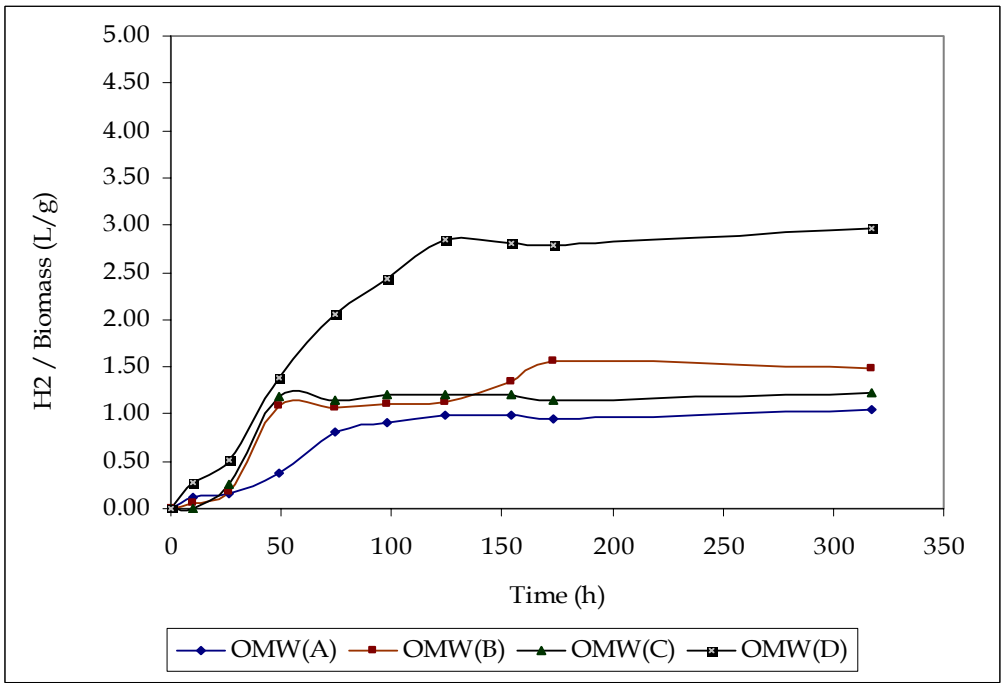


Figure 5.3 Total amount of hydrogen gas production per weight of bacteria grown at different OMW samples. [The volumetric ratio of raw OMW, fed to the photobioreactors was 4% (v/v)]

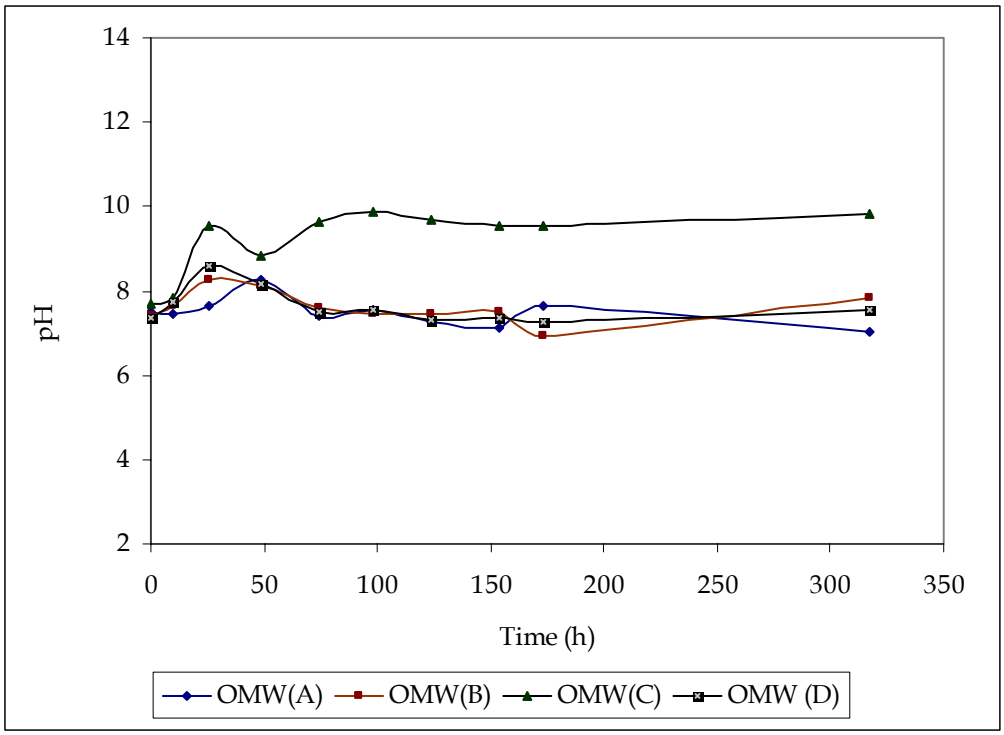


Figure 5.4 pH change versus time for different OMW samples. [The volumetric ratio of raw OMW, fed to the photobioreactors was 4% (v/v)]

Figures 5.5 and 5.6 represent the light absorption spectra of raw OMW samples and *Rhodobacter sphaeroides* O.U.001, respectively. As observed in Figure 5.6, this bacterium absorbs light energy at a wide range of light spectrum including the non-visible parts (i.e., 400 to 950 nm). It is also known that *R. sphaeroides* contains the photosynthetic pigments of bacteriochlorophyll a (absorption maxima: 372-375, 586-588, 800-805, 850-852 and 870-875 nm for living cells), and carotenoids of spheroidene series (absorption maxima: 414-416, 446-450, 474-481 and 507-508 nm for living cells) (Pellerin and Gest, 1983). Nonetheless, these wavelengths are also absorbed by the dark OMW samples (especially OMW-A and OMW-D), which would have a shadowing effect on the photosynthetic bacteria (Figure 5.5). Accordingly, color depletion (i.e., by dilution, or by

various pretreatment techniques) is extremely essential for an effective photofermentation process.

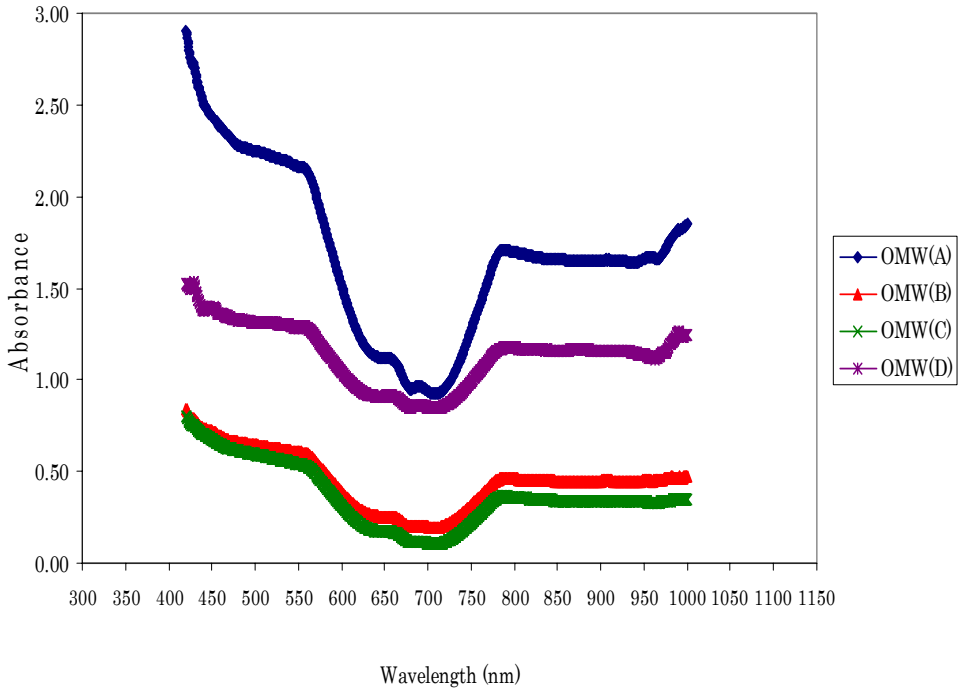


Figure 5.5 Light absorption spectra of different raw OMW samples (100% (v/v))

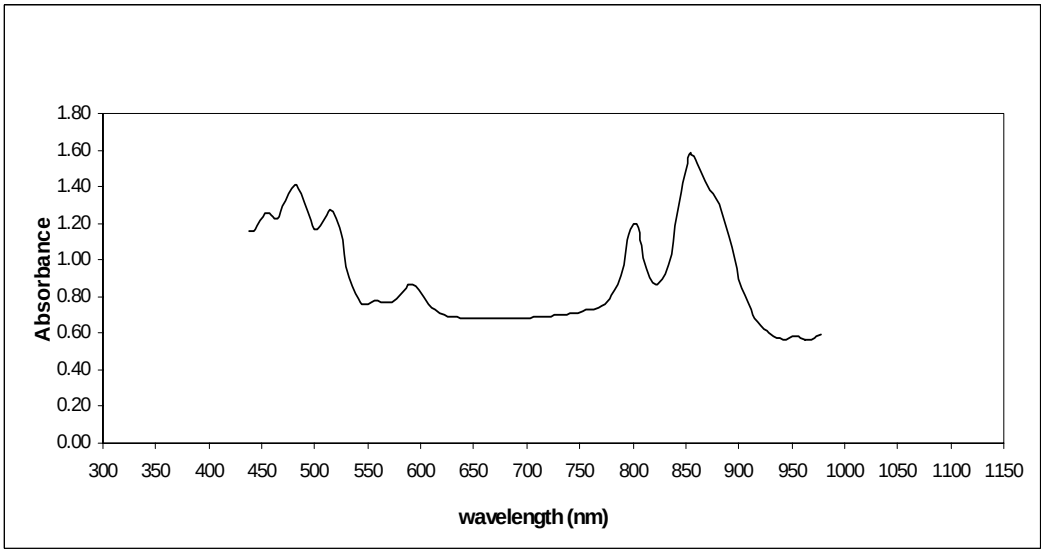


Figure 5.6 Light absorption spectrum of *Rhodobacter sphaeroides* O.U.001

The results of photofermentative H_2 production with different OMW samples are summarized in Table 5.2. One of the important aspects on the photobioreactor design is the light conversion efficiency (η), which depends on light intensity, irradiated area, duration time of H_2 production and total amount of H_2 production (Miyake and Kawamura, 1987). It is the ratio of the total energy value of the obtained hydrogen (heat of combustion) to the total energy input of the photobioreactor by solar radiation. The light conversion efficiency (η) is calculated with the following formula (Miyake and Kawamura, 1987):

$$\eta\ (\%) = \frac{33.61 \,.\, \rho_{H2} \,.\, V_{H2}}{I \,.\, A \,.\, \Delta t_{H2}} \qquad (5.1)$$

where V_{H2} is the volume of produced H_2 in liters, 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 Δt_{H2} is the duration of hydrogen production in hours. Photofermentation with OMW-C sample resulted higher light conversion efficiency (0.33 %) due to its brightest color that increases its light absorption capacity.

Two expressions were used for the determination of gas production rate within the present study. The first is the average gas production rate per culture volume (r_g), which is calculated by dividing the total volume of gas produced by the volume of the culture and by the duration of gas production, with the unit of L/L/h. The second expression is the average gas production rate per bacterial dry weight (r_g^1) that is obtained by the time averaging of individual rates and has the unit of L/g/h. The individual rates for a certain period were calculated by dividing the volume increment of gas produced by the average cell concentration and by the duration of that period.

Rate values of the current study are comparable with the rates mentioned in the literature. In the present study, r_g and $r_g{}^1$ results are within a range of 0.007-0.014 L/L/h, and 0.012-0.040 L/g/h, respectively. Sunita and Mitra (1993) found a rate of 0.002 L/L/h from the sewage wastewater by the free cells and by the immobilized cells with a rate of 0.003 L/L/h. Sasikala *et al.* (1991) observed a hydrogen production rate of 0.005 L/L/h and 0.002 L/g/h with 50% wastewater of lactic acid fermentation plant, using free *R. sphaeroides* O.U.001. Also, Zhu *et al.* (1999) calculated a relatively higher hydrogen production rate of 0.059 L/L/h with tofu wastewater. Immobilization process and the utilization of different strain (i.e., *R. sphaeroides* RV) might be the reasons for their higher rates. Türkarslan *et al.* (1998) obtained 0.006 L/g/h with the mixture of malate and diary plant wastewater. Koku *et al.* (2003) reported a maximum rate of 0.018 L/g/h, in malic acid and sodium glutamate media. Eroğlu *et al.* (2004) observed the maximum rates (0.009 L/L/h and 0.021 L/g/h) for the case of 1% OMW containing media, using *R. sphaeroides* O.U.001 in 400 mL column photobioreactors.

The hydrogen production potential (HPP), which is defined as total hydrogen gas produced per volume of OMW, has a highest value of 20 L/L for the OMW-D sample in addition to its lowest lag time (10 h). The hydrogen production potentials determined in this study, are relatively higher than the literature. For example, Sasikala *et al.* (1991) reported a potential of 4.5 L /L for 10% lactic acid fermentation plant wastewater. In another study of the same group (Sasikala *et al.*, 1992), 1.0 L/L and 3.0 L/L was obtained for the case with free cells, and with immobilized cells in 10% distillery wastewater, respectively. Zhu *et al.* (1999) determined HPP as 1.9 L/L for tofu wastewater, utilizing

immobilized cells in agar gels. In our previous study with olive mill wastewater, we reported the highest potential as 14 L/L in a 400 mL column photobioreactors (Eroğlu *et al.*, 2004). Yetiş *et al.* (2000) observed 8.6 L/L HPP values by the supplementation of malate into sugar refinery wastewater. As a consequence, hydrogen production from OMW appears to be a favorable process.

As regards to its relatively highest amount of hydrogen production in parallel to its organic acid rich medium, OMW-D sample was chosen to be utilized during further photofermentative hydrogen production studies reported at the rest sections of Chapter 5.

Table 5.2 Comparison of different OMW samples on the context of hydrogen production parameters

Photofermentation Broth	**Property**						
	X_{max} (g/L)	t_{lag} (h)	Δt_{H2} (h)	HPP (L / L)	r_g (L L^{-1} h^{-1})	r_g^1 (L g^{-1} h^{-1})	η (%)
4% raw OMW (A)	0.64	12	95	14.9	0.007	0.012	0.18
4% raw OMW (B)	0.35	12	158	12.2	0.014	0.044	0.14
4% raw OMW (C)	0.34	16	33	9.8	0.012	0.041	0.33
4% raw OMW (D)	0.32	10	92	19.9	0.011	0.040	0.25

5.2. Effect of Pretreatment Process on Photofermentative Hydrogen Production

The previous results showed that the enhancement of the system is noticeably needed due to the dark color and inhibitory effects of raw OMW samples. These kinds of properties lead to the depletion of light absorbance and H_2 production capability of the photosynthetic bacteria. The characteristic black-brownish color of this effluent is due to the slowly biodegradable compounds such as polyphenols, which are difficult to remove. Hence, an important step in the decolorization of the olive oil wastewater is the breakdown of colored polymeric phenolics to monomers (Yeşilada *et al.*, 1999).

The aim of this part of the work is to search different pretreatment techniques for both color and phenol removal, and to investigate their relative effects on photofermentative hydrogen production by *Rhodobacter sphaeroides* O.U.001. At first, either physicochemical (i.e., clay, zeolite 4A, ozone, Fenton's reagent and UV radiation) or biological (dark fermentation) pretreatment processes are compared with each other on the basis of their OMW remediation capability in Section 5.2.4. Then, photofermentative hydrogen production results obtained from the effluents of physicochemical and biological pretreatment processes are given in Sections 5.2.2 and 5.2.3, respectively. To sum up, all of these results are comparatively discussed in Section 5.2.4.

5.2.1. Decolorization of Olive Mill Wastewater with Different Pretreatment Techniques

Some physicochemical methods such as physical adsorption with clay or Zeolite-4A, chemical oxidation with strong oxidants like Fenton's reagent or ozone, and photooxidation by UV radiation were

investigated for the decolorization of OMW. On the other hand, dark fermentation with sewage sludge (either acclimated or not) was chosen as a biological pretreatment technique. As previously stated in Section 5.1, raw OMW-D sample was utilized throughout these studies.

Pretreatment processes with ozone or with Fenton's reagent are alike as regards to their highest removal efficiencies for color (85% and 78%, respectively), total phenol (91%) and COD (%67 and 62%, respectively) as shown in Table 5.3. This was observed, because these strong oxidants selectively oxidized phenols and other polymeric complexes present in OMW. Clay adsorption (65%) and dark fermentation processes (67%) are also effective for color and phenol removal.

The acclimation of sewage sludge to olive mill wastewater was performed to provide the adaptation of microorganisms to hardly biodegradable compounds such as phenols. It was observed that the acclimation of biomass to the wastewater components improves the efficiency of biological pretreatment process. In other words, total phenol removal (78%) and COD degradation (43%) efficiencies were significantly increased (Table 5.3) as a result of relatively higher organic matter consumption by several microorganisms inside the sewage sludge culture.

For a visual consideration of these pretreatment results, the changes in the color of raw OMW sample(4) after treated with Fenton's reagent(1), clay adsorption(2), and UV radiation(3) processes are given in a photography (Figure 5.7).

Table 5.3 Color, COD and total phenol changes after pretreatment of raw OMW (100% (v/v)) with different processes

Pre-trt. Process	$Color_i$ (PtCo APHA)	$Color_f$ (PtCo APHA)	Color Remv. (%)	COD_i (g/L)	COD_f (g/L)	COD Remv. (%)	Total $phenol_i$ (g/L)	Total $phenol_f$ (g/L)	Total phenol remv. (%)
Clay	61500	21500	65	52.1	35.9	31	12.2	2.3	81
Zeolite-4A	61500	35600	42	52.1	37.0	29	12.2	3.8	69
Ozone	61500	9200	85	52.1	17.2	67	12.2	1.1	91
Fenton's Reagent	61500	13500	78	52.1	19.8	62	12.2	1.1	91
UV Radiation	61500	47300	23	52.1	31.8	39	12.2	6.6	46
Dark ferme. (sewage sludge)	61500	22100	64	52.1	33.9	35	12.2	4.8	61
Dark ferm. (acclimated sewage sludge)	61500	20300	67	52.1	29.7	43	12.2	2.7	78

(1)	(2)	(3)	(4)
Fenton's Reagent	Clay	UV	Raw OMW

Figure 5.7 Changes in the color of raw OMW (100% (v/v)) samples after different pretreatment processes

5.2.2. Photofermentative Hydrogen Production with the Effluents of Physicochemical Pretreatment Processes

Rhodobacter sphaeroides O.U.001 was grown in the effluents of different physicochemical pretreatment processes for photofermentative hydrogen production. Volumetric ratio of either pretreated or raw OMW (as a control) samples in the photofermentor inlets was 4%. The experimental data are given in Appendix M2.

The total volume of hydrogen gas production, bacterial growth, hydrogen to bacterial weight ratio and pH changes of these media are all given in consecutive figures (Figure 5.8, 5.9, 5.10 and 5.11,

respectively). As can be seen from Figure 5.8, clay pretreatment processes significantly enhance hydrogen production. Compared to the photofermentation with raw OMW (32 mL), the amount of hydrogen production was improved by %100 as a result of photofermentation with the effluent of clay pretreatment process (63 mL). Despite the fact that strong chemical oxidants like ozone and Fenton's reagent had a powerful effect on the color removal, their effluents were observed to be unsuitable for both hydrogen production and bacterial growth. Trace amounts of ozone or H_2O_2 (in Fenton's Reagent) remaining in the media appears to be toxic for the bacteria. Besides, the final oxidation products might have additional inhibition effects on the photosynthetic bacteria.

According to the total amount of hydrogen gas production (Figure 5.8) and its ratio to bacterial mass (Figure 5.10), photofermentation with the effluent of clay treatment showed a two-step hydrogen production mechanism. Between the 50^{th} and 90^{th} hours, hydrogen was produced exponentially. However, hydrogen production was stopped till the end of 160^{th} hour, following a second hydrogen evolution stage between 160^{th} and 200^{th} hours. The latest stage is mainly caused by the self utilization property of bacteria for both growing and hydrogen production purposes. It usually happens when the carbon source in the media is significantly depleted for hydrogen production (Koku, 2001).

Compared to the untreated-raw OMW case, all pretreatment effluents had lower bacterial growth (Figure 5.9) due to the removal of some biodegradable carbon sources together with the colorful and hardly biodegradable compounds. No significant pH variation was observed during photofermentation processes with the pretreatment effluents (Figure 5.11).

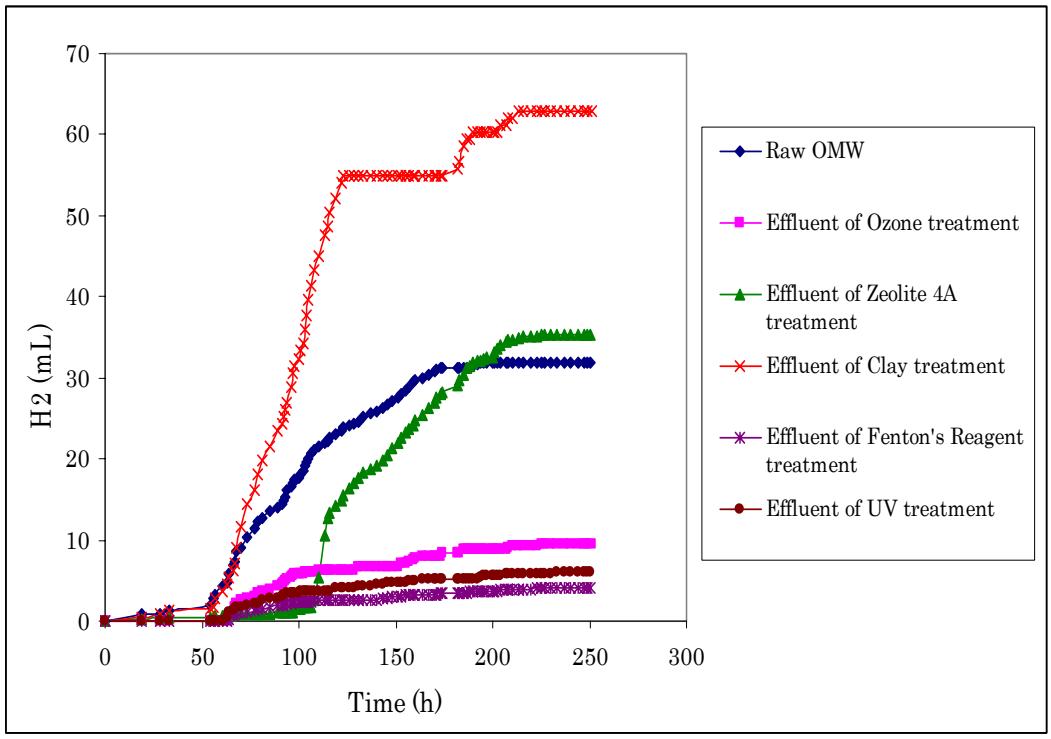


Figure 5.8 Total volume of hydrogen gas production versus time for the effluents of different pretreatment processes. [The volumetric ratio of either the effluents of pretreatment processes or raw OMW, fed to the photobioreactors was 4% (v/v)]

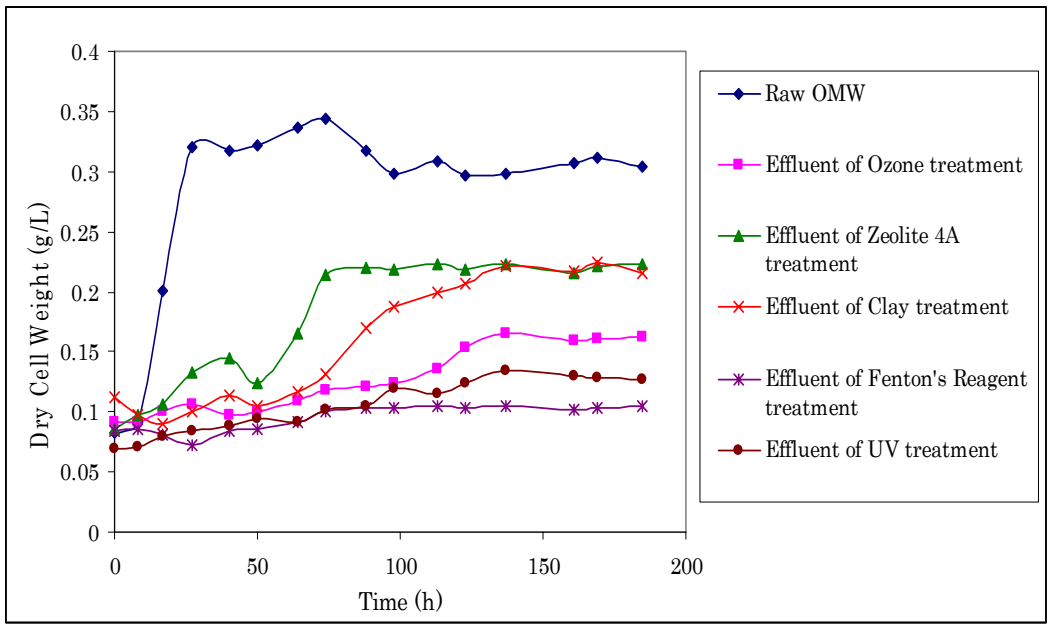


Figure 5.9 Bacterial growth curves for the effluents of different pretreatment processes. [The volumetric ratio of either the effluents of pretreatment processes or raw OMW, fed to the photobioreactors was 4% (v/v)]

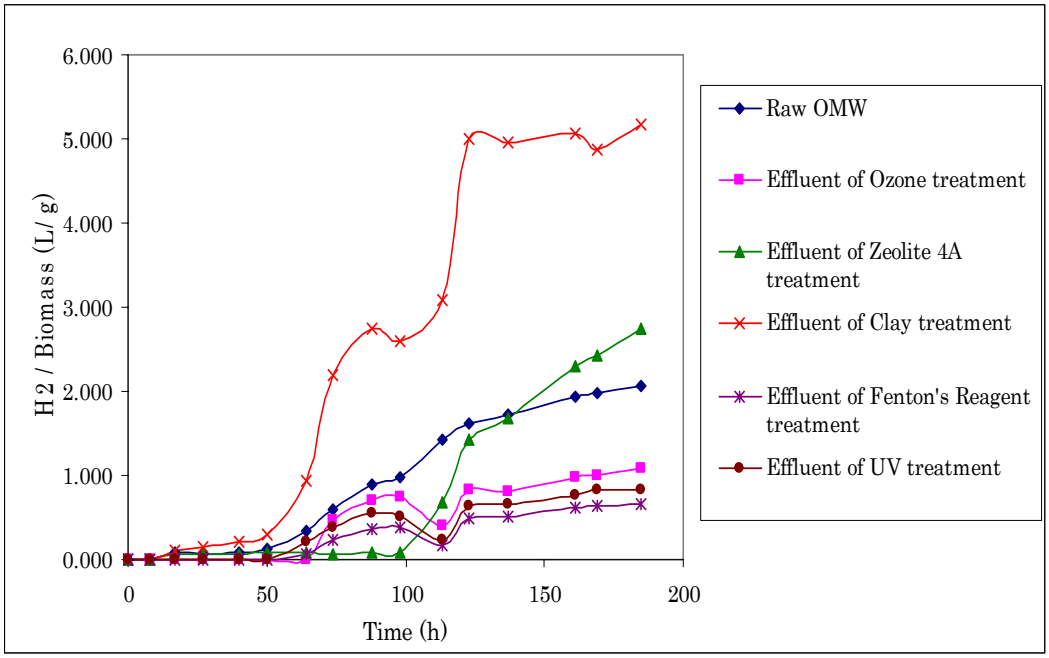


Figure 5.10 Total amount of hydrogen gas production per weight of bacteria grown at the effluents of different pretreatment processes.[The volumetric ratio of either the effluents of pretreatment processes or raw OMW, fed to the photobioreactors was 4% (v/v)]

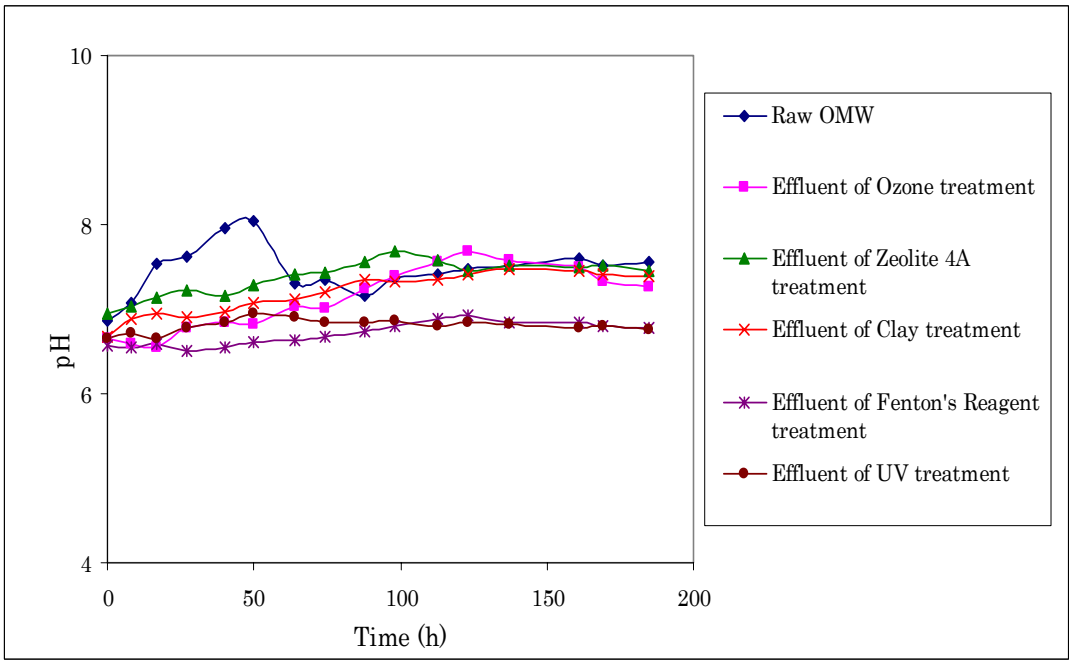


Figure 5.11 pH change versus time for the effluents of different pretreatment processes. [The volumetric ratio of either the effluents of pretreatment processes or raw OMW, fed to the photobioreactors was 4% (v/v)]

5.2.3. Photofermentative Hydrogen Production with the Effluents of Dark Fermentation

This two-stage process involves dark-fermentation followed by a photofermentation process. Dark-fermentation by sewage sludge culture and photofermentation by *Rhodobacter sphaeroides* O.U.001 were both performed under anaerobic conditions. In some cases of dark-fermentation, sewage sludge was initially acclimatized to the olive mill wastewater to provide the adaptation of microorganisms to the extreme conditions of OMW. The experimental data are given in Appendix M3.

Figures 5.12 and 5.13 illustrate the changes in the total volume of hydrogen gas production and bacterial growth at different times for two-stage bioprocesses. In addition pH variations are given in Figure 5.14.

Compared to the photofermentation with 4 % (v/v) OMW (31 mL) containing media, hydrogen production was enhanced by %50 (52 mL) for the case of photofermentation with 4 % (v/v) effluent of dark fermentation with sewage sludge (Figure 5.12). This might be a result of organic acid production during dark fermentation step that is known to be a good substrate by the photosynthetic bacteria for hydrogen production.

As a result of acclimation method, the utilization of concentrated OMW samples (100%) for hydrogen production could be achieved by two-step processes. Compared to the photofermentation results obtained with 50 % (v/v) effluent of dark fermentation, the concentrated media (100% (v/v) effluent of dark fermentation with acclimated sewage sludge)

resulted in lower hydrogen production and bacterial growth (Figure 5.13). This can be caused by the presence of relatively higher amounts of toxic compounds in the feedstock due to the lower dilution rates. Their pH values were around 7 and not an important fluctuation was observed during photofermentation processes with the dark fermentation effluents (Figure 5.14).

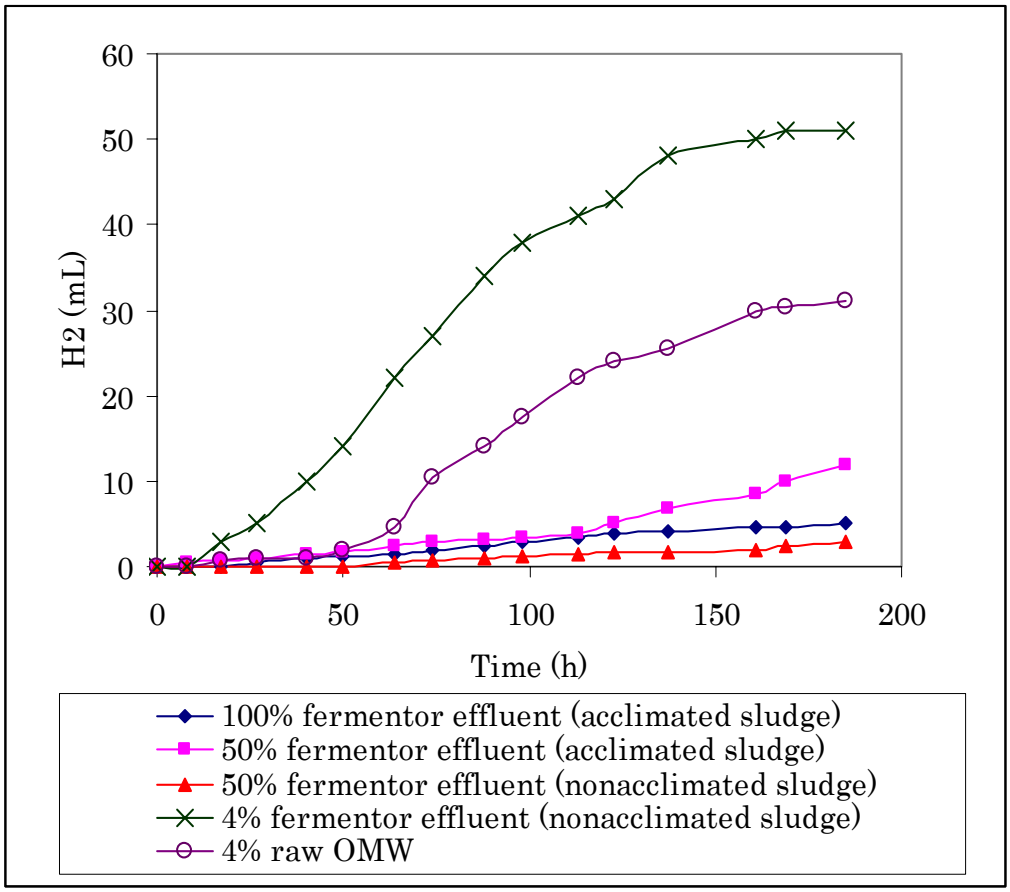


Figure 5.12 Total volume of hydrogen gas production versus time for the effluents of dark fermentation

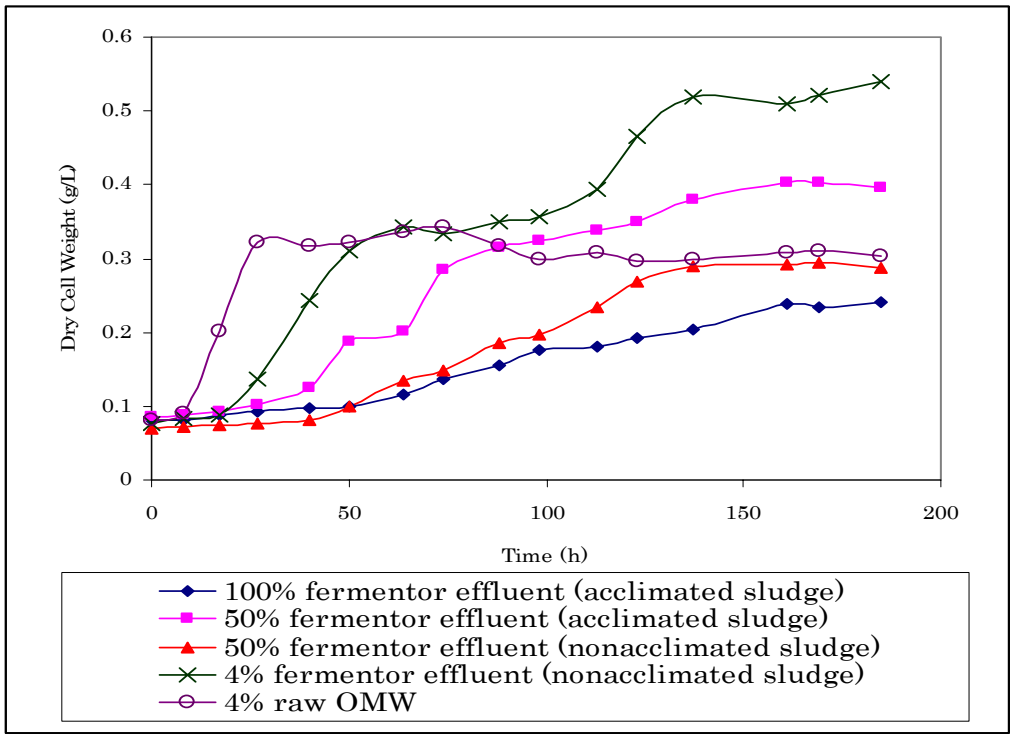


Figure 5.13 Bacterial growth curves for the effluents of dark fermentation

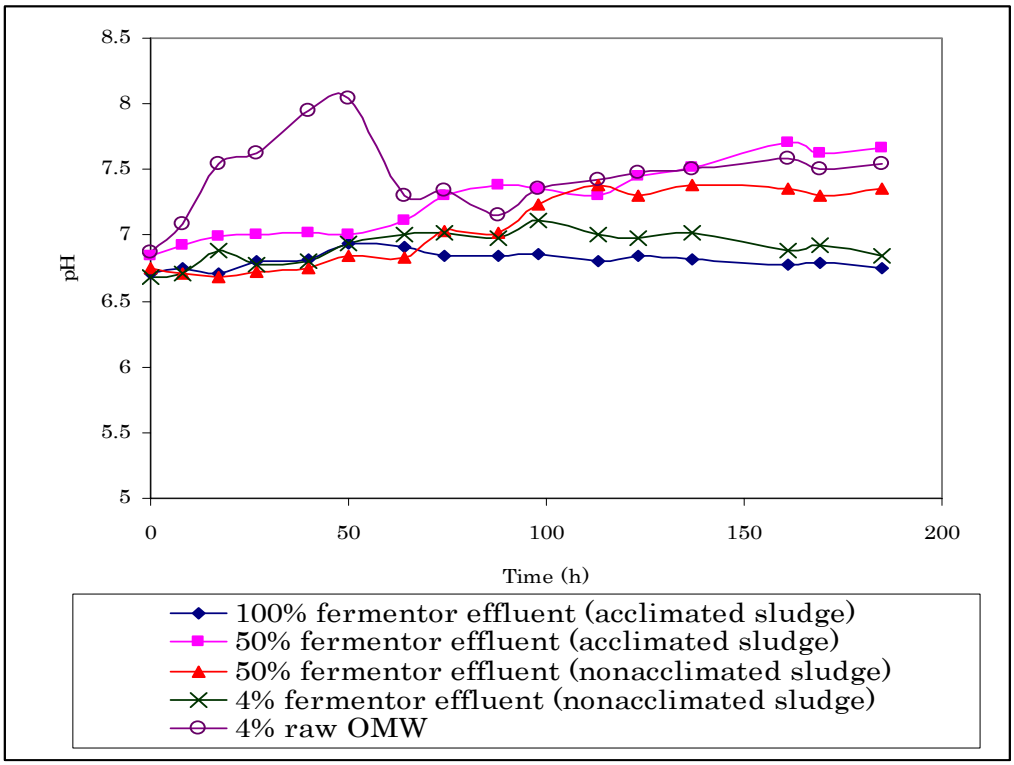


Figure 5.14 pH change versus time for the effluents of dark fermentation

5.2.4. Photofermentative Hydrogen Production with the Effluents of Pretreatment Processes

The results of photofermentative H_2 production either with raw OMW and the effluents of different pretreatment processes are given in Table 5.4. According to the highest hydrogen production (32 L/L) and hydrogen production rates (0.016 L/L/h and 0.084 L/g/h); two stage processes with clay pretreatment appears to be a good method for the hydrogen production techniques alone.

Although treatment with ozone and Fenton's reagent significantly removed the dark color of OMW, it has inhibitory effects on the photosynthetic bacteria. Besides, photofermentation with the effluents of clay (0.23%) and dark fermentative (0.39%) pretreatment processes had higher light conversion efficiency results due to the depletion of their color intensity and enhancement on the light absorption capability of these relatively non-toxic media.

Photofermentative hydrogen production results determined in this study are quite comparable with the results present in the literature for two-stage bioprocesses. For example, dark fermentation with *Clostridium butyricum* NCIB 9576 followed by photofermentation with immobilized cells of *R. sphaeroides* E15-1 resulted in H_2 production rates of 0.008 L/L/h for tofu and 0.016 L/L/h for makkoli wastewater during continuous H_2 production studies (Kim *et al.*, 2001). In another study, Fascetti *et al.* (1998) observed a hydrogen evolution rate of 0.01 L/g/h by *Rhodobacter sphaeroides* RV cells while the cells were cultivated on lactate containing solutions derived from the dark fermentation of municipal solid wastes. After the external addition of some nutrients and trace elements to the dark fermentation effluent,

they achieved a higher H_2 production rate (0.1 L/g/h) compared to the results obtained without external additions.

Compared to the other effluents of dark fermentative pretreatment processes, photofermentation with 50 % (v/v) effluent of dark fermentation with acclimated sewage sludge produced the highest amount of hydrogen in addition to the highest light conversion efficiency (0.39%). As can be seen from Table 5.4, photofermentation with 50 % (v/v) effluent of dark fermentation with acclimated sewage sludge also enhances the hydrogen production rates and shortens the lag time of H_2 production to 8 hours which is much shorter than the ones obtained for non-acclimated samples (around 30 h).

Table 5.4 The results of photofermentative H_2 production with raw OMW and the effluents of different pretreatment processes

Process	Characteristics							
	OMW * (%)	X_{max} (g/l)	t_{lag} (h)	Δt_{H2} (h)	HPP (l_{H2}/l_{OMW})	r_g ($l\ l^{-1}\ h^{-1}$)	r_g^1 ($l\ g^{-1}\ h^{-1}$)	η (%)
Photoferm. with the effluent of Clay pretreatment	4	0.23	18	195	31.5	0.016	0.084	0.23
Photoferm. with the effluent of Zeolite-4A pretreatment	4	0.22	18	198	17.6	0.007	0.037	0.10
Photoferm. with the effluent of Ozone pretreatment	4	0.17	65	145	4.7	0.002	0.017	0.04
Photoferm. with the effluent of Fenton's Reagent pretreatment	4	0.10	64	159	2.1	0.001	0.012	0.02
Photoferm. with the effluent of UV pretreatment	4	0.13	62	171	3.0	0.001	0.011	0.02
Photoferm. with raw OMW	4	0.34	18	155	16.0	0.006	0.021	0.12
Photoferm. with the effluent of dark fermentation	4	0.56	13	150	26	0.006	0.024	0.35
	50	0.30	30	155	0.1	0.003	0.016	0.18
Photoferm. with the effluent of dark fermentation (acclimated sewage sludge)	50	0.40	8	145	0.5	0.008	0.030	0.39
	100	0.24	15	95	0.1	0.003	0.015	0.15

*volumetric ratio (%) of either pretreated or raw OMW, in the photobioreactor inlet

5.3. Developments in the Clay Pretreatment Process

According to the previous results (Section 5.3), both pretreatment with clay adsorption and dark fermentation with acclimated sewage sludge processes are found to enhance the photofermentative hydrogen production. According to its highest hydrogen production potential, ease of operation and comparable low cost of application; clay pretreatment technique appears to be a good alternative for a two stage hydrogen production process. For this reason, clay treatment step followed by photofermentation by *R. sphaeroides* O.U.001 will be explored throughout the rest studies given in subsequent sections from 5.3 to 5.5.

The aim of this part of the work is to gain further insight into optimize the clay treatment process for an efficient photofermentative hydrogen production. In order to do that, detailed analysis of the clay sample was investigated (Section 5.3.1). Afterwards, a comparative study for the determination of suitable clay quantity (Section 5.3.2) was carried out with five different fractions of clay. After the investigations on the clay quantity; the proper dilution rates of the clay treatment effluents were also investigated (Section 5.3.3). Subsequently, the regeneration of spent clay and the possibility of its reutilization for OMW pretreatment were tested (Section 5.3.4).

5.3.1. Properties of Clay

Cloisite® Na+ was used as natural, white colored, and fine-grained clay material. Regarding to its characteristic properties given in Table 5.5, Cloisite® Na+ is essentially composed of silica (54%) and alumina (19%); and considerable quantities of iron (8.3% Fe_2O_3), alkalies and alkaline earths (especially Na_2O, MgO, and CaO) . Another important

property of a clay material is its layer thickness (basal spacing), showing the efficiency of its adsorption property. Basal spacing of Cloisite® Na+ was obtained as 8 Å (Appendix L), which is consistent with the literature data (9.6 Å) given for a natural montmorillonite molecule (i.e., Cloisite® Na+) (Işık, 2002).

Table 5.5 Characteristics of clay material, utilized throughout the current study

Property	Amount
Trade Name	Cloisite® Na+
Color	White
Organic Modifier	None
Density	2.86 g/ cm^3
Particle Size (Dry)	Less than 1 μ (10%)
	Less than 5 μ (30%)
	Less than 11 μ (70%)
Basal spacing (d_{001})	8 Å
SiO_2, %	54
Al_2O_3, %	19
Fe_2O_3, %	8.3
Na_2O, %	5.8
MgO, %	0.7
CaO, %	0.14
TiO_2, %	0.11
NiO, %	0.071
K_2O, %	0.023
ZnO, %	0.021
PbO, %	0.021
CuO, %	0.014
P_2O_5, %	0.0012
MnO, %	0.0010
Cr_2O_3, %	0.0032

5.3.2. Effect of Clay Quantity on the Pretreatment of OMW

Five different fractions of clay (0.1, 1, 10, 20 and 50 grams of clay per liters of OMW) were investigated to perform a comparative study on the determination of the effect of clay quantity. *Rhodobacter sphaeroides* OU001 was grown in the effluents of clay treatment process at different clay fractions. Volumetric ratio of these pretreated samples in the photofermentor inlets was 4%. The experimental data are given in Appendix M4.

Table 5.6 gives the color, COD and total phenol removals after pretreatment of raw OMW sample with 5 different fractions of clay. It is remarkable that the elevations on the clay quantity proportionally increase the color, COD and phenol removal efficiencies (Table 5.6). The highest color (69%), COD (38%) and total phenol (83%) removal efficiencies were achieved after clay treatment process with 50 grams of clay added to 1 L of raw OMW sample. In the same way, removal efficiencies of a lower quantity (20 g /L) were quiet comparable with the results obtained with 50 g /L of clay.

Table 5.6 Color, COD and total phenol changes after clay pretreatment of OMW with different amounts of clay

Amount of clay (g) added to 1 L of raw OMW	0.1	1	10	20	50
$Color_i$ (PtCo APHA)	61500	61500	61500	61500	61500
$Color_f$ (PtCo APHA)	37500	35600	25800	21500	19000
Color removal (%)	39	42	58	65	69
COD_i (g/L)	52.1	52.1	52.1	52.1	52.1
COD_f (g/L)	42.7	42.2	40.6	35.9	32.3
COD removal (%)	18	19	22	31	38
Total $phenol_i$ (g/L)	12.2	12.2	12.2	12.2	12.2
Total $phenol_f$ (g/L)	6.3	5.5	3.9	2.3	2.1
Total phenol removal (%)	48	55	68	81	83

The photofermentative hydrogen production, bacterial growth, and pH changes of these clay treatment effluents are given in Figures 5.15, 5.16 and 5.17, respectively. According to Figure 5.15, clay pretreatment process with the 20 grams of clay per 1 liters of OMW had the highest hydrogen production capacity (58 mL). In contrast, it generated the lowest bacterial mass (Figure 5.16).

Up to a certain saturation concentration (20 g/L), a strong proportionality between the hydrogen production and the color removal efficiencies of different clay fractions was observed (Table 5.6). As the clay quantity was increased; color, COD and phenol removal efficiencies as well as hydrogen production capacities are directly increased. After the consumption of higher clay fractions (i.e., 50 g/L); hydrogen production, bacterial growth and wastewater treatment efficiencies (Table 5.6) were found to be similar to the ones obtained with 20 g/L. This shows that the addition of 20 grams of clay into 1 liters of OMW sample is the saturation point for an efficient clay treatment process.

For the case of lower clay fractions (0.1, 1 and 10 g/L), bacterial growth is relatively higher. Lower bacterial growth results of higher clay fractions (20 and 50 g/L) might be caused by the probable removal of some biodegradable carbon sources together with the colorful and hardly biodegradable compounds. No significant pH variation was observed during photofermentation processes for the effluents of clay pretreatment process with different quantities of clay (Figure 5.19).

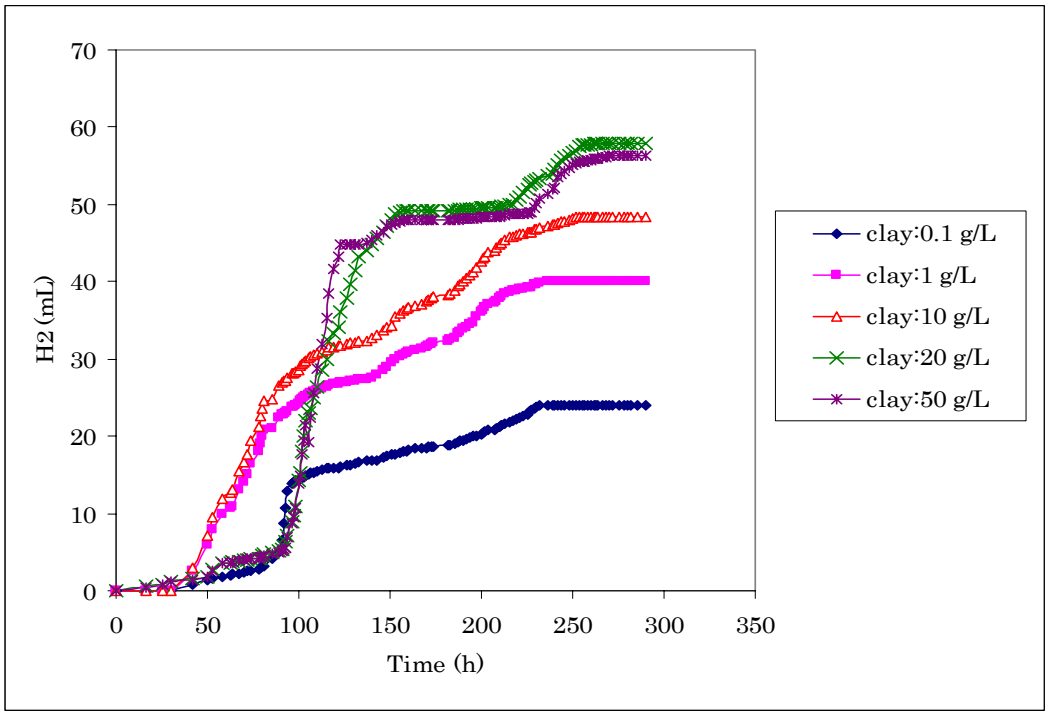


Figure 5.15 Total volume of hydrogen gas production versus time for the effluents of clay pretreatment process with different quantities of clay. [The volumetric ratio of the clay pretreatment effluents, in the photobioreactors was 4% (v/v)]

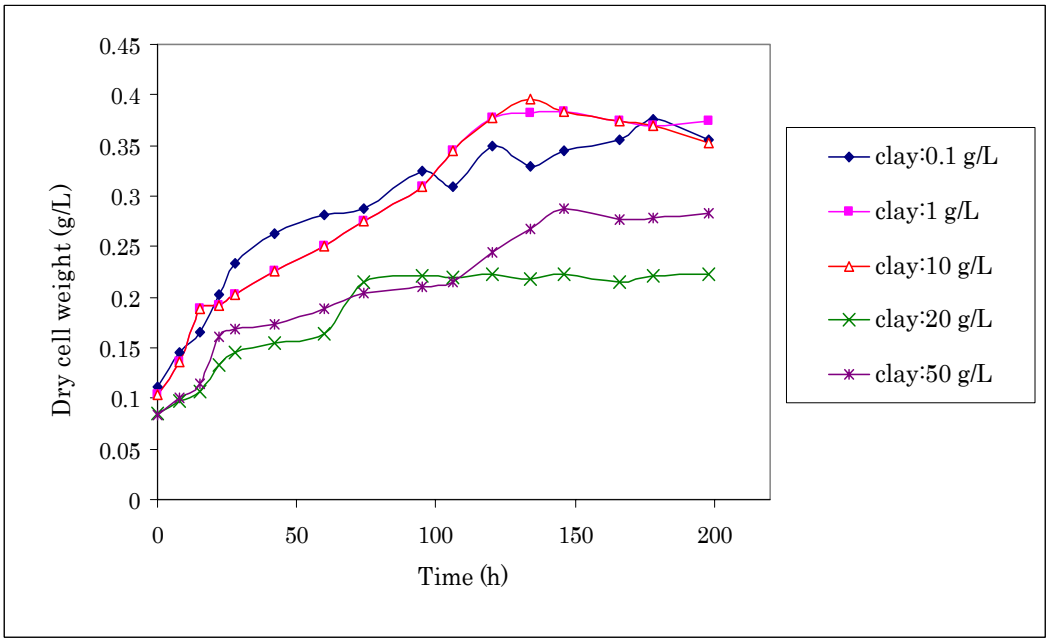


Figure 5.16 Bacterial growth curves for the effluents of clay pretreatment process with different quantities of clay. [The volumetric ratio of the clay pretreatment effluents, in the photobioreactors was 4% (v/v)]

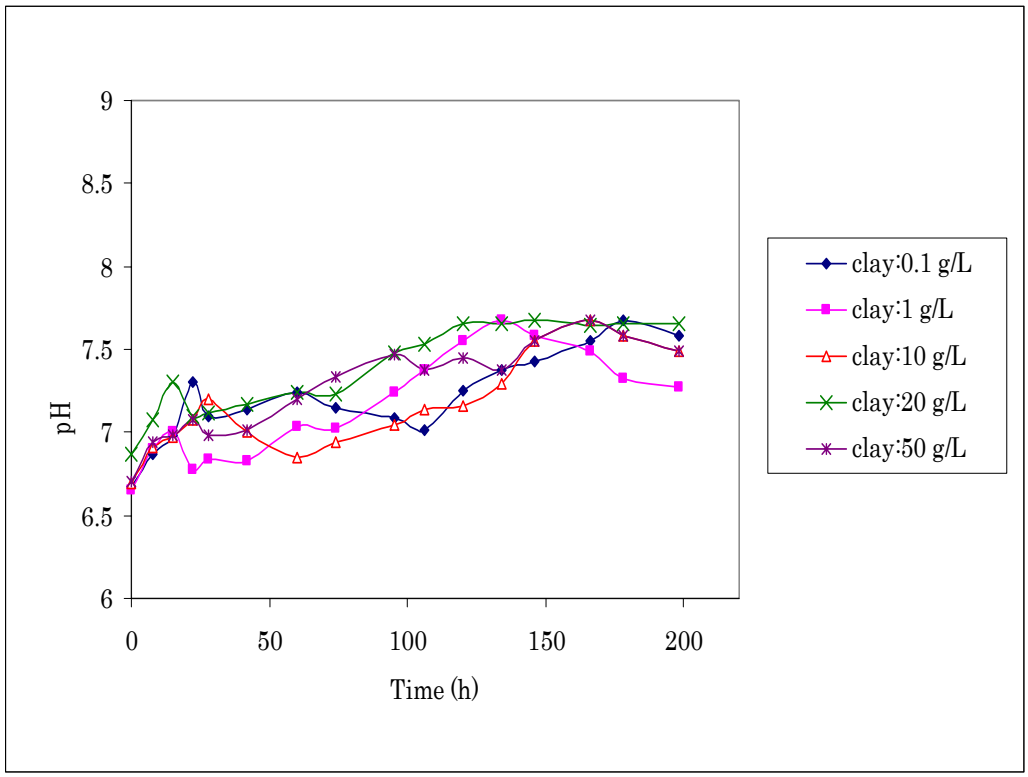


Figure 5.17 pH change versus time for the effluents of clay pretreatment process with different quantities of clay. [The volumetric ratio of the clay pretreatment effluents, in the photobioreactors was 4% (v/v)]

Table 5.7 represents the overall results of photofermentative H_2 production parameters, with the effluents of clay pretreatment processes, using different quantities of clay. According to its relatively higher hydrogen gas production potential (29 L/L), hydrogen production rates (0.012 L/L/h and 0.071 L/g/h) and light conversion efficiency (0.13%) results, pretreatment with 20 g/L of clay appear to be the optimum clay amount. That quantity was also observed to be the saturation concentration for an effective clay pretreatment process. For this reason, the following clay treatment processes in the subsequent sections will be explored with the addition of 20 grams of clay into 1 liters of raw OMW sample.

Table 5.7 The results of photofermentative H_2 production with the effluents of clay pretreatment processes, using different quantities of clay

Amount of clay (g) added to 1 L of raw OMW	X_{max} (g/l)	t_{lag} (h)	Δt_{H2} (h)	HPP (l / l)	r_g (l l^{-1} h^{-1})	r_g^1 (l g^{-1} h^{-1})	η (%)
0.1	0.38	40	192	12.1	0.006	0.018	0.07
1	0.38	38	198	20.1	0.005	0.014	0.11
10	0.40	37	217	24.2	0.005	0.015	0.13
20	0.22	15	247	29	0.012	0.071	0.13
50	0.29	15	257	28.2	0.021	0.094	0.12

5.3.3. Effect of Dilution Rates on the Photofermentative Hydrogen Production

Three different volumetric ratios of clay treatment effluents in the photofermentor inlets (4%, 50% and 100%) and their controls with untreated raw OMW samples were carried out to determine the effect of dilution percentage on photofermentative hydrogen production. According to the results given in Section 5.3.2, clay pretreatment process was done with the addition of 20 g/L clay into raw OMW sample. The experimental data are given in Appendix M5.

Total volume of hydrogen gas production and the bacterial growth for the effluents of clay pretreatment process and raw olive mill wastewater samples at different dilution rates are given in Figures

5.18 and 5.19, respectively. Lastly, pH changes of these photofermentor liquids are shown in Figure 5.20.

Although H_2 production yields of diluted OMW samples gave satisfactory results, dilution process has some problems on the aspects of environmental logic by means of increasing the quantity of wastewater. In this part of the study, the possibility of concentrated OMW utilization was examined.

As can be realized from Figure 5.18; pretreatment with clay gave result to the production of hydrogen with concentrated OMW (50% and 100%). Photofermentation with 50% effluent of clay pretreatment process (27 mL) produced similar amounts of hydrogen compared with 4% untreated raw OMW media (32 mL).This is mainly attributable to the removal of color and non-biodegradable compounds as a result of clay treatment. On the other hand, untreated and concentrated media (50% and 100% raw OMW) had failure in photofermentative hydrogen production due to their very dark color and the presence of toxic compounds at relatively higher amounts.

Although the concentrated effluents (50% and 100%) of clay treatment resulted relatively lower amounts of hydrogen production (27 mL and 13 mL, respectively) than with diluted (4%) ones (58 mL), it is a significant achievement for the photosynthetic bacteria to be grown (Figure 5.19) and evaluate hydrogen in such a highly concentrated media.

All pH values are quiet comparable with each other at values around 7, in which concentrated media has slightly lower pH values coming from the acidic environment of OMW itself (Figure 5.20). For 50% raw OMW

containing media, significant depletion in pH was observed as a result of dark fermentative acidic end products since dark fermentation seems to be the sole metabolism responsible for the bacterial growth (Figure 5.19) without hydrogen evolution.

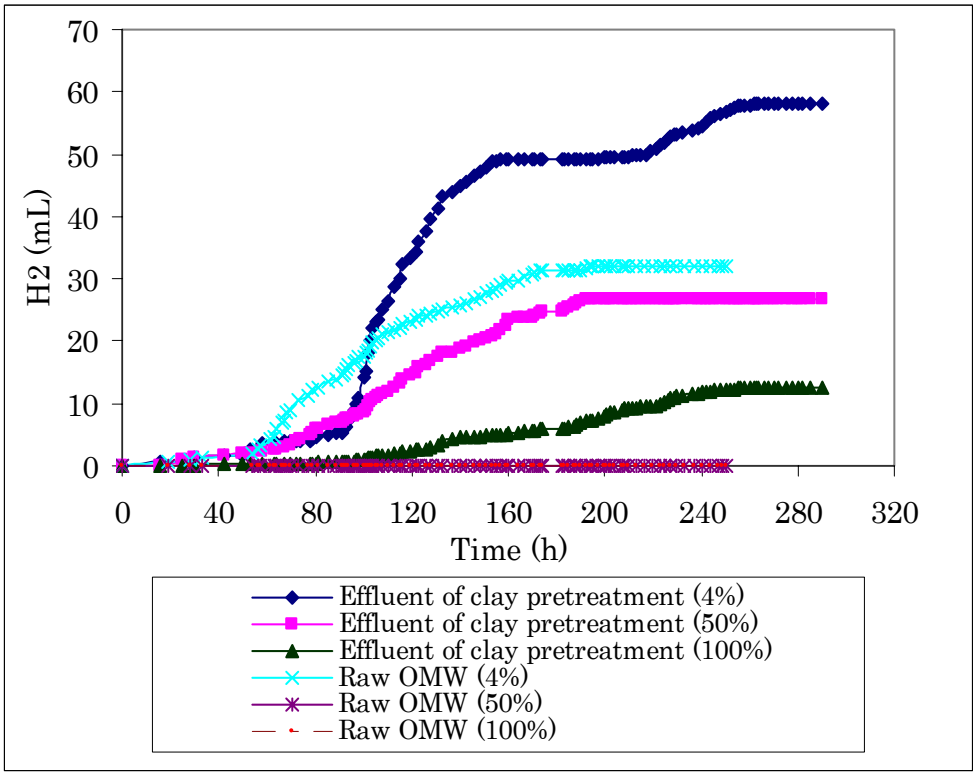


Figure 5.18 Total volume of hydrogen gas production versus time for the effluents of clay pretreatment process with different dilution rates

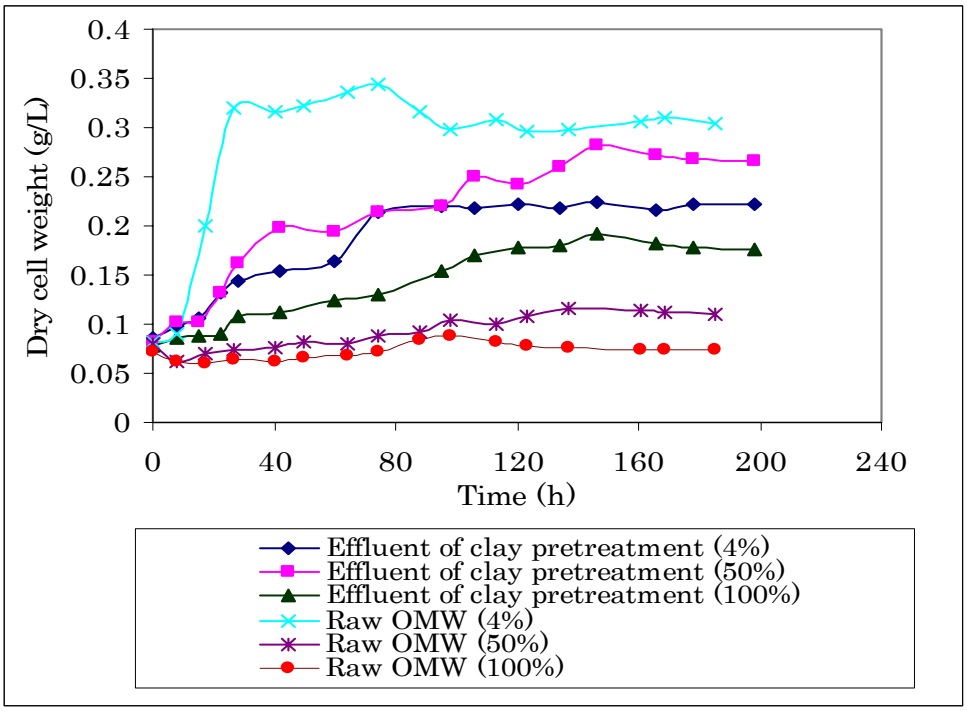


Figure 5.19 Bacterial growth curves for the effluents of clay pretreatment process with different dilution rates

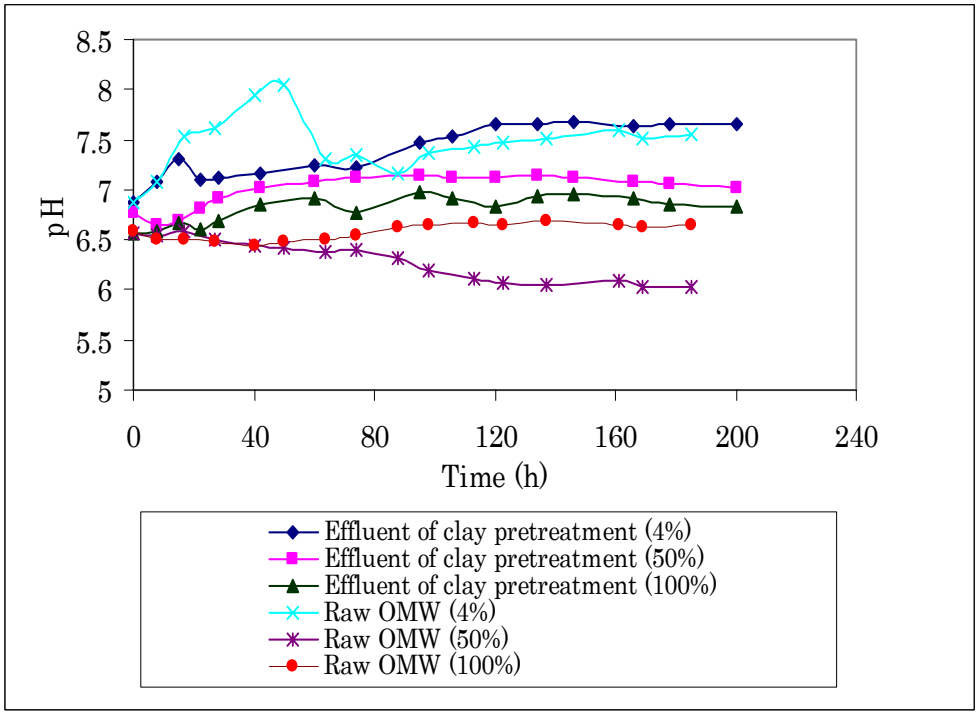


Figure 5.20 pH change versus time for the effluents of clay pretreatment process with different dilution rates

The overall results of photofermentative H_2 production with raw OMW and the effluents of clay pretreatment processes at different dilution rates are all tabulated in Table 5.8. As previously stated, the utilization of concentrated media for hydrogen production became available by clay pretreatment process. As a matter of fact that more dilute (4%) clay pretreatment effluent resulted the highest hydrogen gas production potential (29 L/L), hydrogen production rates (0.012 L/L/h and 0.071 L/g/h) and light conversion efficiency (0.13%) results, it appears to be the most suitable dilution rate for an efficient photofermentation process.

Table 5.8 The results of photofermentative H_2 production with raw OMW and the effluents of clay pretreatment processes at different dilution rates

Process	X_{max} (g/l)	t_{lag} (h)	Δt_{H2} (h)	HPP (l / l)	r_g (l l^{-1} h^{-1})	r_g^l (l g^{-1} h^{-1})	η (%)
Photofermentation with the effluent of clay pretreatment (4%)	0.22	15	247	29	0.012	0.071	0.13
Photofermentation with the effluent of clay pretreatment (50%)	0.28	22	170	1.1	0.005	0.022	0.09
Photofermentation with the effluent of clay pretreatment (100%)	0.19	28	235	0.3	0.002	0.013	0.03
Raw OMW (4%)	0.34	18	155	16	0.006	0.021	0.12
Raw OMW (50%)	0.12	-0-	-0-	-0-	-0-	-0-	-0-
Raw OMW (100%)	0.09	-0-	-0-	-0-	-0-	-0-	-0-

5.3.4. Spent Clay Regeneration

Spent clay regeneration was investigated on the grounds that very significant amount of clay was utilized throughout the OMW treatment process (20 g/L_{OMW}). Besides, the solid waste minimization gains further importance. In order to regenerate spent-clay after OMW pretreatment process, clay-OMW complexes were washed with water prior to a drying step. Then, reutilization of these regenerated samples for the OMW pretreatment process was inspected in this part of the study. As a result of optimization studies, *Rhodobacter sphaeroides* OU001 was grown in the effluents of either fresh clay or regenerated clay treatment process with the addition of 20 g/L clay into raw OMW sample. Volumetric ratio of these pretreated samples in the photofermentor inlets was 4%. The experimental data are given in Appendix M6.

The photosynthetic hydrogen production, bacterial growth, hydrogen per bacterial weight and pH changes of these experiments are given in Figures 5.21, 5.22 and 5.23, respectively. According to Figure 5.21, regenerated clay (46 mL) was found to gave higher hydrogen production compared to untreated-raw OMW samples (32 mL), although not as good as the fresh clay (58 mL). Either fresh or regenerated clays were utilized, similar bacterial growth (Figure 5.22) and pH variations (Figure 5.23) were observed.

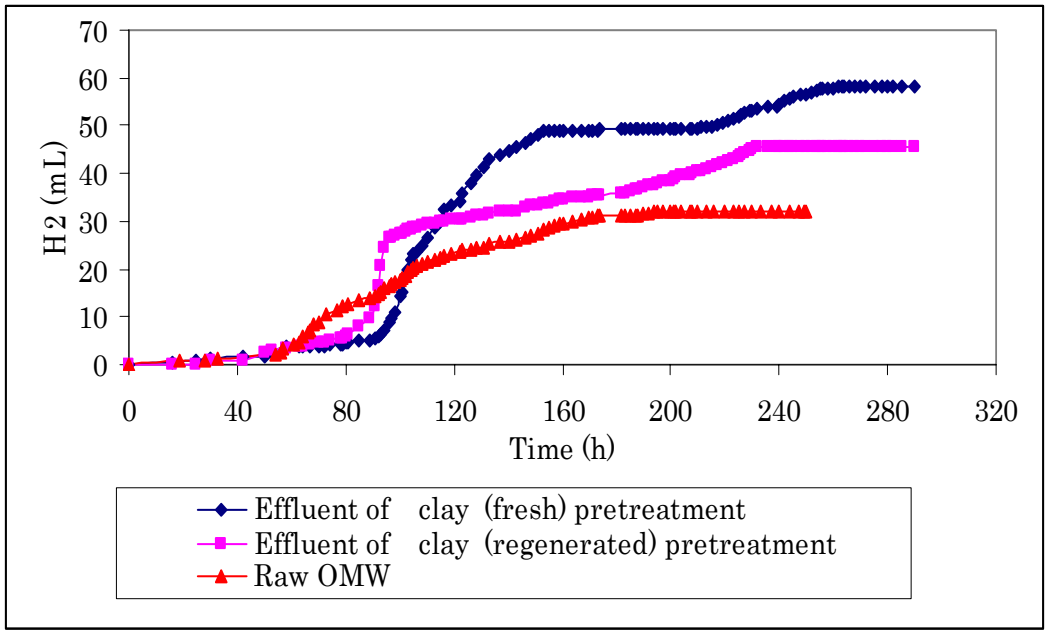


Figure 5.21 Total volume of hydrogen gas production versus time for the effluents of either fresh or regenerated clay treatment process. [The volumetric ratio of the clay pretreatment effluents, in the photobioreactors was 4% (v/v)]

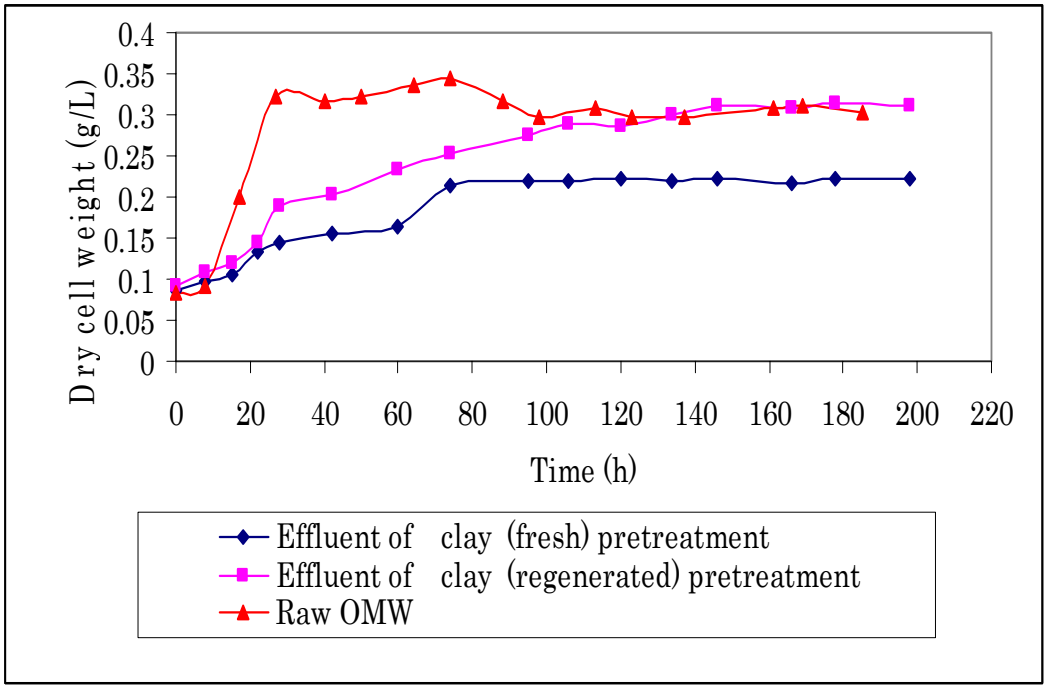


Figure 5.22 Bacterial growth curves for the effluents of either fresh or regenerated clay treatment process. [The volumetric ratio of the clay pretreatment effluents, in the photobioreactors was 4% (v/v)]

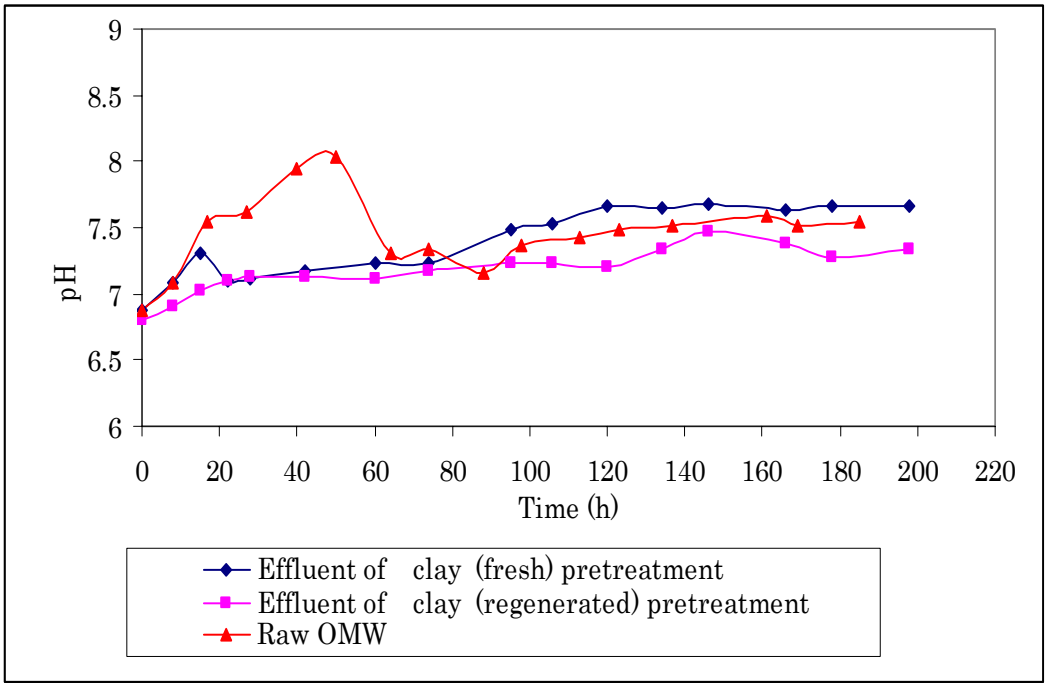


Figure 5.23 pH change versus time for the effluents of either fresh or regenerated clay treatment process. [The volumetric ratio of the clay pretreatment effluents, in the photobioreactors was 4% (v/v)]

A list of hydrogen production results for regenerated and fresh clay samples are tabulated in Table 5.9. Compared to the photofermentation process with raw OMW sample, relatively higher hydrogen gas production potential (23 L/L) and the hydrogen production rates (0.005 L/L/h and 0.024 L/g/h) with regenerated clay were observed. This might give rise to the solution of solid waste reduction, which is essential on the economical and environmental basis.

Table 5.9 The results of photofermentative H_2 production with effluents of either fresh or regenerated clay treatment process

Process	X_{max} (g/l)	t_{lag} (h)	Δt_{H2} (h)	HPP (l / l)	r_g (l l^{-1} h^{-1})	r_g^1 (l g^{-1} h^{-1})	η (%)
Photofermentation with the effluent of clay (fresh) pretreatment (4%)	0.22	15	247	29	0.012	0.071	0.13
Photofermentation with the effluent of clay (regenerated) pretreatment (4%)	0.31	30	202	22.8	0.005	0.024	0.12
Photofermentation with raw OMW (4%)	0.34	18	155	16.0	0.006	0.021	0.12

5.4. Comparison of Single-stage and Two-stage Biohydrogen Production Processes

5.4.1. Results of Photofermentative Hydrogen Production with the Effluent of Clay Pretreatment

According to the results of the previous sections, clay pretreatment technique was found to be a good alternative for a two stage hydrogen production process. Main reasons for the enhancement of photofermentative hydrogen production following a clay treatment process are especially attributed to the high color removal efficiency (65%) and its non-toxic environment. To be more precise, detailed analysis of photofermentation stage with the effluent of clay treatment process is considerably required.

For this reason, the aim of this part of the work is to gain further insight into photofermentation stage that was carried out with the effluent of clay treatment process (4% (v/v)). In addition to hydrogen, bacterial weight and pH analysis; further determinations including

total phenol, total sugar, color, light absorption spectra, specific organic acids, phenols, amino acids, sugars and alcohols were investigated at different time intervals of photofermentation process.

The total volume of hydrogen gas production, bacterial growth and pH changes of the effluent of clay treatment (4% (v/v)) and the raw OMW (4% (v/v)) media are given in the following figures (Figure 5.24, 5.25 and 5.26, respectively). Correspondingly, changes in the color, total phenol and total sugar content versus time data for photofermentor liquids are shown Figures 5.27, 5.28, and 5.29, respectively. Light absorption spectra of the photofermentor liquid at different time intervals are also present (Figures 5.35 and 5.36).

According to the detailed HPLC and GC analysis, it becomes possible to determine the main organic constituents of each photofermentor liquid. Changes in organic acids, sugars, phenolics, amino acids and alcohol content of photofermentor liquid are all shown in the consecutive figures (Figure 5.33, 5.34, 5.35, 5.36 and 5.37, respectively).

As given in Figure 5.24, clay pretreatment processes significantly enhance the hydrogen production. Compared to the photofermentation with raw OMW (32 mL), the amount of hydrogen production was improved by %100 as a result of clay pretreatment process (63 mL). Besides, clay pretreatment effluent had lower bacterial growth (Figure 5.25) due to the removal of some biodegradable carbon sources such as biologically digestible meta and orto substitutions of phenolics (Figure 5.35); and total sugars (Figure 5.29) together with the colorful and hardly biodegradable compounds such as para-cresol. No significant pH variation was observed during photofermentation processes with the clay effluents (Figure 5.26).

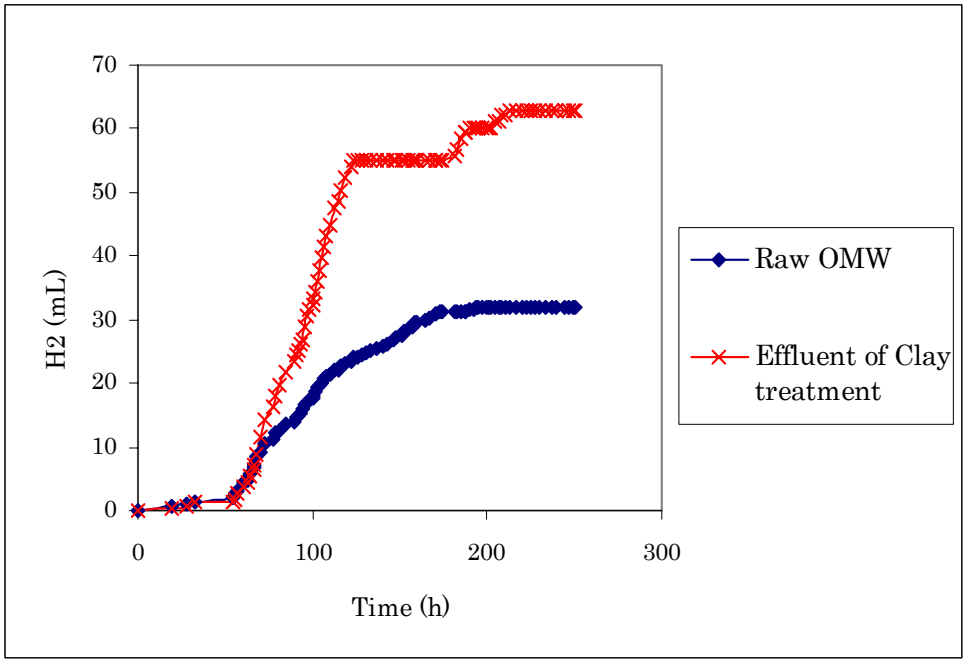


Figure 5.24 Total volume of hydrogen gas production versus time for the effluent of clay treatment process and for raw OMW sample. [The volumetric ratio of either the effluents of pretreatment processes or raw OMW, fed to the photobioreactors was 4% (v/v)]

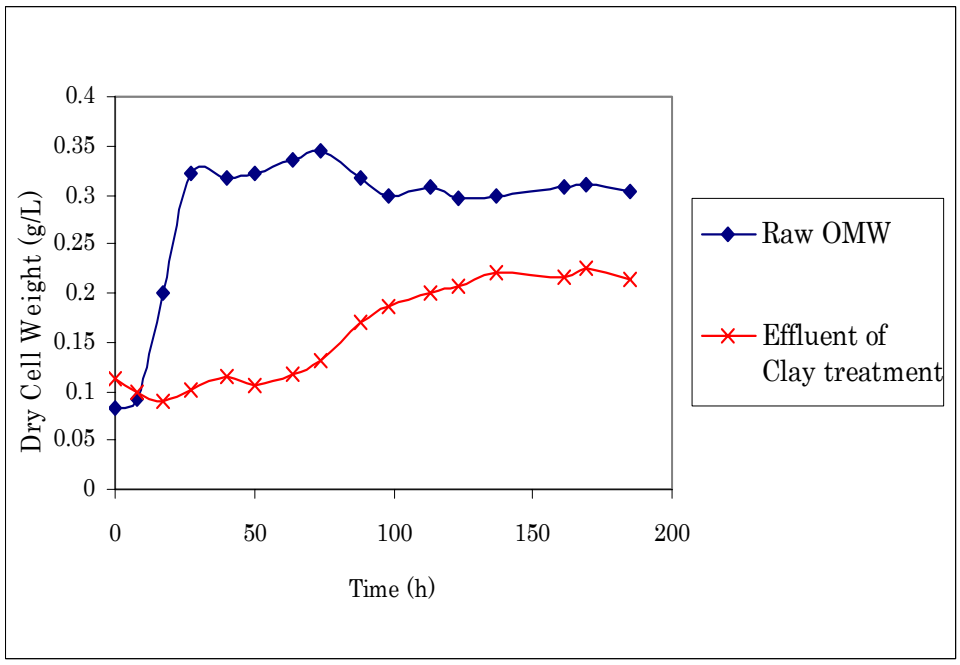


Figure 5.25 Bacterial growth curves for the effluent of clay treatment process and for raw OMW sample. [The volumetric ratio of either the effluents of pretreatment processes or raw OMW, fed to the photobioreactors was 4% (v/v)]

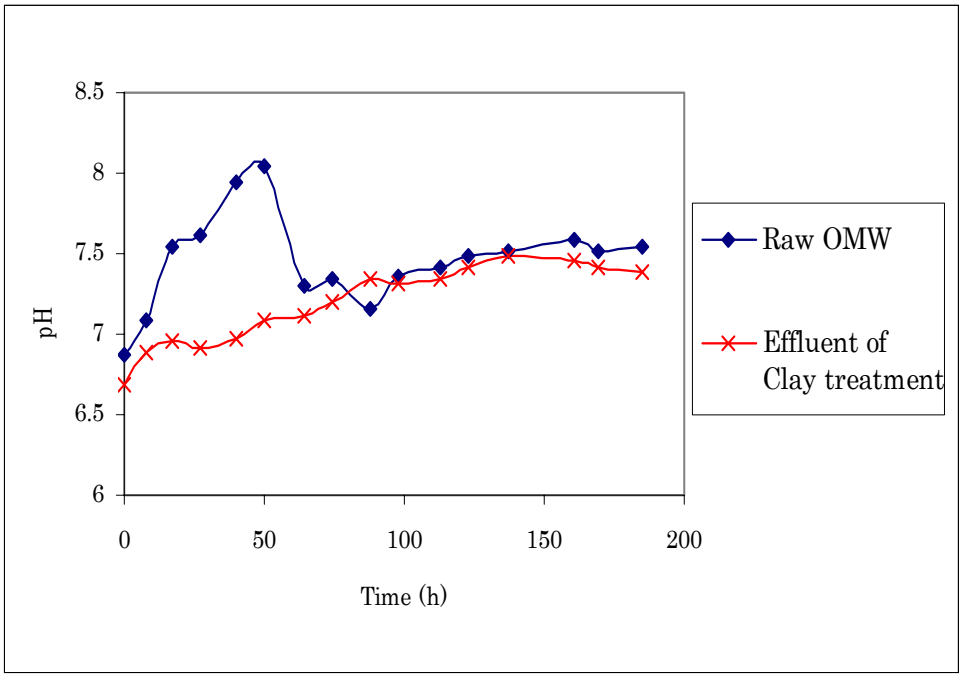


Figure 5.26 pH change versus time for the effluent of clay treatment process and for raw OMW sample. [The volumetric ratio of either the effluents of pretreatment processes or raw OMW, fed to the photobioreactors was 4% (v/v)]

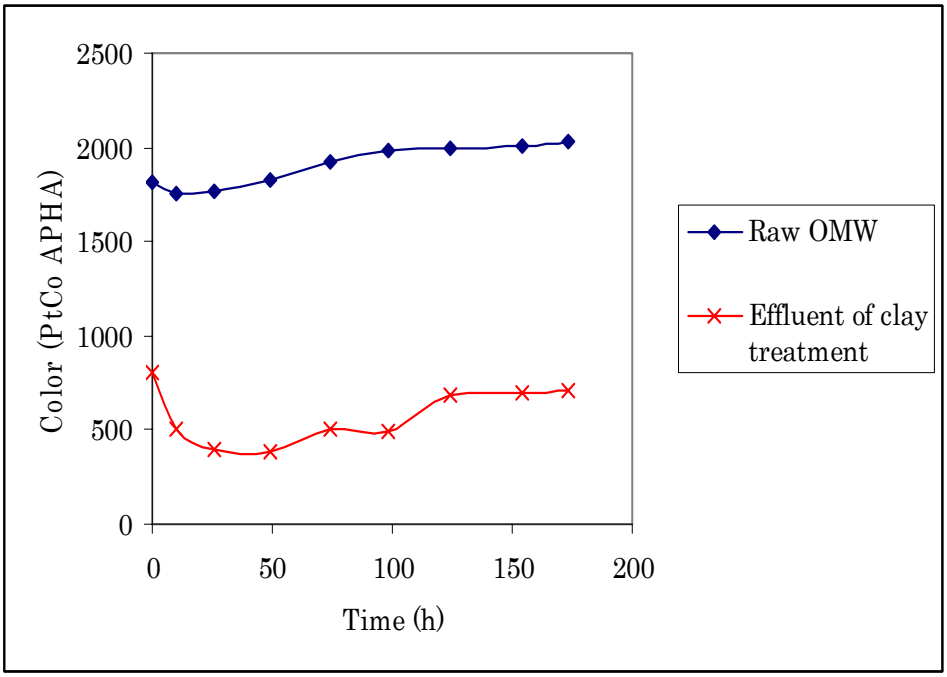


Figure 5.27 Color change versus time for the effluent of clay treatment process and for raw OMW sample, during photofermentation. [The volumetric ratio of either the effluents of pretreatment processes or raw OMW, fed to the photobioreactors was 4% (v/v)]

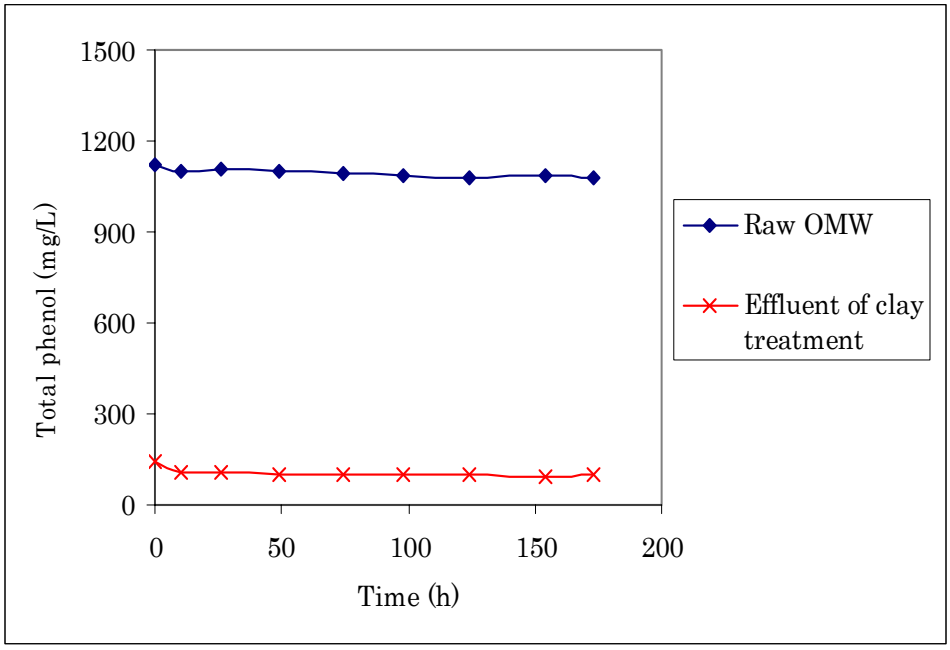


Figure 5.28 Total phenol change versus time for the effluent of clay treatment process and for raw OMW sample, during photofermentation. [The volumetric ratio of either the effluents of pretreatment processes or raw OMW, fed to the photobioreactors was 4% (v/v)]

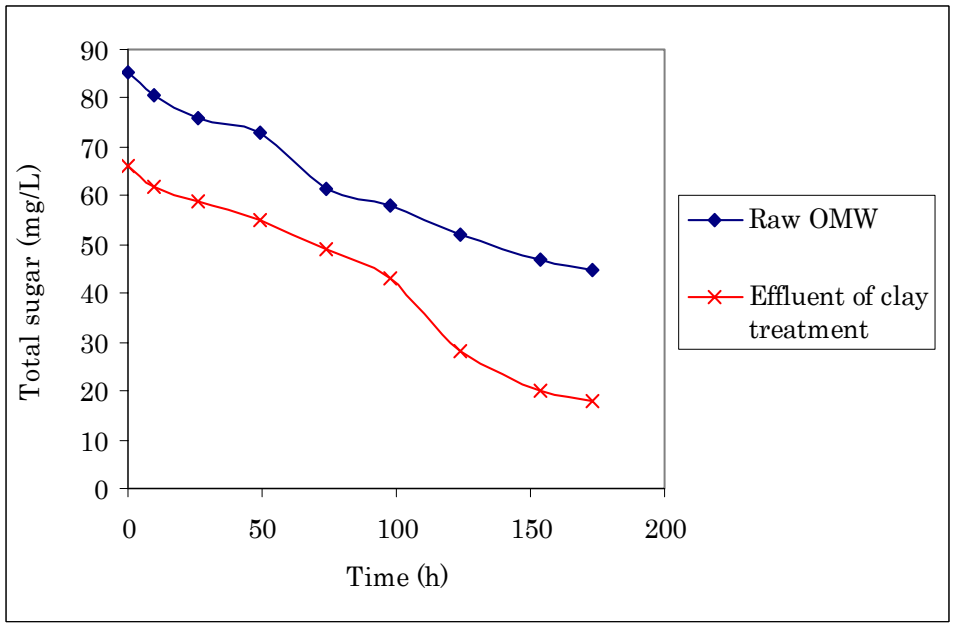


Figure 5.29 Total sugar change versus time for the effluent of clay treatment process and for raw OMW sample, during photofermentation. [The volumetric ratio of either the effluents of pretreatment processes or raw OMW, fed to the photobioreactors was 4% (v/v)]

It can be clearly observed from the light spectrum of the photofermentor liquids (Fig. 5.30 and 5.31) that clay treatment process resulted a significant depletion in the light absorbance of raw OMW sample which was found to have a shadowing effect on the photosynthetic bacteria (Section 3.1; Figures 3.5 and 3.6). These results are in accordance with the high removal of phenolics and other aromatic complexes which are responsible for the dark color and higher light absorbance values. It is known that *R. sphaeroides* contains has an absorption maxima at 850 nm due to the photosynthetic pigments of bacteriochlorophyll-a as shown in Figure 3.6. Thus, Figure 5.32 represents the variations in the absorbance values of photofermentor liquids at 850 nm.

A strong relation was observed between the color (Figure 5.27) of the fermentor liquid and its light absorption capacity at this specific wavelength (850 nm). Phenol, m-cresol and o-cresol consumption rates (Figure 5.35) were highest during the exponential phase of bacterial growth (around first 25 hours). This statement also gives rise to the color depletion during first 25 h (Figure 5.27), and the significant decrease in the light absorbance (Figure 5.32). The results of photofermentation experiment with untreated-raw OMW sample showed that the hardly degradable para-cresols were not utilized during photofermentation process (Figure 5.35 a). After the exponential phase of growth, at which phenolics were significantly consumed, the photofermentor liquids gets darker with higher light absorbance values due to the cell growth and the probable colorful pigment (such as carotenoids) formation property of the photosynthetic bacteria.

As a result of clay treatment process, high removal of total phenolic content (90%); and relatively lower removal of total sugar (20%) and other organic content (i.e., organic acid, alcohol, and amino acids) was observed. Owing to the high depletion of its phenolic content (Figure 5.28), the main organic constituent of the clay treatment effluent is the acetic acid; upon which aspartic, glutamic acid, ethanol, normal-phenol and meta-cresol are also present at higher concentrations.

There is not a significant change on the organic acid consumption behaviors of the photofermentor liquid, either with raw OMW or with the effluent of clay. The main difference is attributable to the prolonged consumption of acetic acid throughout the exponential hydrogen production for the experiments with clay effluents (Fig. 5.33 b), due to the higher light penetration capability of the media at which acetate utilization through photosynthetic pathway was gained. For raw OMW; glucose consumption rate was significantly increased (Fig. 5.34a) after stationary phase of the bacterial growth and hydrogen production (around 100th hour). For each processes, highest portion of the organic compounds were consumed during the exponential phase of bacterial growth (around first 25 hours).

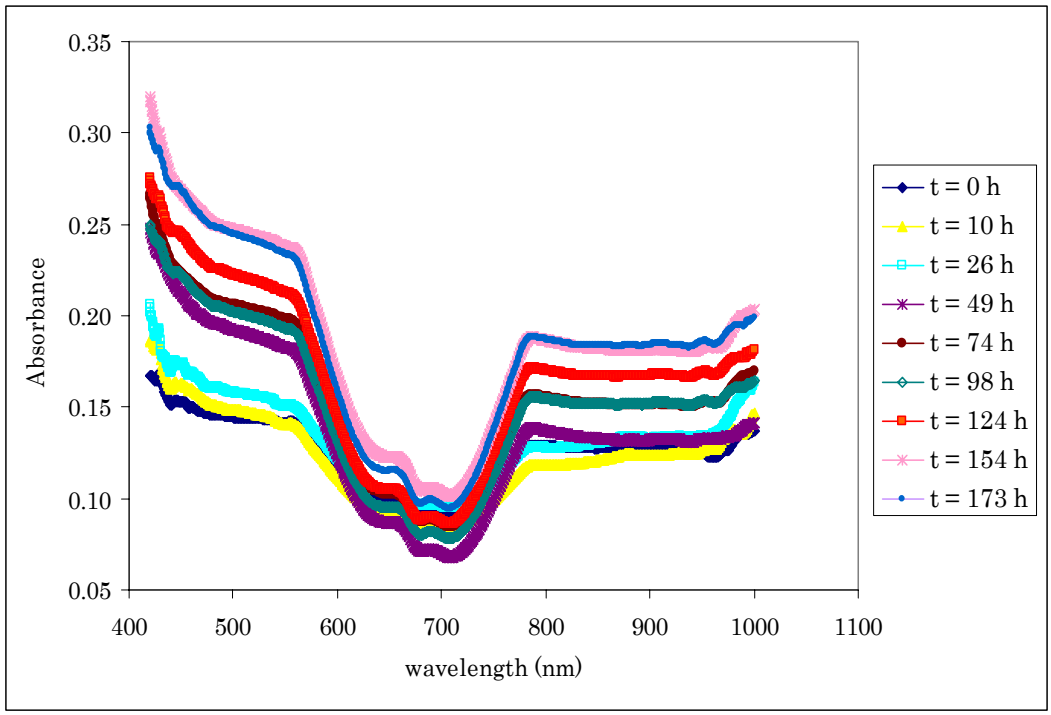


Figure 5.30 Light absorption spectra of the photobioreactor liquid at different time intervals (raw OMW containing sample (4% v/v) is the substrate source)

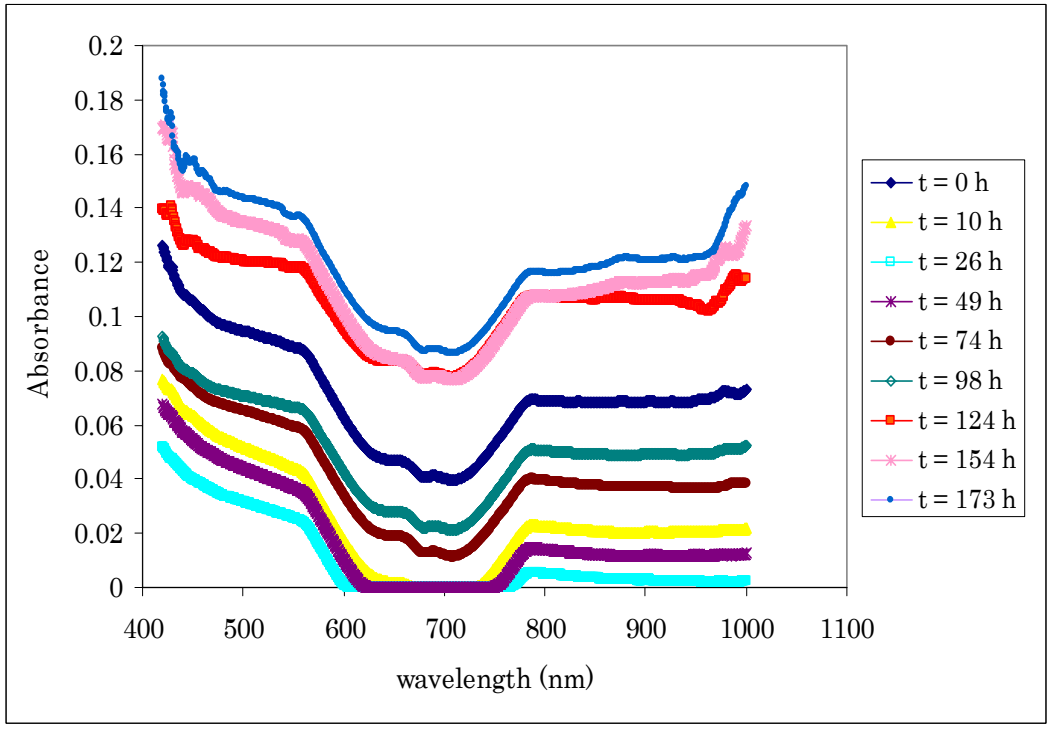


Figure 5.31 Light absorption spectra of the photobioreactor liquid at different time intervals (effluent of clay treatment process (4% v/v) is the substrate source)

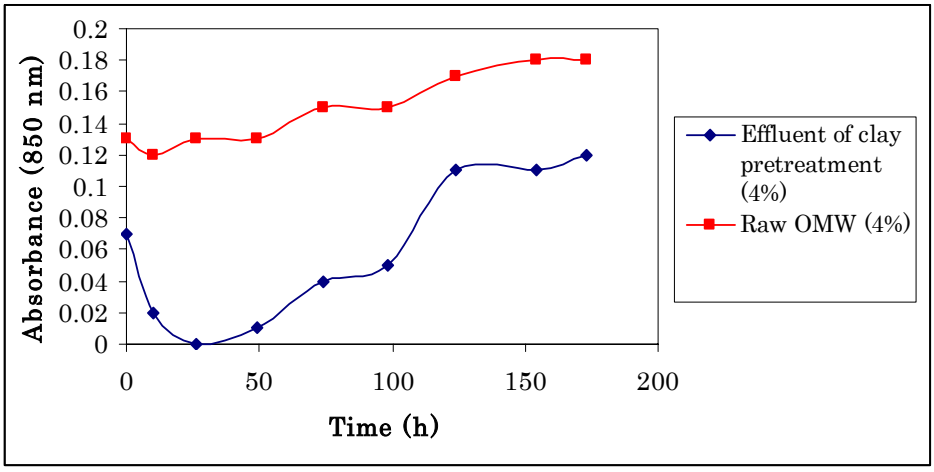
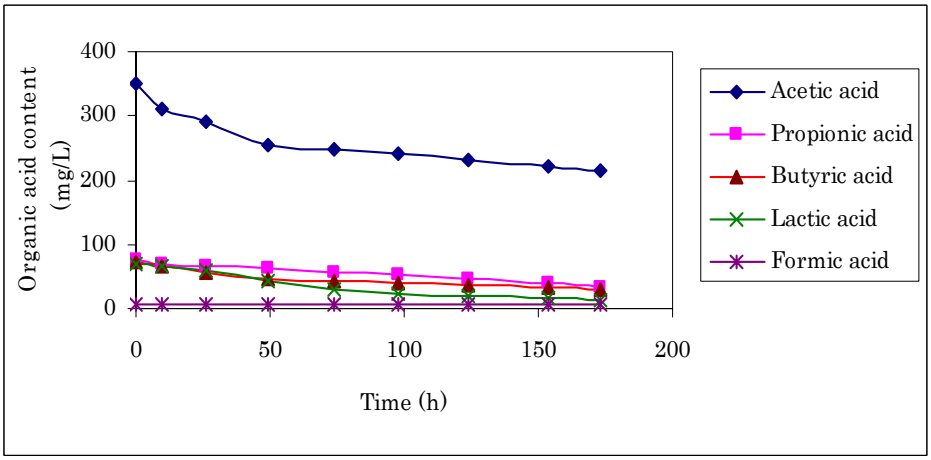
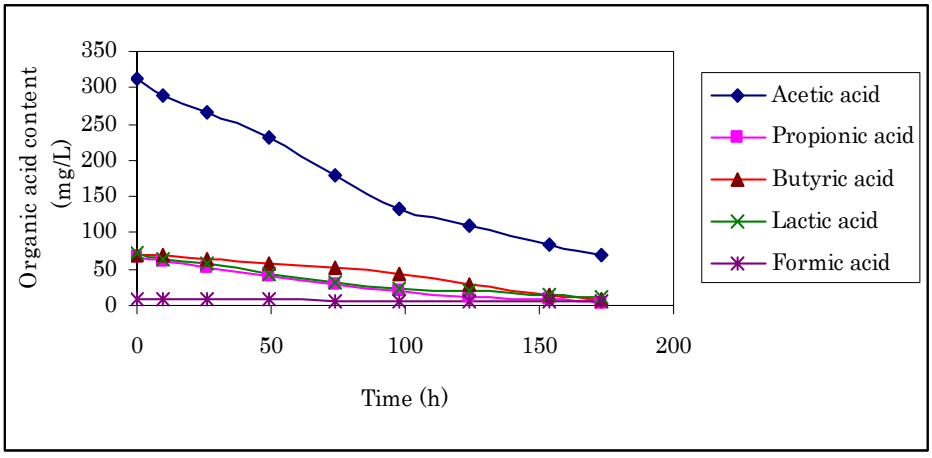


Figure 5.32 Light absorbance of the photobioreactor liquids at 850 nm

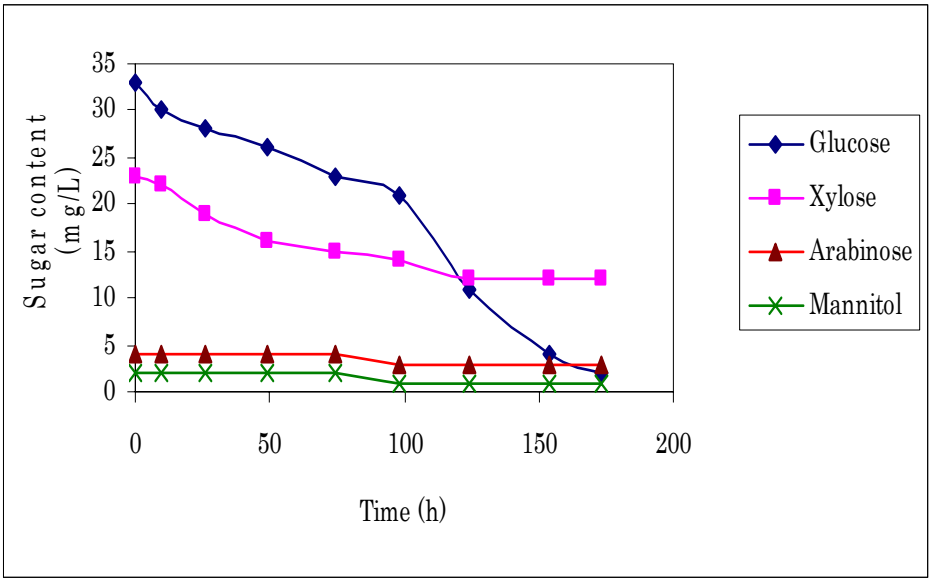


(a)

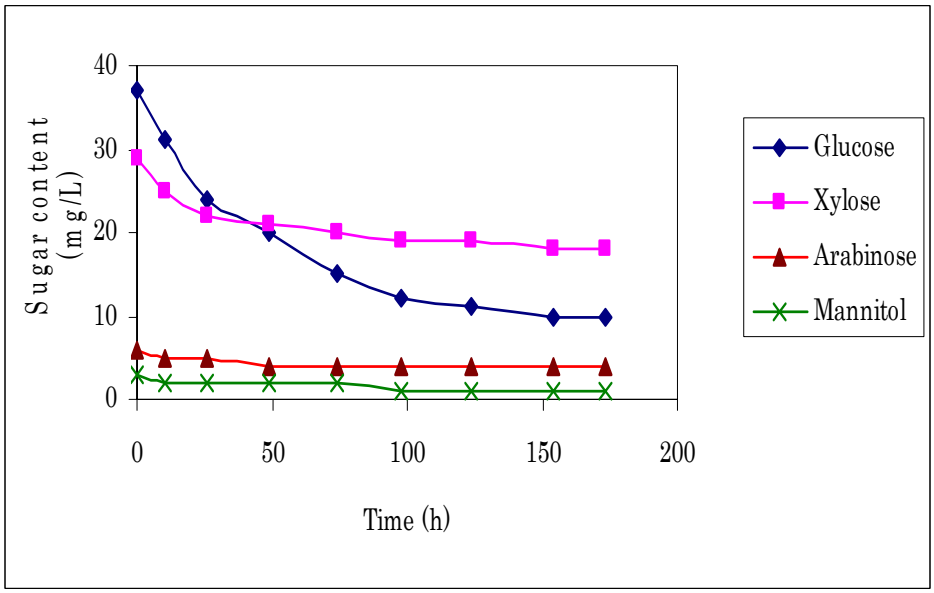


(b)

Figure 5.33 Organic acid content change versus time for raw OMW sample (a), and for the effluent of clay treatment process (b), during photofermentation. [The volumetric ratio of either the effluents of pretreatment processes or raw OMW, fed to the photobioreactors was 4% (v/v)]

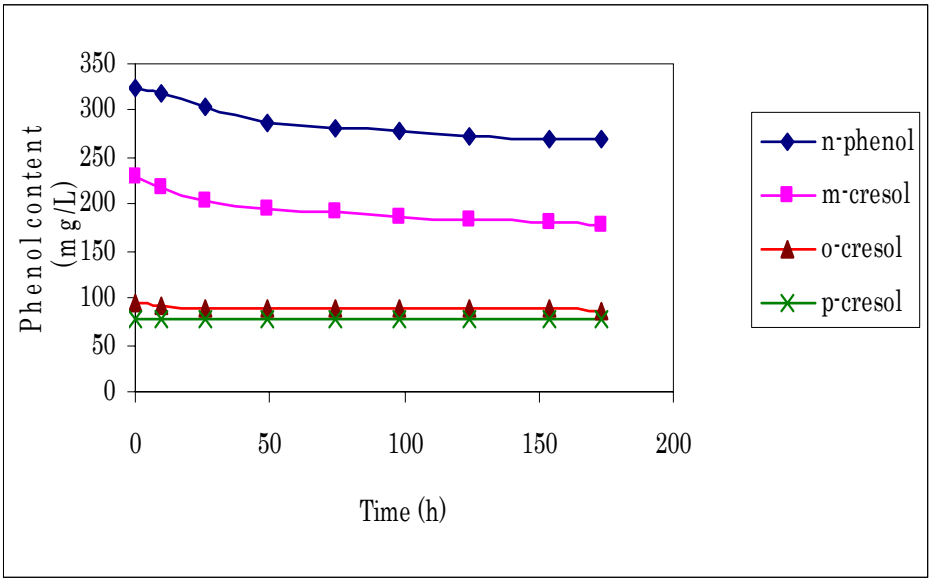


(a)

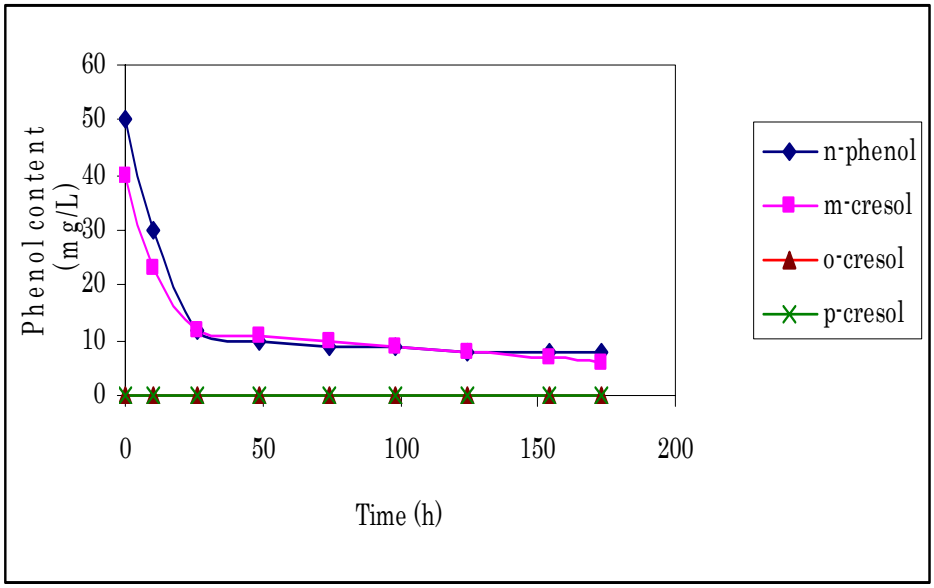


(b)

Figure 5.34 Sugar content change versus time for raw OMW sample (a), and for the effluent of clay treatment process (b), during photofermentation. [The volumetric ratio of either the effluents of pretreatment processes or raw OMW, fed to the photobioreactors was 4% (v/v)]

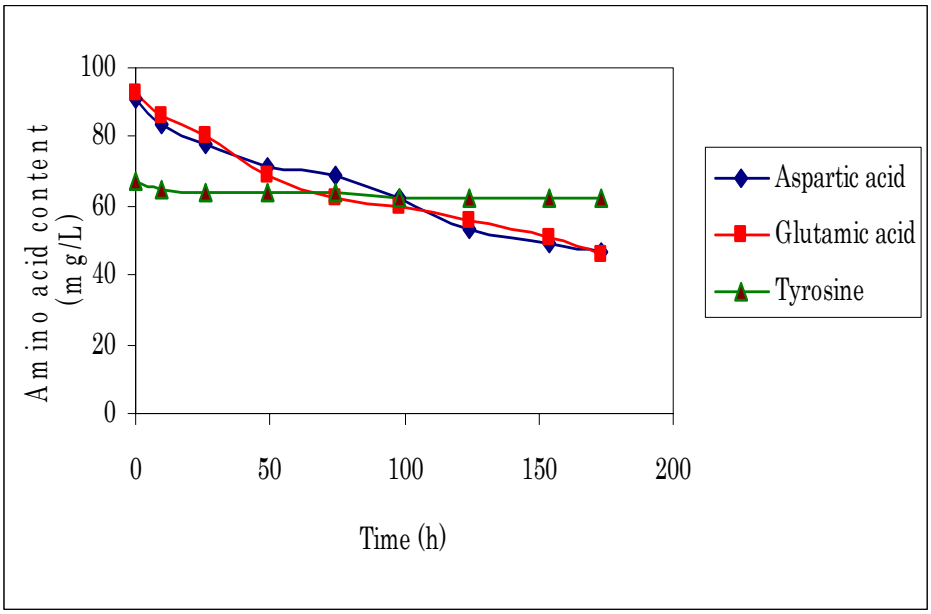


(a)

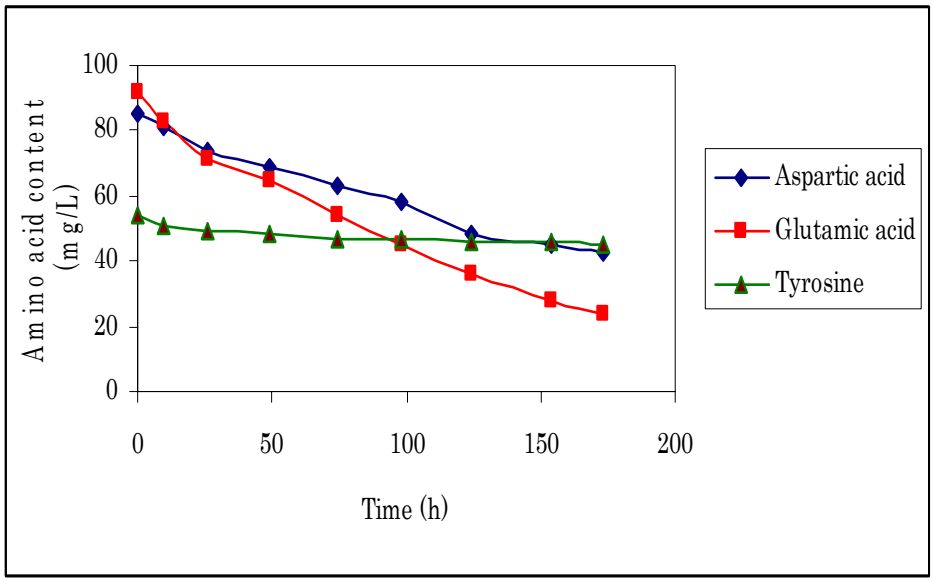


(b)

Figure 5.35 Phenolic content change versus time for raw OMW sample (a), and for the effluent of clay treatment process (b), during photofermentation. [The volumetric ratio of either the effluents of pretreatment processes or raw OMW, fed to the photobioreactors was 4% (v/v)]

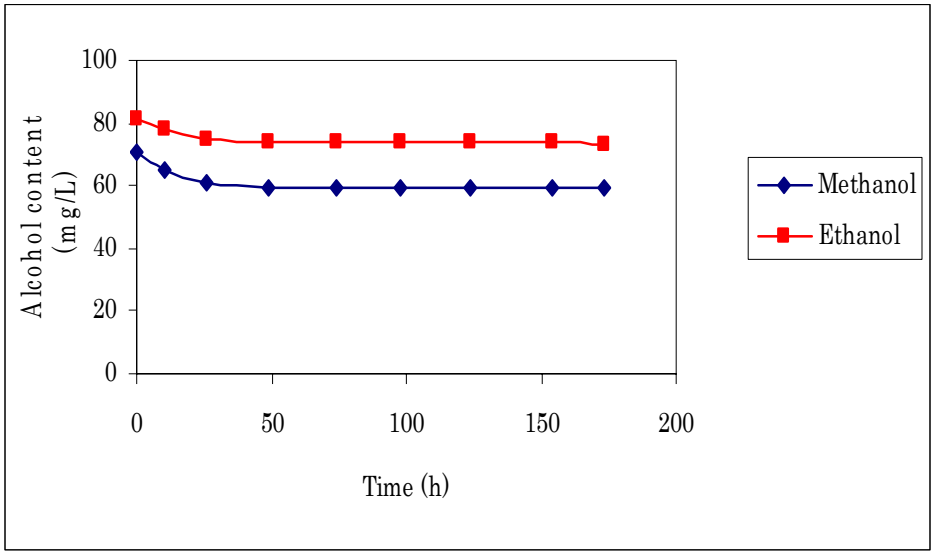


(a)

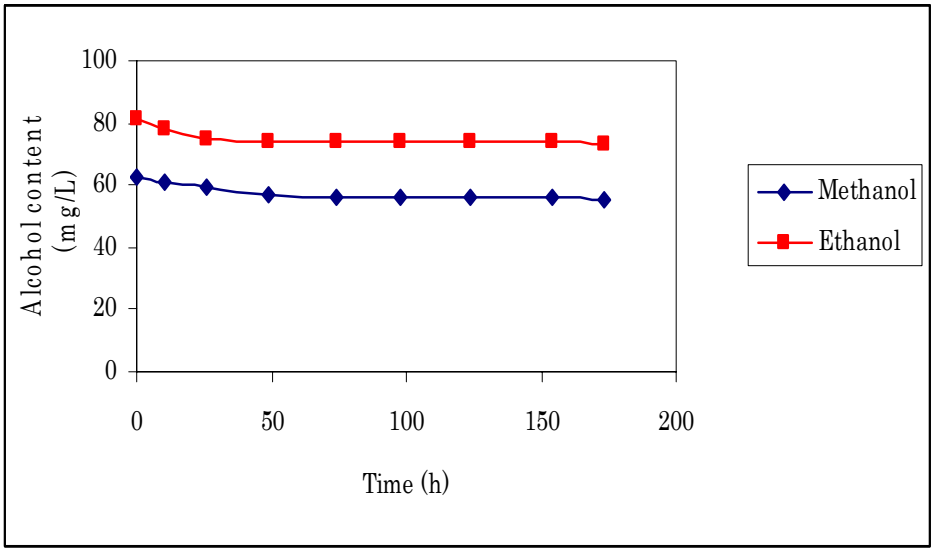


(b)

Figure 5.36 Amino acid content change versus time for raw OMW sample (a), and for the effluent of clay treatment process (b), during photofermentation. [The volumetric ratio of either the effluents of pretreatment processes or raw OMW, fed to the photobioreactors was 4% (v/v)]



(a)



(b)

Figure 5.37 Alcohol content change versus time for raw OMW sample (a), and for the effluent of clay treatment process (b), during photofermentation. [The volumetric ratio of either the effluents of pretreatment processes or raw OMW, fed to the photobioreactors was 4% (v/v)]

Results of a single-stage and a two-stage process are compared on the context of hydrogen production and substrate consumption in Tables 5.10 and 5.11, respectively. In Table 5.11, concentrations of organics at the inlet and at the outlet of photobioreactors (PBR) were given to illustrate the substrate consumption at the photofermentation step. According to Table 5.10; two stage process appears to be a good alternative, in accordance with its significantly higher hydrogen production potential (32 L/L), hydrogen production rates (0.016 L/L/h and 0.084 L/g/h), and light conversion efficiency (0.23%) results.

Purple non-sulphur bacteria are known to be metabolically the most diverse species among the prokaryotes (Tabita, 1995), with the capability of utilizing a wide variety of substrates as carbon and nitrogen sources (Koku *et al.*, 2002). According to the previous experimental studies on photoheterotrophic growth, *R. sphaeroides* is known to utilize several carbon sources, such as lactate, butyrate, pyruvate, acetate, citrate, fumerate, malate, succinate, glucose, fructose and glycerol (Koku *et al.*, 2002).

In the present study, significant utilization of acetic, butyric, lactic and propionic acids as well as glutamic, and aspartic acids, glucose, mannitol, n-phenol and m-cresol constituents were observed (Table 5.11). For two-stage process, much higher amounts of organic acids were consumed in parallel to enhanced hydrogen production. Although n-phenol and m-cresol removal percentages (85%) were obtained to be higher for the clay effluent case, total amount of their consumption is much higher during the experiments with raw-OMW sample that is initially rich in phenolic content. With regard to the clay treatment process, phenolic compounds in the photofermentor inlet composition were highly depleted. As a matter of fact, bacteria can easily utilize

these biologically degradable phenolic compounds present at lower concentrations.

Within our knowledge, consumption of phenol, m-cresol, o-cresol, arabinose and mannitol by the photosynthetic bacteria has not been reported in the literature.

Table 5.10 Comparison of single-stage and two-stage processes on the context of hydrogen production parameters.

Characteristics	**Process**	
	Single stage	**Two-stage**
Photobioreactor liquid	Raw OMW -4% (v/v)-	Effluent of Clay treatment -4% (v/v)-
X_{max} (g/L)	0.34	0.23
t_{lag} (h)	18	18
Δt_{H2} (h)	155	195
HPP (L_{H2}/L_{OMW})	16.0	31.5
r_g ($L\ L^{-1}\ h^{-1}$)	0.006	0.016
r_{g^1} ($L\ g^{-1}\ h^{-1}$)	0.021	0.084
η (%)	0.12	0.23

Table 5.11 Comparison of single-stage and two-stage processes on the context of substrate consumption.

Process	**Single stage**			**Two-stage**		
Photobioreactor liquid	Raw OMW -4% (v/v)-			Effluent of Clay treatment -4% (v/v)-		
SUBSTRATES						
Organics	PBR inlet (mg/L)	PBR outlet (mg/L)	Cons. (%)	PBR inlet (mg/L)	PBR outlet (mg/L)	Cons. (%)
Acetic acid	352	214	39.2	312	68	78.2
Butyric acid	74	30	59.5	70	9	87.1
Propionic acid	76	32	57.9	67	3	95.5
Lactic acid	71	12	83.1	71	12	83.1
Formic acid	8	7	12.5	8	7	12.5
Aspartic acid	91	47	48.4	85	43	49.4
Glutamic acid	93	46	50.5	92	24	73.9
Tyrosine	67	62	7.5	54	45	16.7
n-phenol	325	270	16.9	50	8	84.0
m-cresol	230	179	22.2	40	6	85.0
o-cresol	95	86	9.5	-0-	-0-	-0-
p-cresol	78	78	-0-	-0-	-0-	-0-
Glucose	33	2	94.0	37	10	73.0
Xylose	23	12	47.8	29	18	37.9
Arabinose	4	3	25.0	6	4	33.3
Mannitol	2	1	50.0	3	1	66.7
Methanol	71	59	16.9	63	55	12.7
Ethanol	78	78	11.4	81	73	9.9

5.4.2. Process Evaluation

All through the Chapter 5, principally investigated two stage process includes the clay treatment of OMW prior to the utilization of pretreatment effluents for photofermentative hydrogen production. In order to check the accuracy of the measurements given in previous sections, a material balance on this two stage process becomes necessary. The first step in the solution of material balance problems is generally to apply the principles of conservation of mass to the whole system or to the individual parts of the system. Simple mass balance equation is known as:

$$\text{Input} = \text{Output} + \text{Accumulation} \quad (5.2)$$

Overall scheme of this two-stage photofermentation process is given in Figure 5.38. During the first stage (clay pretreatment); raw OMW sample (100 mL) was mixed with clay material (2 grams). Solid outlet of this process was washed with water (100 mL), resulting same amounts of liquid effluent (100 mL). On the other hand, small part (2 mL) of clay pretreatment effluent was channeled into the photofermentative hydrogen production path by storing the rest liquid effluent (98 mL) in a refrigerator. It was subjected to some manipulations (i.e, pH change, autoclaving and dilution with 48 mL of distilled water to yield 4% (v/v)) before fed into the photobioreactor (PBR). Other than this liquid substrate source, 10% (v/v) of bacterial culture (5 mL) is inoculated into PBR. Outputs of this photofermentation stage are the off- gases (H_2 and CO_2) and the liquid effluent of the photobioreactor (55 mL).

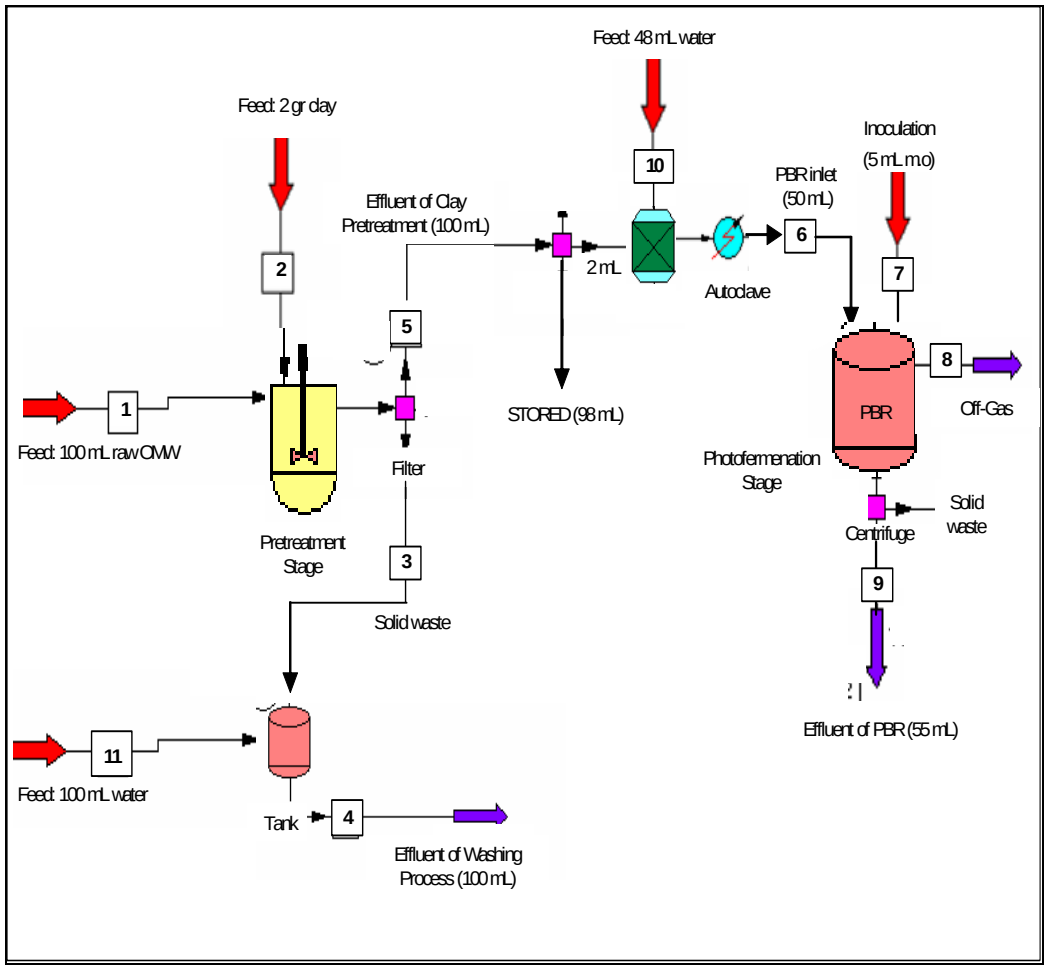


Figure 5.38 Overall scheme of two-stage photofermentation process with the effluent of clay pretreatment

Clay pretreatment and photobioreactor (PBR) stages were chosen as two separate systems in which the mass balance equation will be applied, individually. After the elimination of the accumulation term in Equation (5.2), overall mass balance on each system must provide the condition that "the total amount of inlet is equal to the total amount of outlet".

Amounts of elements and organics at different streams are given in Tables 5.12 and 5.13, respectively. The chemical compositions of each stream are given in Appendix M7.

To sum up these results, Table 5.14 represents the overall summation of the constituents at different stages. During the application of overall material balance on each system, the following components were summed up.

1. Elemental totals (Subtotal-1 in Table 5.12)
2. Total sugar contents (Table 5.14)
3. Total phenol contents (Table 5.14)
4. Alcohol contents (Subtotal-3 given in Table 5.13)
5. Organic acids (Subtotal-4 given in Table 5.13)
6. Amino acids (Subtotal-6 given in Table 5.13)
7. Total solids (Table 5.14)
8. Hydrogen gas (Table 5.14)
9. Carbondioxide gas (Table 5.14)

Although the inputs are not strictly equal to the outputs, there is not a large dissimilarity between them (Table 5.14). Main difference (1362 g) for the pretreatment stage is caused by the probable material losses during solid separation processes including filtration and centrifugation techniques. On the other hand, slight difference (11 mg) of the photofermentation stage is mainly attributable to the losses occurred during the collection of samples at certain time intervals for individual photofermentative hydrogen production analysis and the constituents inside the grown bacteria.

Table 5.12 Elemental content of different streams within a two-stage hydrogen production process

Stage		Clay Pretreatment					Photofermentation		
Stream Number		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**9**
Stream Name		Raw OMW	Fresh Clay	Spent clay	Effluent of washing	Effluent of clay pret.	PBR Inlet	Bacterial Inoc.	Effluent of PBR
Total Basis		100 mL	2 gr	2 gr	100 mL	100 mL	50 mL	5 mL	55 mL
ELEMENTS (mg)	K	350	0.33	1.8	36	300	0.52	0.0082	0.12
	Na	14	86	16	9.2	70	0.98	0.0031	0.12
	Fe	3.9	116	85	11	16	0.23	0.0024	0.094
	Ca	10	2.0	1.2	0.89	8.5	0.11	-0-	0.014
	Mg	9.6	8.7	8.0	1.2	7.8	0.14	0.0012	0.064
	B	0.47	-0-	-0-	-0-	0.42	0.011	-0-	0.0072
	Pb	0.46	0.37	0.32	0.11	0.23	-0-	-0-	-0-
	Zn	0.18	0.32	0.42	-0-	0.12	-0-	-0-	-0-
	Mn	0.091	0.022	0.02	-0-	0.11	-0-	-0-	-0-
	Co	0.025	-0-	-0-	-0-	0.02	-0-	-0-	-0-
	Mo	0.0037	-0-	-0-	-0-	0.0033	-0-	-0-	-0-
	Ni	0.020	1.10	0.74	0.35	0.032	-0-	-0-	-0-
	Cr	0.023	0.041	0.035	-0-	0.011	-0-	-0-	-0-
	Cu	0.0052	0.16	0.14	-0-	0.007	-0-	-0-	-0-
	Subtotal (1)	**389**	**215**	**114**	**59**	**403**	**2.0**	**0.015**	**0.42**

*Gaseous stream (8) does not contain these elements.

Table 5.13 Organic content of different streams within a two-stage hydrogen production process

Stage		Clay Pretreatment			Photofermentation		
Stream Number		1	4	5	6	7	9
Stream Name		Raw OMW	Effluent of washing	Effluent of clay pret.	PBR Inlet	Bacterial Inoc.	Effluent of PBR
Total Basis		100 mL	100 mL	100 mL	50 mL	5 mL	55 mL
Sugars (mg)	Glucose	91	-0-	88	1.65	-0-	0.12
	Xylose	67	2	64	1.14	-0-	0.61
	Arabinose	13	-0-	12	0.21	-0-	0.16
	Mannitol	7	-0-	6	0.10	-0-	0.04
	Subtotal (2)	**178**	**2**	**172**	**3.1**	**-0-**	**0.93**
Alcohol (mg)	Methanol	210	6	199	3.14	-0-	2.78
	Ethanol	232	5	224	4.08	-0-	3.64
	Subtotal (3)	**442**	**11**	**423**	**7.22**	**-0-**	**6.42**
Organic Acids (mg)	Acetic Acid	971	7	962	15.58	0.08	3.40
	Formic acid	27	-0-	24	0.41	-0-	0.35
	Prop. acid	180	4	171	3.39	-0-	0.15
	Butyric Acid	195	3	189	3.48	-0-	0.44
	Lactic Acid	114	9	102	3.51	0.01	0.59
	Subtotal (4)	**1487**	**23**	**1448**	**26.37**	**0.09**	**4.93**
Phenol (mg)	Phenol	234	89	132	2.5	-0-	0.4
	p-cresol	138	109	12	-0-	-0-	-0-
	m-cresol	256	99	117	2	-0-	0.3
	o-cresol	97	58	14	-0-	-0-	-0-
	Subtotal (5)	**725**	**355**	**275**	**4.5**		**0.7**
Amino acid (mg)	Aspartic acid	732	7	723	4.2	0	2.2
	Glutamic acid	765	11	753	4.6	0.01	1.2
	Tyrosine	245	28	229	2.7	0	2.3
	Subtotal (6)	**1742**	**46**	**1705**	**11.5**	**0.01**	**5.7**

* Streams 2, 3 and 8 do not contain organic compounds.

Table 5.14 Overall material balances of different streams within a two-stage hydrogen production process

Stage		Clay Pretreatment					Photofermentation			
Stream Number		1	2	3	4	5	6	7	8	9
Stream Name		Raw OMW	Fresh Clay	Spent clay	Effluent of washing	Effluent of clay pret.	PBR Inlet	Bacterial Inoc.	Off gas	Effluent of PBR
Total Basis		100 mL	2 gr	2 gr	100 mL	100 mL	50 mL	5 mL	62 mL	55 mL
PROPERTY (mg)	Elements (Subtotal-1)	389	215	114	59	403	2	0.015	-	0.42
	Alcohols (Subtotal-3)	442	-	-	11	423	7.22	0	-	6.42
	Organic Acids (Subtotal-4)	1487	-	-	23	1448	26.37	0.09	-	4.93
	Amino acids (Subtotal-6)	1742	-	-	29	1705	11.5	0.01	-	5.7
	Total Sugar	1500	-	-	60	1200	72	0	-	64
	Total Phenol	1200	-	-	490	400	7	0	-	5
	Total solids	4070	1620	5010	120	600	7.5	2	-	21
	H2	-	-	-	-	-	-	-	5.43	-
	CO2	-	-	-	-	-	-	-	1.98	-
	TOTAL	10830	1835	5124	792	6179	121	2	7.4	101.5
	Mass Balances	Inputs: 10830+ 1835= 12665 mg Outputs: 5124 + 6179 = 11303 mg Difference: 12665 – 11303 = 1362 mg					Inputs: 121 + 2 = 123 mg Outputs: 7+ 102 = 109 mg Difference: 123 – 109 = 11 mg			

In order to check the accuracy of the amount of hydrogen production (58 mL); Comparison of experimental and theoretical yield of hydrogen that could be produced from the consumption of organic substrates present in OMW (Table 5.15). Theoretical amounts were obtained by considering that all of the substrate was used for hydrogen and carbon dioxide production according to the following hypothetical reaction (Sasikala *et al.*, 1993):

$$C_xH_yO_z + (2x\text{-}z)\ H_2O \rightarrow (y/2 + 2x - z)\ H_2 + x\ CO_2 \qquad (5.2)$$

According to Table 5.15, theoretical yield of hydrogen was obtained as 2.40 and 2.83 mg/L_{OMW} for the single-stage and two-stage processes, respectively. Both of these values are relatively higher than the experimental results (1.50 mg/L_{OMW} for one-stage and 2.72 mg/L_{OMW} for two-stage).

In relation to the elemental analysis; single-stage and two-stage processes were obtained to have 2.57 mol_C/L_{OMW} (30.8 g/L) and 1.34 mol_C/L_{OMW} (16.1 g/L), respectively. According to the inlet compositions of PBR (given in Table 5.11), analysed carbon content was 25.4 g/L for single-stage processes and 12.3 g/L for two-stage processes which are close to the total amounts calculated by the elemental analysis results.

However, it should not be overlooked that the additional amounts, resulting from the non-detected organic substrates (such as low chain fatty acids, citric acids, complex sugars, etc) would have a rising effect on the theoretical values. These calculations showed that the amount of experimental hydrogen gas production is in a reasonable range when compared with the theoretical one. In particular, the main organic compounds for higher hydrogen production are acetic, lactic, propionic,

glutamic and butyric acids as well as phenol and meta-cresol; if they were not utilized during bacterial growth or alternative biosynthetic pathways. By applying clay pretreatment technique, it was observed that higher amounts of substrate were converted into hydrogen. Lower substrate conversion was observed for one-stage process due to the probable shift of the metabolism for bacterial growth and PHB synthesis in parallel to hydrogen evolution.

Table 5.15 Comparison of experimental and theoretical yield of hydrogen that could be produced from the consumption of organic substrates present in OMW. (Consumption values are taken from Table 5.11)

Organics	Single-stage Process	Two-stage Process
	Theoretical H2 Yield (g $_{H2}$ / L $_{OMW}$)	
Acetic acid (C2H4O2)	0.46	0.81
Butyric acid (C4H8O2)	0.25	0.35
Propionic acid (C3H6O2)	0.15	0.22
Lactic acid (C3H6O3)	0.20	0.20
Formic acid (CH2O2)	0.00	0.00
Aspartic acid (C4H7NO4)	0.10	0.09
Glutamic acid (C5H9NO4)	0.14	0.21
Tyrosine (C9H11NO3)	0.03	0.05
Phenol (C6H5O)	0.40	0.30
m-cresol (C7H8O)	0.40	0.27
o-cresol (C7H8O)	0.07	0.00
p-cresol (C7H8O)	0.00	0.00
Glucose (C6H12O6)	0.10	0.09
Xylose (C5H10O5)	0.04	0.04
Arabinose (C5H10O5)	0.00	0.01
Mannitol (C6H14O6)	0.00	0.01
Methanol (CH4O)	0.06	0.04
Ethanol (C2H6O)	0.00	0.05
Total Theoretical H2 Yield (g $_{H2}$ / L $_{OMW}$)	**2.40**	**2.83**
Total Experimental H2 Yield (g $_{H2}$ / L $_{OMW}$)	**1.50**	**2.72**

5.5. Outdoor Hydrogen Production in a Solar Bioreactor for Scale-up

Rhodobacter sphaeroides O.U.001 is a kind of bacteria that can be able to make dark fermentation in addition to photo-fermentation (Koku *et al.*, 2002). By using proper experimental conditions, this property can be used to make a coupled system of its own. In this part of the study, photobiological hydrogen production experiments were carried in a solar bioreactor (8L) under the illumination of sun which is not a continuous illumination process.

It has been known that under limited illumination with sunlight, bacteria follow the dark fermentative pathway and produce organic acids (Tabanoğlu, 2002). When light is sufficient, then the bacteria shifts its metabolism into photofermentation mode. As a result of these light/dark cycles, process behaves like a two-step coupled system as experimented in the previous section (Section 5.2.3) by the coupling of *R.sphaeroides* O.U.001 with sewage sludge culture.

Outdoor experiment within an 8 L solar bioreactor was carried out from August 21th to August 30th, 2003. 4% (v/v) OMW-A containing media was utilized as a substrate source, because of the fact that OMW-A is the only raw wastewater present in our laboratories. Its physicochemical properties are given in Section 5.1 (Table 5.1). The experimental data for outdoor hydrogen production study is given in Appendix M8.

Figure 5.39 shows the illumination pattern of the solar bioreactor during that time period (21-23 August 2003). It can be observed from this figure that, except for the sixth day (160-180 h) which was partially cloudy, illumination of the sunlight fits to a

sinusoidal pattern with a peak value at around 95,000 lux. In the partially cloudy day variable light intensity was observed due to the light scattering effects. Approximately 14 h light/10 h dark periods were observed throughout the whole experiment (Figure 5.39). In this figure; the dark periods are symbolized by gray-blocks.

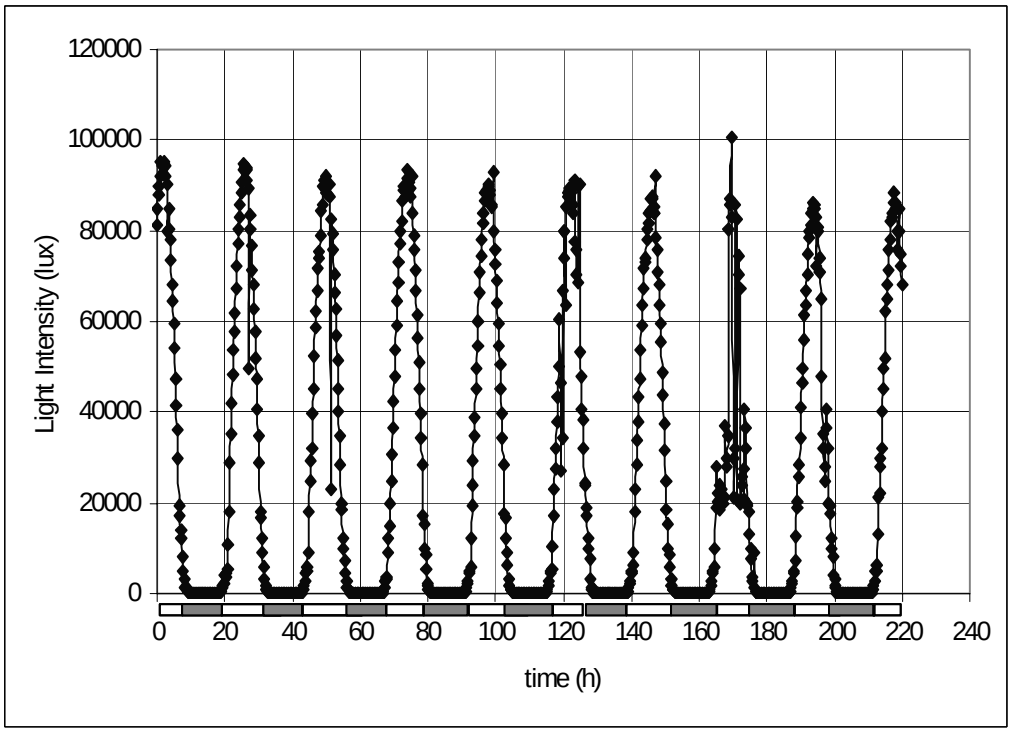


Figure 5.39 Light density distribution profile for the outdoor experiment (21-23 August 2003)

Figures 5.40, 5.41, 5.42 and 5.43 illustrate the total hydrogen production, bacterial growth, the amount of hydrogen production per bacterial weight and pH changes of an outdoor hydrogen production experiment, respectively. Dark periods are again symbolized by gray-blocks.

As can be observed from Figure 5.40, hydrogen is continuously produced throughout the experiment (2560 mL). Meanwhile, bacterial

growth was observed to be slightly depleted during dark periods (Figure 5.41). These kind of bacterial actions resulted exponential increases on the total amount of hydrogen production per bacterial weight (Figure 5.42) at dark periods, i.e. under limited illumination. On the other hand, this ratio was kept as stationary during available light conditions. It was also observed that pH values generally tended to decrease during dark periods (Figure 5.43), because of the probable accumulation of dark-fermentative end products having acidic properties.

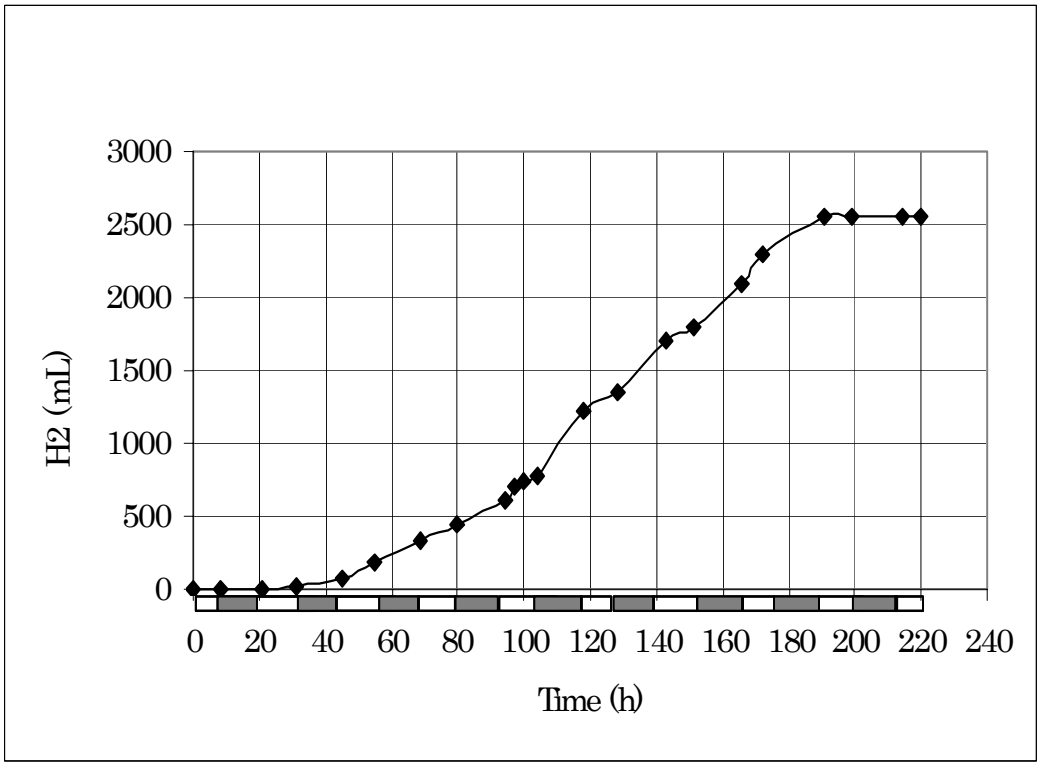


Figure 5.40 Total volume of hydrogen gas production versus time for raw OMW (4% v/v) containing media in a solar bioreactor

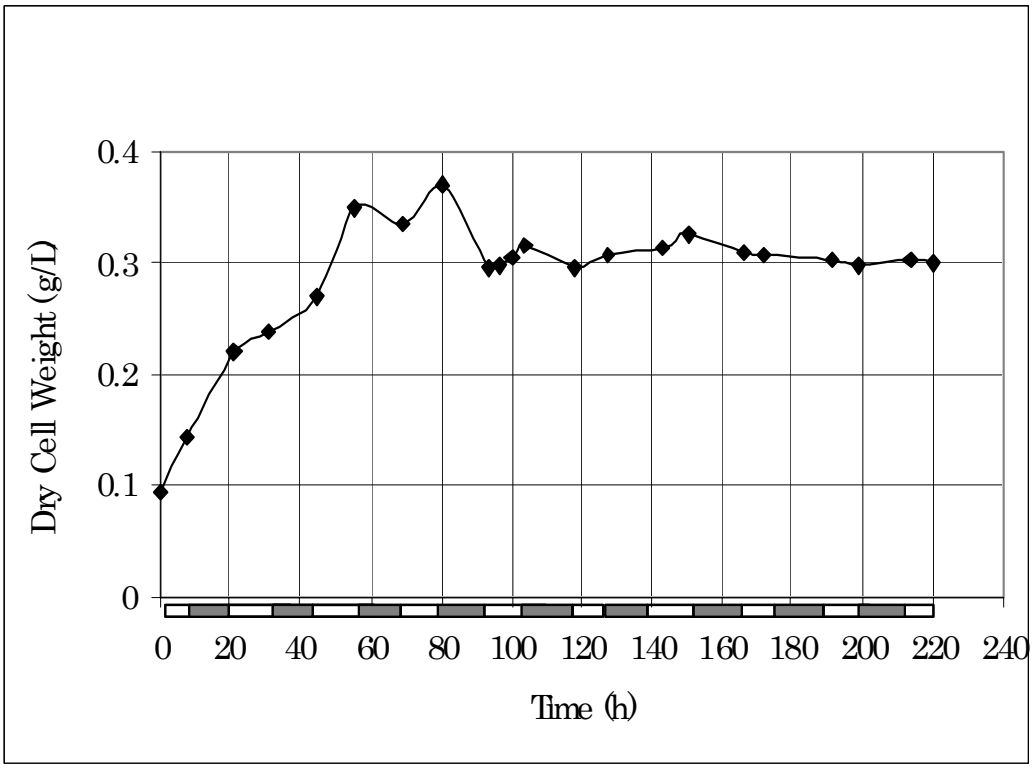


Figure 5.41 Bacterial growth curves for raw OMW (4% v/v) containing media in a solar bioreactor

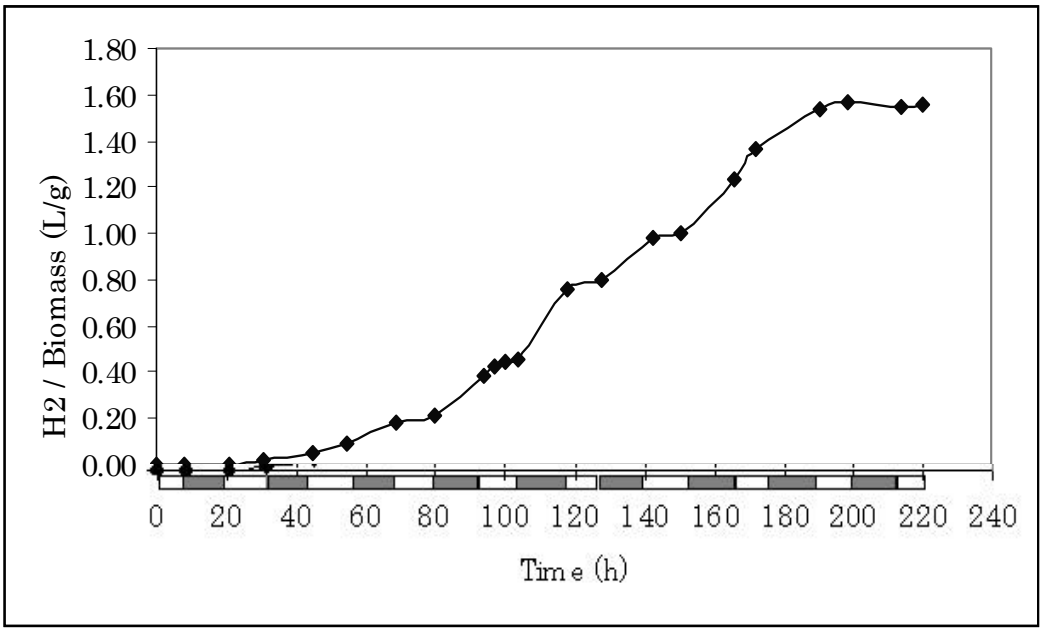


Figure 5.42 Total amount of hydrogen gas production per weight of bacteria grown in raw OMW (4% v/v) containing media in a solar bioreactor

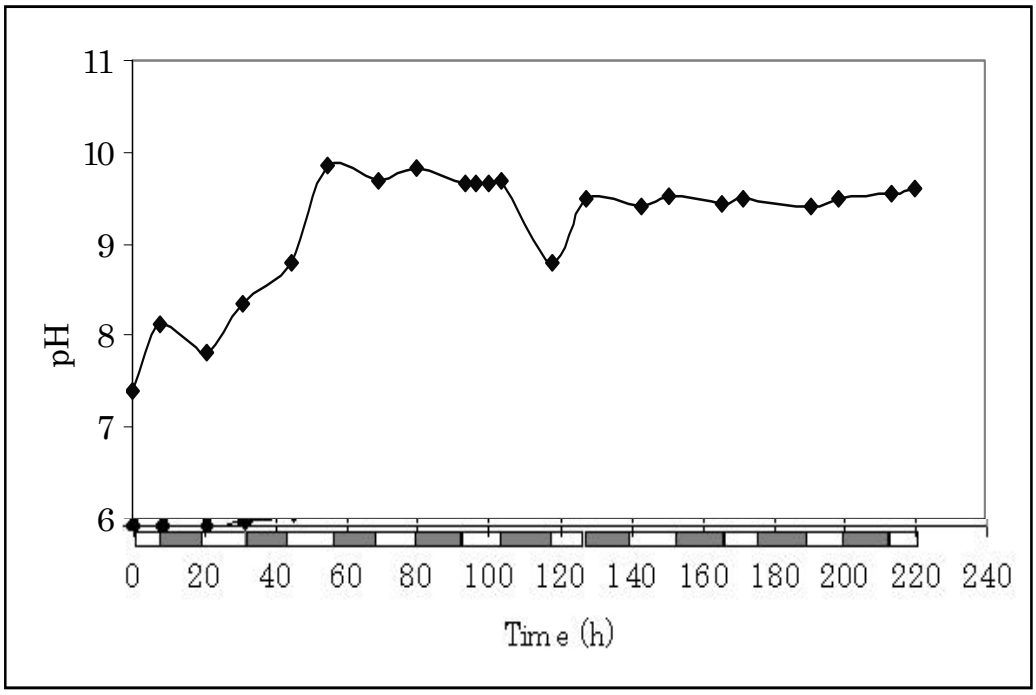


Figure 5.43 pH change versus time for raw OMW (4% v/v) containing media in a solar bioreactor

Hydrogen production parameters obtained for different bioreactors, having different geometries and illumination patterns, are compared with each other in Table 5.16. In all of these bioreactors, same substrate source was utilized (4% raw OMW-A). Indoor photobioreactor with smaller scale (55 mL) resulted the highest hydrogen gas production potential (15 L/L) and hydrogen production rates (0.007 L/L/h).

On the basis of valuable by-products, both outdoor bioreactor (8L) and indoor bioreactor (400mL) outlets were analyzed for polyhydroxybutyrate and carotenoid contents. In the case of outdoor experiments, relatively higher amounts of by-product formation (0.78 mg carotenoid and 1.21 mg PHB per g wet weight of bacteria) was observed. The present study showed that microorganisms tend to protect themselves from the high light intensity by forming carotenoid

pigments and they produce PHB as energy storage material for dark periods. Therefore, it can be concluded that by-product formation support the feasibility of large scale outdoor biological hydrogen production from olive mill wastewater.

Table 5.16 Comparison of different bioreactors on the context of hydrogen production parameters

Characteristics	**Outdoor bioreactor**	**Indoor bioreactor**	**Indoor bioreactor** (Eroğlu *et al.*, 2004)
Size of the reactor	8 L	55 mL	400 mL
Light source	Sun light	Tungsten lamp	Tungsten lamp
Substrate source	4% OMW	4% OMW	4% OMW
X_{max} (g/l)	0.35	0.64	0.552
t_{lag} (h)	20	12	18
Δt_{H2} (h)	194	95	80
HPP (l_{H2}/l_{OMW})	9.9	14.9	10.1
r_g (l l^{-1} h^{-1})	0.003	0.007	0.005
r_{g^1} (l g^{-1} h^{-1})	0.011	0.012	0.014
η (%)	0.47	0.18	0.38
PHB (mg/g wet weight)	1.21	Not analyzed	0.23
Carotenoid (mg/g wet weight)	0.78	Not analyzed	0.25

CHAPTER 6

CONCLUSIONS

It can be drawn as a main conclusion that, the efficiency of photobiological hydrogen production from OMW as well as its remediation is significantly enhanced by developing appropriate two-stage processes such as clay pretreatment followed by photofermentation.

Rhodobacter sphaeroides O.U.001 absorbs light energy at a wide range of light spectrum including the non-visible parts (i.e., 400 to 950 nm), and contains the photosynthetic pigments of bacteriochlorophyll and carotenoids of spheroidene series (Pellerin and Gest, 1983). Nonetheless, most of the raw OMW samples absorb these wavelengths and have a shadowing effect on the photosynthetic bacteria. Accordingly, decolorization (i.e., by dilution, or by various pretreatment techniques) becomes essential for an effective photofermentation process.

Among different pretreatment processes; clay pretreatment technique was found to be the best alternative for a two stage hydrogen production process. Main reasons for the enhancement of photofermentative hydrogen production following a clay pretreatment process are especially attributed to the following achievements:

1. High color depletion (65%)
2. High phenol removal (81%)
3. Minor removal (%20) of organic acids, sugars and amino acids from the media that are known to be mainly utilized for photofermentative hydrogen production
4. Significant depletion in the light absorbance of raw OMW sample, having a shadowing effect on the photosynthetic bacteria
5. Ease of operation and availability
6. Materials at lower costs
7. Give rise to the utilization of more concentrated OMW sample (50% raw-OMW)
8. Non-toxic environment of the process
9. Possibility of spent-clay regeneration

During photofermentative hydrogen production with the effluent of clay pretreatment; significant amounts of acetic, butyric, lactic, propionic, glutamic and aspartic acids, as well as glucose, mannitol, n-phenol and m-cresol constituents were utilized. Compared to the photofermentation with raw OMW (16 L H_2/L $_{OMW}$), the amount of hydrogen production was improved by %100 during photofermentation with the effluent of clay pretreatment process (29 L H_2/L $_{OMW}$).

Phenol, m-cresol and o-cresol consumption rates were highest during the exponential phase of bacterial growth (around first 25 hours). This statement also gives rise to the color depletion during first 25 h, and the significant decrease in the light absorbance values. These results are in accordance with the high removal of phenolics and other aromatic complexes which are responsible for the dark color and higher light absorbance values. Glucose consumption rate was significantly increased after stationary phase of the bacterial growth and hydrogen

production (around 100th hour). Highest portion of the organic compounds were consumed during the exponential phase of bacterial growth. Main organic compounds resulting higher hydrogen production rates are mainly acetic, lactic, propionic, and butyric acids.

A strong proportionality between the hydrogen production and the color removal efficiencies of different clay fractions was also observed. As the clay quantity was increased (up to a certain saturation concentration: 20 g clay per 1 L OMW); color, COD and phenol removal efficiencies as well as hydrogen production capacities are directly increased. Thus, high amounts of clays should be used which will result solid waste accumulation. Accordingly, their reutilization during the post treatment of the liquid waste is promising for the reduction of solid waste quantity and the liquid waste quality. Alternative to regeneration, spent clay can be utilized as a raw material for glass-ceramics; consumed either in the brick or cement industries; burned in the furnaces or added to animal feeds (Toya *et al.*, 2004).

Based on these hydrogen production results with the effluent of clay pretreatment process (HPP_{max} is around 30 L hydrogen per 1L OMW), the biological hydrogen production potential of olive mill wastewater for the Mediterranean countries (generating more than 30 million m^3 of OMW per year) would be circa $1x10^9$ m^3 H_2 per year, which would result in approximately 2500 GWh/y electricity through a suitable fuel cell application having more than 50% conversion efficiency.

Other explored pretreatment methods include chemical oxidation with ozone or Fenton's reagent, UV radiation, and dark fermentation with sewage sludge. Despite the fact that strong chemical oxidants like

ozone and Fenton's reagent had a powerful effect on the color removal (90%), their effluents were observed to be unsuitable for both hydrogen production and bacterial growth. Trace amounts of ozone or H_2O_2 (in Fenton's Reagent) remaining in the media appears to be toxic for the bacteria. Besides, the final oxidation products might have additional inhibitory effects on the photosynthetic bacteria.

During the biological pretreatment process, the acclimation of sewage sludge to olive mill wastewater was performed to provide the adaptation of microorganisms to the hardly biodegradable compounds such as phenols. Subsequently, hydrogen production was enhanced by %50 for the case of photofermentation with 4 % (v/v) effluent of dark fermentation with sewage sludge. This might be a result of organic acid production during dark fermentation step that is known to be efficiently utilized by the photosynthetic bacteria for hydrogen production.

It was also proven in the present study that the concentrated OMW samples (i.e., 50% or 100 %) can be utilized for photofermentative hydrogen production, after being subjected to a pretreatment step such as dark fermentation with acclimated sewage sludge or clay treatment. The utilization of concentrated OMW during hydrogen production processes will have a positive effect on the solution of the current wastewater dilution problem.

Within our knowledge, consumption of phenol, m-cresol, o-cresol, arabinose and mannitol by the photosynthetic bacteria has not been reported in the literature before.

According to the outdoor experiments with raw OMW (4%); hydrogen is continuously produced in a solar bioreactor. Meanwhile, bacterial growth was observed to be slightly depleted during dark periods. It was also realized that pH values generally tended to decrease during dark periods because of the probable accumulation of dark-fermentative end products having acidic properties. Relatively higher amounts of by-product formation were realized in this study. These data revealed that microorganisms tend to protect themselves from the high light intensity by forming carotenoid pigments and producing PHB as an energy storage material for dark periods. As a matter of fact, by-product formations will support the feasibility of large scale outdoor biohydrogen studies with olive mill wastewater.

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APPENDIX A

PREPARATION OF PRE-ACTIVATION MEDIUM

Preparation and composition of standard Pre-activation Medium, Trace Element Solution, Vitamin Solution and Fe-citrate Solution

Table A1 The composition of standard pre-activation medium

Composition	Amount
KH_2PO_4	0.5 g/L
$MgSO_4 \cdot 7H_2O$	0.2 g/L
NaCl	0.4 g/L
Na-glutamate (10 mM)	1.8 g/L
$CaCl_2 \cdot 2H_2O$	0.05 g/L
L-Malic Acid (7.5 mM)	1.0 g/L
Vitamin Solution	1 mL/L
Trace Element Solution SL7	1 mL/L
Fe-citrate Solution	1 mL/L

Table A2 The composition of trace element solution

Composition	Amount
HCl (25% v/v)	1 mL/L
$ZnCl_2$	70 mg/L
$MnCl_2 \cdot 4H_2O$	100 mg/L
H_3BO_3	60 mg/L
$CoCl_2 \cdot 6H_2O$	200 mg/L
$CuCl_2 \cdot 2H_2O$	20 mg/L
$NiCl_2 \cdot 6H_2O$	20 mg/L
$NaMoO_4 \cdot 2H_2O$	40 mg/L

The components of the trace element solution were dissolved in 1000 mL distilled water and sterilized in an autoclave.

Table A3 The composition of vitamin solution

Composition	Amount
Thiamine	500 mg/L
Niacin (Nicotinate)	500 mg/L
Biotin	15 mg/L

Fe-citrate Solution: Within 100 ml distilled water, 0.5 g Fe-citrate was dissolved and sterilized by autoclaving.

APPENDIX B

OD-DRY CELL WEIGHT CALIBRATION CURVE

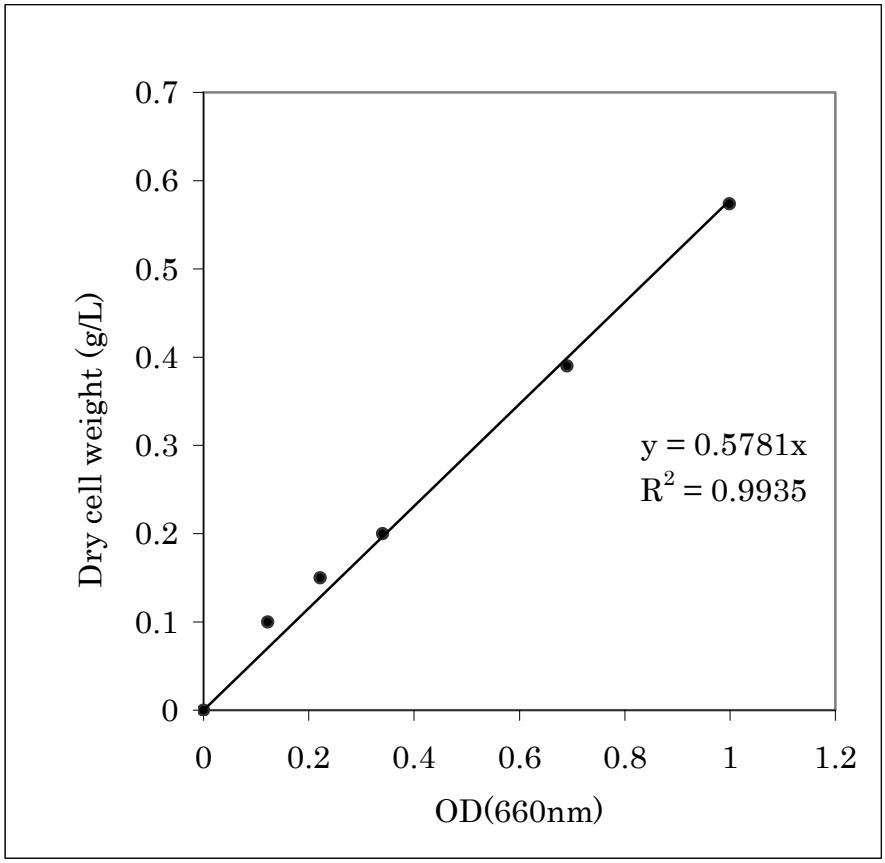

Figure B1 Calibration curve and the regression trend line for dry weight versus OD_{660}

APPENDIX C

SAMPLE GAS CHROMATOGRAM

A sample gas chromatogram, for a typical hydrogen production experiment with OMW containing medium is given in Figure C1. The first largest peak is hydrogen and the smaller peak at the end belongs to the carbon dioxide gas.

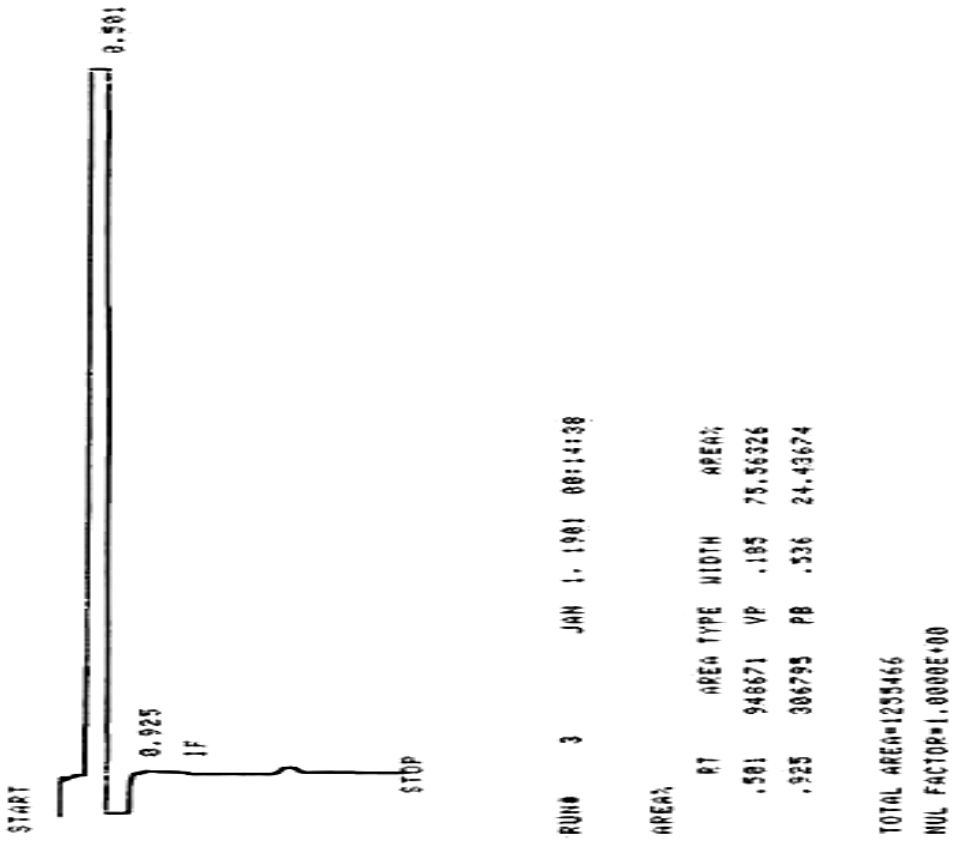

Figure C1 Gas chromatogram of a hydrogen production experiment

APPENDIX D

TOTAL PHENOL CALIBRATION CURVE

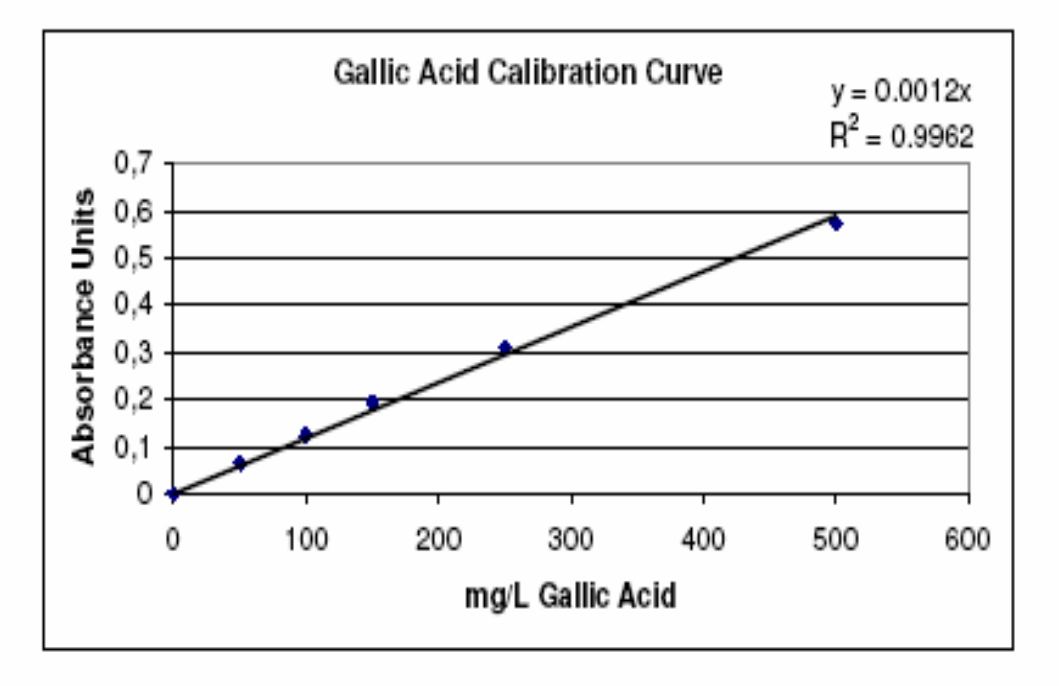

Figure D1 Calibration curve and the regression trend line for absorbance at 765 nm versus gallic acid equivalent of total phenol concentration (Özcan, 2006)

APPENDIX E

TOTAL SUGAR CALIBRATION CURVE

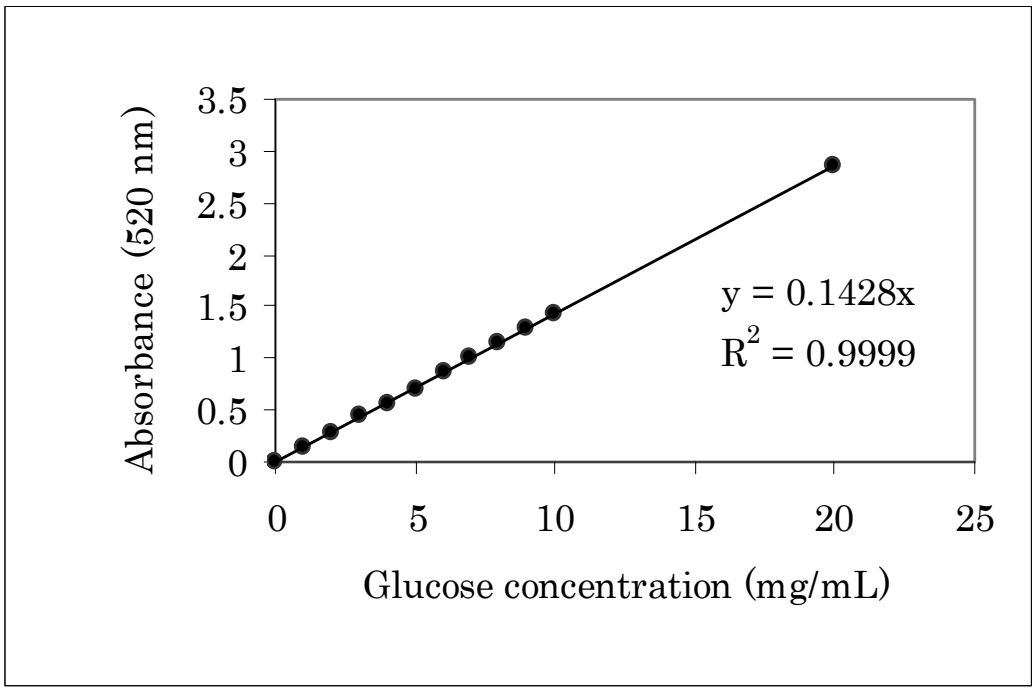

Figure E1 Calibration curve and the regression trend line for absorbance at 520 nm versus glucose equivalent of total sugar concentration (Yılmaz, 2005)

APPENDIX F

SAMPLE HPLC CHROMATOGRAM FOR ORGANIC ACIDS

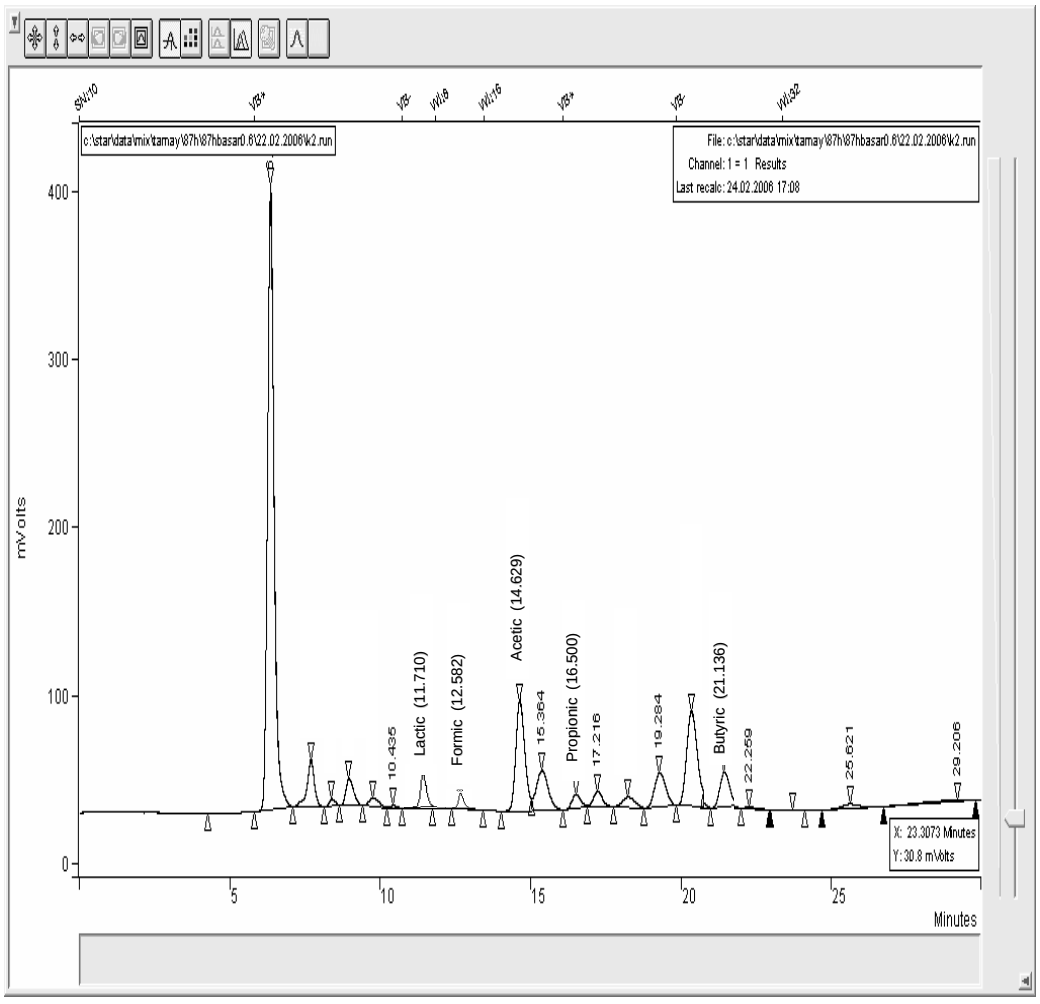

Figure F1 Sample HPLC chromatogram for organic acids

APPENDIX G

SAMPLE HPLC CHROMATOGRAM FOR SUGARS AND ALCOHOLS

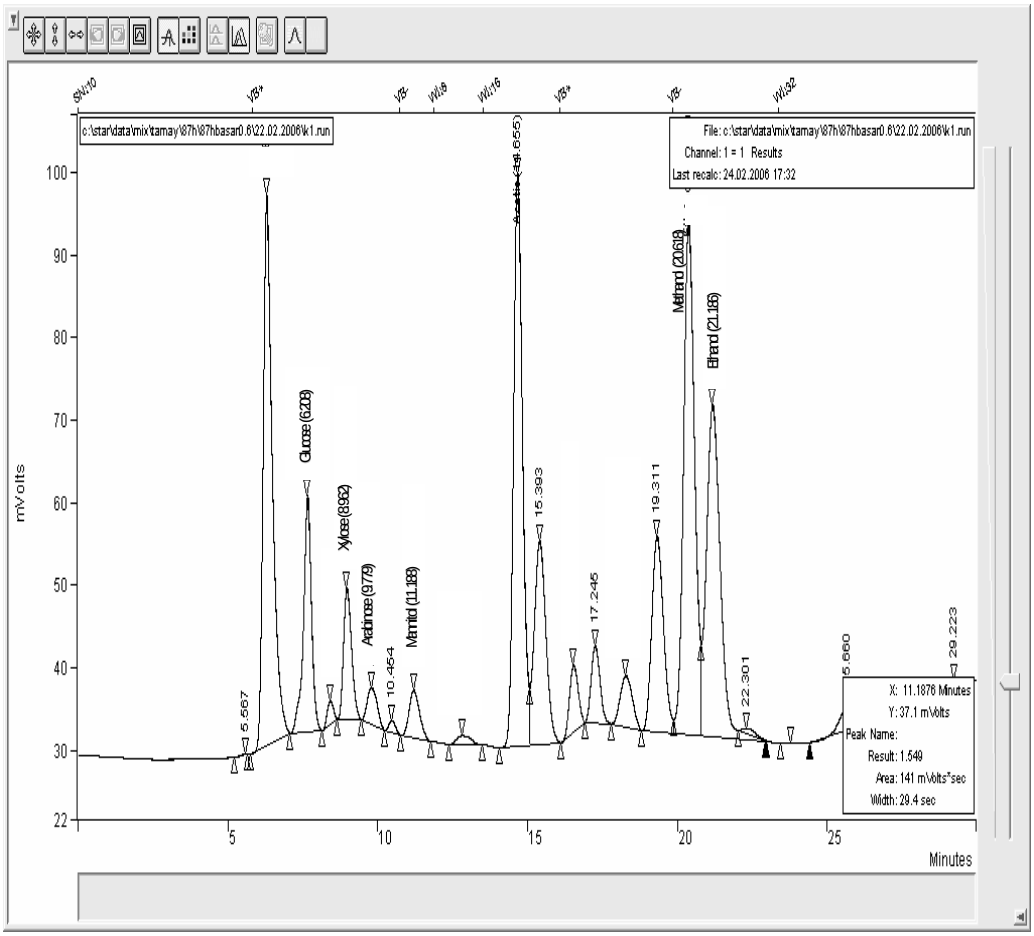

Figure G1 Sample HPLC chromatogram for sugars and alcohols

APPENDIX H

SAMPLE HPLC CHROMATOGRAM FOR AMINO ACIDS

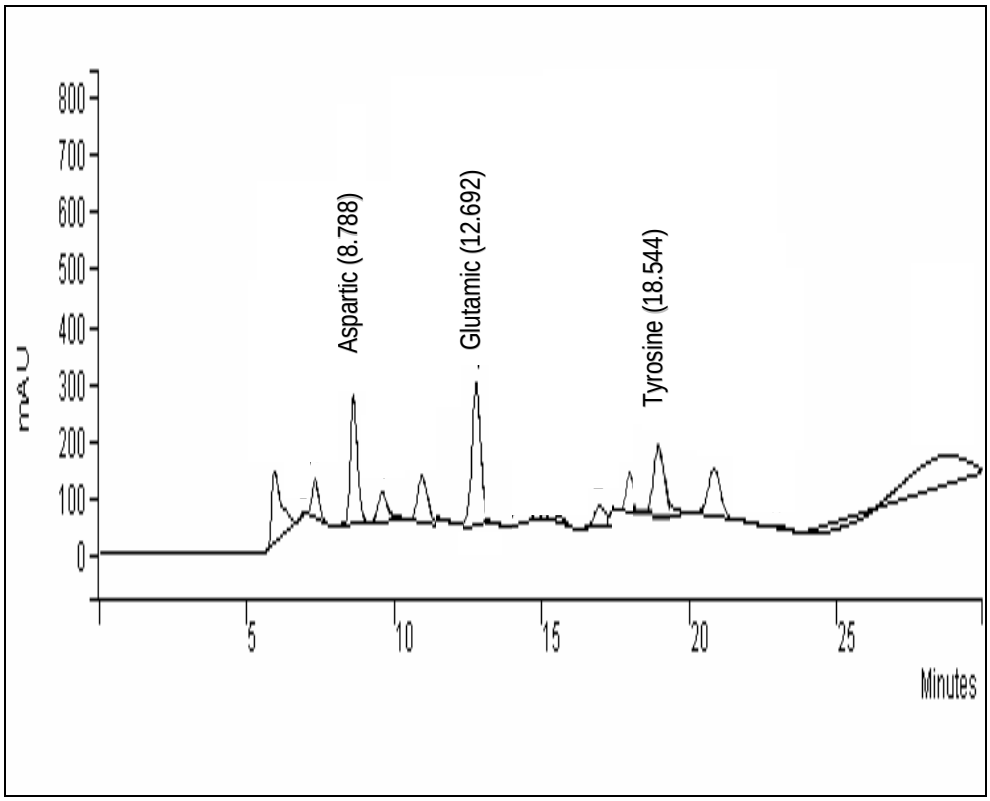

Figure H1 Sample HPLC chromatogram for amino acids

APPENDIX I

SAMPLE GC CHROMATOGRAM FOR PHENOLS

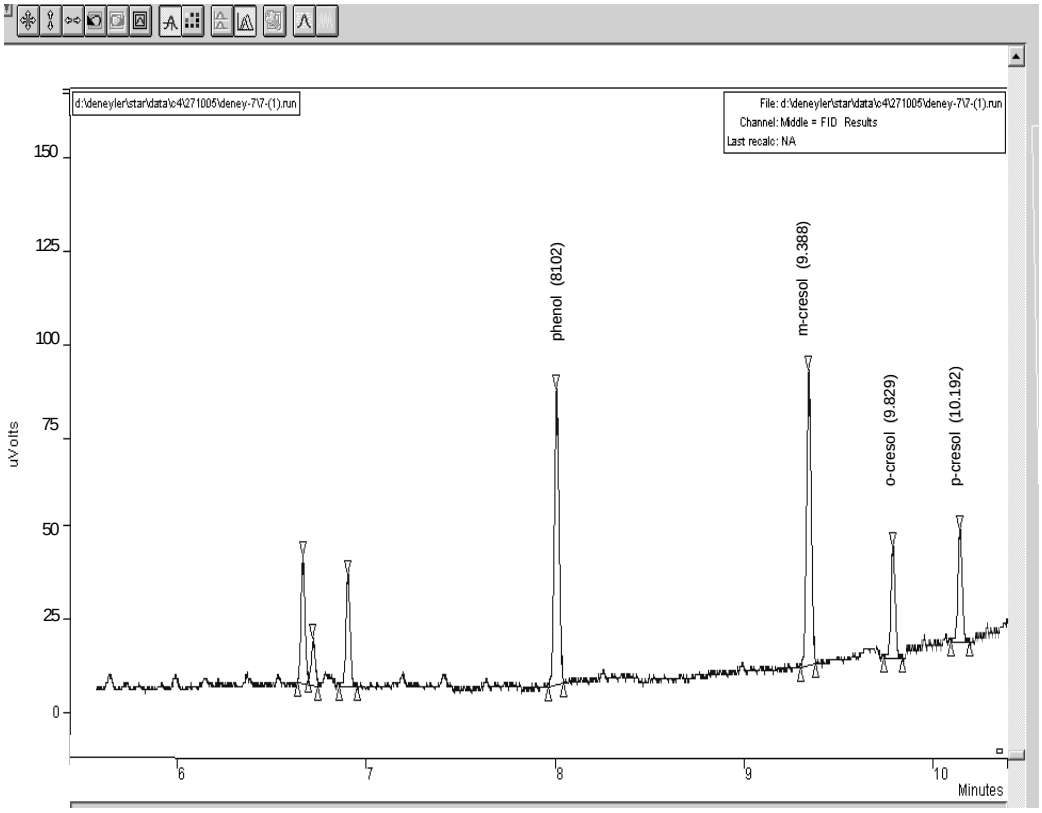

Figure I1 Sample GC chromatogram for phenols

APPENDIX J

DETERMINATION OF PHB CONCENTRATION

In order to determine PHB concentration of the cells grown at different compositions of OMW containing media; a standard curve shown in Figure K1 was used for calculations. The slope of the curve was calculated to be 0.1452 and the concentration of PHB was determined according to the following formula:

$$y = 0.1452x \tag{J1}$$

where, y is the average absorbance of 10 samples that were distributed into 10 different test tubes, and x is the concentration of PHB in μg/mL.

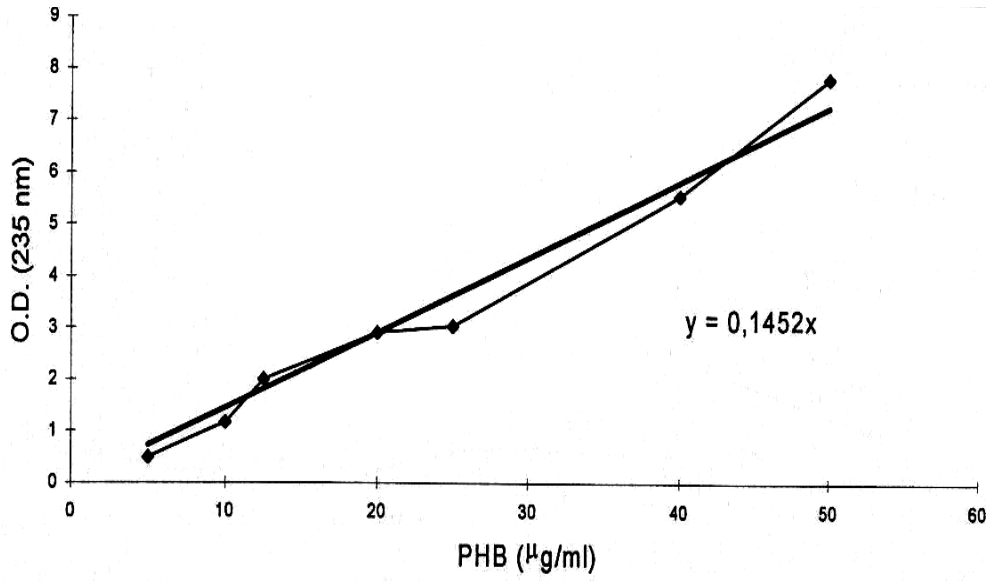

Figure J1 Standard curve for the calculation of PHB concentration (Yiğit, 1999)

APPENDIX K

ABSORPTION SPECTRA OF CAROTENOIDS

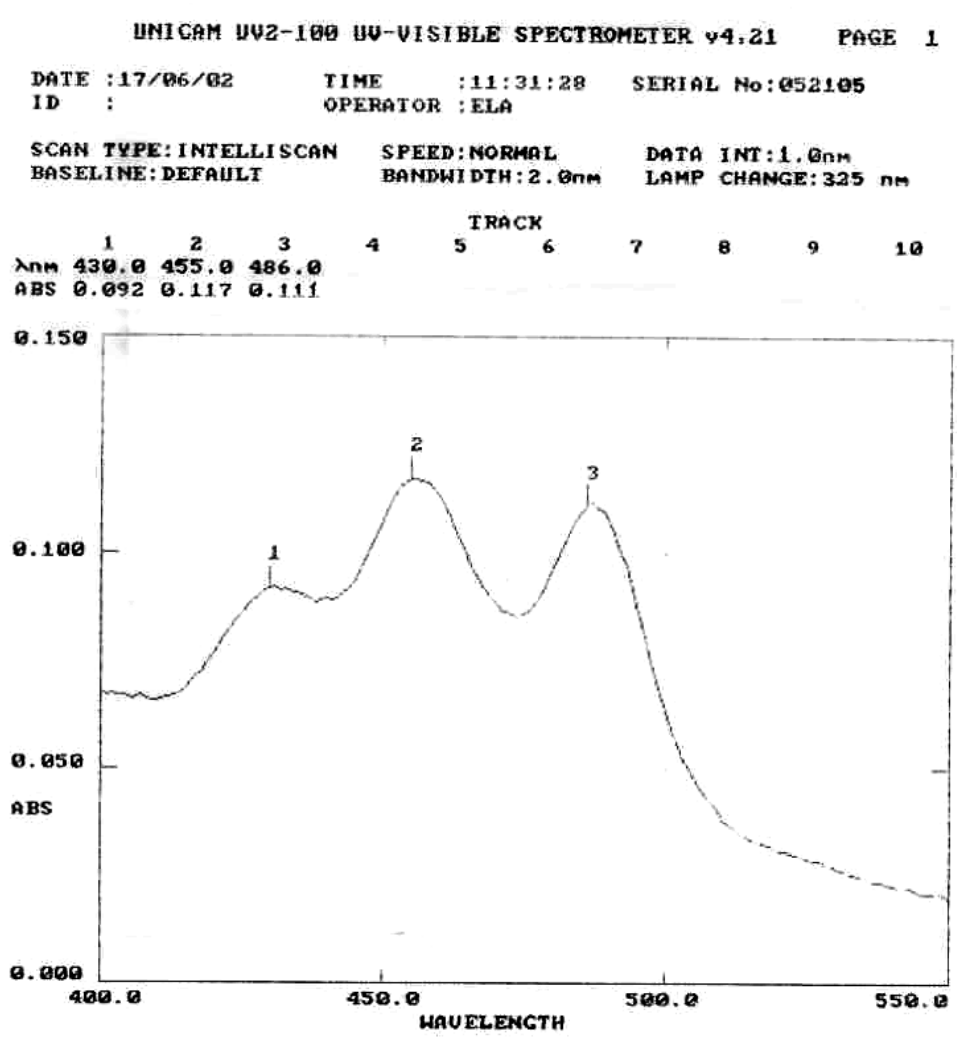

Figure K1 Absorption spectra of the carotenoid extracted from outdoor hydrogen production experiment with 4% OMW containing media

APPENDIX L

XRD RESULTS OF CLAY (CLOISITE® NA+)

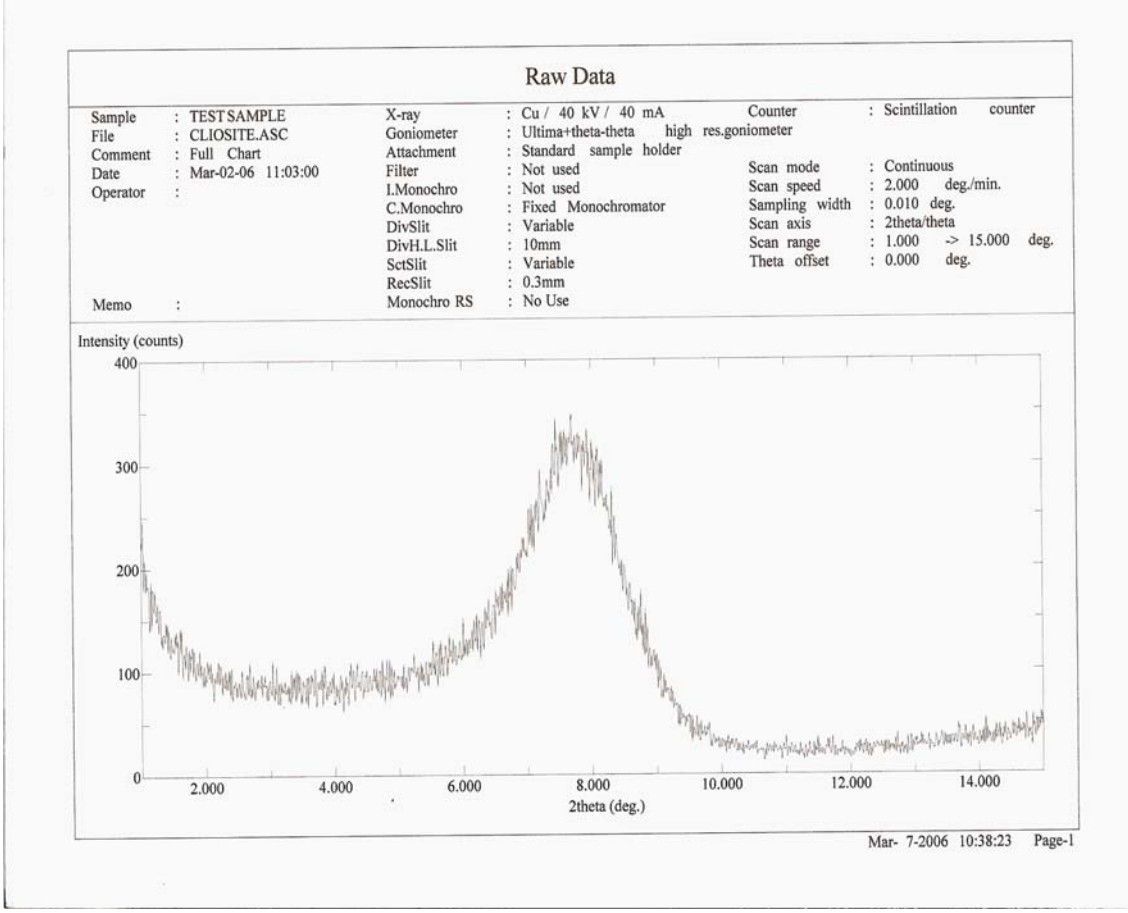

Figure L1 X-ray diffraction graph of Cloisite® Na^+

APPENDIX M

EXPERIMENTAL RESULTS

M1. Experimental data for Section 5.1 (Comparison of Photofermentative Hydrogen Production by OMW Coming from Different Sources)

Table M1.1 Total amount of hydrogen production results for the runs within Section 5.1

H2 (mL)				
Time (h)	OMW(A)	OMW(B)	OMW(C)	OMW(D)
0.0	0.0	0.0	0.0	0.0
13.0	0.8	0.3	0.0	1.1
14.0	1.0	0.3	0.0	1.1
16.0	1.1	0.3	0.1	1.7
17.0	1.7	0.3	0.3	2.5
19.0	2.1	0.3	0.4	3.4
20.0	3.2	0.3	0.9	4.0
22.0	4.4	0.3	1.4	5.4
24.0	4.6	0.8	2.0	5.9
26.0	4.7	1.7	4.1	7.4
28.0	4.7	5.1	6.3	8.5
29.0	4.7	6.2	7.4	9.0
30.0	4.8	7.4	8.7	10.7
32.0	4.8	8.2	9.8	11.3
33.0	5.7	9.9	11.9	13.0
34.0	6.1	11.0	13.0	14.1
36.0	6.1	12.2	12.5	15.3
37.0	6.1	12.7	15.2	15.8
38.0	6.7	13.3	16.8	17.0
39.0	6.7	13.9	17.0	17.5
40.0	6.7	14.7	17.3	18.1
41.0	6.7	15.6	17.6	18.7
42.0	6.7	16.1	17.9	19.2
43.0	7.8	17.0	18.0	20.1
45.0	9.4	17.5	18.2	20.6
46.0	9.8	17.8	18.4	21.2
47.0	11.1	17.8	19.0	21.8
49.0	12.0	17.8	19.6	22.1
50.0	12.4	18.1	19.6	22.6

Table M1.1 Total amount of hydrogen production results for the runs within Section 5.1 (cont'd)

H2 (mL)				
Time (h)	OMW(A)	OMW(B)	OMW(C)	OMW(D)
51.0	13.3	18.1	19.6	23.2
52.0	13.9	18.1	19.6	24.0
53.0	14.8	18.1	19.6	24.6
55.0	15.7	18.1	19.6	25.2
56.0	16.1	18.1	19.6	25.7
57.0	16.7	18.1	19.6	26.3
59.0	17.6	18.1	19.6	26.9
60.0	18.3	18.1	19.6	27.4
61.0	18.7	18.1	19.6	27.7
62.0	19.1	18.1	19.6	28.3
63.0	19.6	18.1	19.6	28.8
64.0	20.0	18.1	19.6	29.4
65.0	20.4	18.1	19.6	30.0
66.0	20.9	18.1	19.6	30.3
67.0	21.3	18.1	19.6	30.5
68.0	21.8	18.1	19.6	30.8
69.0	22.2	18.1	19.6	31.4
70.0	22.6	18.1	19.6	32.0
71.0	23.1	18.1	19.6	32.2
72.0	23.5	18.1	19.6	32.8
73.0	23.9	18.1	19.6	33.4
74.0	24.4	18.1	19.6	33.9
75.0	24.8	18.1	19.6	34.5
76.0	25.0	18.2	19.6	35.1
77.0	25.4	18.2	19.6	35.6
78.0	25.7	18.2	19.6	36.2
79.0	25.9	18.2	19.6	36.8
80.0	25.9	18.2	19.6	37.0
81.0	26.1	18.2	19.6	37.3
82.0	26.1	18.2	19.6	37.9
83.0	26.5	18.2	19.6	38.5
85.0	27.0	18.2	19.6	38.7
87.0	27.4	18.2	19.6	39.0
89.0	27.8	18.2	19.6	39.0
90.0	28.1	18.2	19.6	39.0
92.0	28.5	18.2	19.6	39.0
94.0	28.7	18.2	19.6	39.0
95.0	28.7	18.2	19.6	39.0
96.0	28.8	18.2	19.6	39.1
98.0	29.1	18.2	19.6	39.3
100.0	29.4	18.2	19.6	39.6
102.0	29.6	19.3	19.6	39.9
105.0	29.8	19.3	19.6	39.9
106.0	29.8	19.3	19.6	39.9
108.0	29.8	19.3	19.6	39.9
110.0	29.8	19.3	19.6	39.9
112.0	29.8	19.3	19.6	39.9

Table M1.1 Total amount of hydrogen production results for the runs within Section 5.1 (cont'd)

Time (h)	OMW(A)	OMW(B)	OMW(C)	OMW(D)
114.0	29.8	19.3	19.6	39.9
117.0	29.8	19.3	19.6	39.9
119.0	29.8	19.6	19.6	39.9
121.0	29.8	19.6	19.6	39.9
123.0	29.8	19.6	19.6	39.9
125.0	29.8	19.6	19.6	39.9
127.0	29.8	20.1	19.6	39.9
129.0	29.8	20.1	19.6	39.9
132.0	29.8	20.4	19.6	39.9
135.0	29.8	20.6	19.6	39.9
138.0	29.8	21.2	19.6	39.9
140.0	29.8	21.5	19.6	39.9
142.0	29.8	21.8	19.6	39.9
144.0	29.8	22.1	19.6	39.9
146.0	29.8	22.3	19.6	39.9
148.0	29.8	22.6	19.6	39.9
150.0	29.8	22.6	19.6	39.9
152.0	29.8	22.6	19.6	39.9
155.0	29.8	22.9	19.6	39.9
157.0	29.8	23.2	19.6	39.9
160.0	29.8	23.5	19.6	39.9
165.0	29.8	23.9	19.6	39.9
170.0	29.8	24.3	19.6	39.9
175.0	29.8	24.3	19.6	39.9
178.0	29.8	24.3	19.6	39.9
181.0	29.8	24.3	19.6	39.9
185.0	29.8	24.3	19.6	39.9
190.0	29.8	24.3	19.6	39.9
195.0	29.8	24.3	19.6	39.9
200.0	29.8	24.3	19.6	39.9
205.0	29.8	24.3	19.6	39.9
210.0	29.8	24.3	19.6	39.9
215.0	29.8	24.3	19.6	39.9
219.0	29.8	24.3	19.6	39.9

Table M1.2 Dry cell weight results for the runs within Section 5.1

Dry Cell Weight (g /L)				
Time (h)	OMW(A)	OMW(B)	OMW(C)	OMW(D)
0	0.072	0.079	0.055	0.069
10	0.133	0.089	0.132	0.079
26	0.610	0.198	0.323	0.293
49	0.650	0.326	0.332	0.316
74	0.620	0.343	0.340	0.330
98	0.640	0.326	0.324	0.324
124	0.613	0.348	0.328	0.284
154	0.608	0.336	0.328	0.296
173	0.632	0.312	0.342	0.301
317	0.566	0.333	0.322	0.271

Table M1.3 pH results for the runs within Section 5.1

pH				
Time (h)	OMW(A)	OMW(B)	OMW(C)	OMW(D)
0	7.49	7.42	7.68	7.36
10	7.45	7.63	7.82	7.74
26	7.63	8.24	9.54	8.57
49	8.25	8.1	8.84	8.16
74	7.42	7.6	9.64	7.5
98	7.54	7.46	9.87	7.55
124	7.26	7.45	9.67	7.32
154	7.11	7.48	9.53	7.34
173	7.65	6.91	9.52	7.28
317	7.03	7.84	9.84	7.57

M2. Experimental data for Section 5.2.2 (Photofermentative Hydrogen Production with the Effluents of Physicochemical Pretreatment Processes)

Table M2.1 Total amount of hydrogen production results for the runs within Section 5.2.2

H2 (mL)						
Time (h)	Raw OMW	Ozone	Zeolite 4-A	Clay	Fenton's Reagent	UV treatment
0	0.00	0.00	0.00	0.00	0.00	0.00
19	0.80	0.00	0.32	0.50	0.00	0.00
28	0.90	0.00	0.40	0.80	0.00	0.00
33	1.40	0.00	0.40	1.25	0.00	0.00
54	2.04	0.00	0.53	1.52	0.00	0.00
56	2.71	0.00	0.53	1.80	0.00	0.00
57	3.17	0.00	0.62	2.70	0.00	0.00
61	4.30	0.00	0.66	3.60	0.00	0.00
63	4.75	0.00	0.66	4.50	0.00	0.38
64	5.88	0.00	0.71	5.40	0.25	1.00
66	6.79	0.90	0.71	6.30	0.67	1.25
67	7.24	1.68	0.80	7.20	0.83	1.50
68	8.60	2.10	0.80	9.00	1.00	1.63
70	9.05	2.52	0.80	11.70	1.08	1.75
73	10.41	2.73	0.80	14.40	1.17	2.00
77	11.31	2.94	0.80	16.20	1.33	2.13
79	12.21	3.36	0.88	18.00	1.42	2.25
81	12.67	3.57	0.88	19.80	1.50	2.50
85	13.57	3.78	0.88	21.60	1.67	2.75
89	14.02	4.20	0.97	23.40	1.83	2.88
91	14.48	4.62	0.97	24.30	1.92	3.00
92	14.93	4.83	0.97	25.20	2.00	3.13
93	15.38	5.04	1.06	26.10	2.08	3.25
94	16.06	5.25	1.06	27.00	2.17	3.38
96	16.51	5.46	1.06	28.80	2.25	3.44
97	16.97	5.67	1.06	30.60	2.29	3.44
98	17.42	5.78	1.42	31.50	2.29	3.50
100	17.64	5.78	1.42	32.40	2.33	3.50
101	18.10	5.88	1.42	33.30	2.33	3.56
102	18.55	5.88	1.77	34.20	2.38	3.56
103	19.23	5.99	1.77	36.00	2.38	3.56
104	19.68	5.99	1.77	37.80	2.38	3.63
105	20.13	5.99	1.77	39.60	2.42	3.63
106	20.58	6.09	1.77	41.40	2.42	3.75
108	21.04	6.09	2.65	43.20	2.50	3.75
110	21.49	6.30	5.31	45.00	2.50	3.75
113	21.94	6.30	10.62	47.70	2.50	3.75
115	22.17	6.30	12.74	48.60	2.50	3.75
116	22.62	6.30	13.45	50.40	2.50	3.75
119	23.07	6.30	14.16	52.20	2.50	4.00

Table M2.1 Total amount of hydrogen production results for the runs within Section 5.2.2 (cont'd)

H2 (mL)						
Time (h)	Raw OMW	Ozone	Zeolite 4-A	Clay	Fenton's Reagent	UV treatment
122	23.52	6.30	14.87	54.00	2.50	4.00
123	23.98	6.30	15.58	54.90	2.50	4.00
126	24.20	6.30	16.28	54.90	2.50	4.00
128	24.43	6.30	16.99	54.90	2.67	4.00
131	24.66	6.72	17.70	54.90	2.67	4.25
133	25.11	6.72	18.41	54.90	2.67	4.25
137	25.56	6.72	18.76	54.90	2.67	4.38
140	25.79	6.72	19.12	54.90	2.67	4.50
143	26.24	6.72	19.82	54.90	2.83	4.63
146	26.69	6.72	20.53	54.90	2.83	4.75
148	27.14	6.72	21.24	54.90	2.92	4.75
151	27.60	6.72	21.95	54.90	3.00	4.75
153	28.05	7.14	22.65	54.90	3.08	4.75
155	28.50	7.14	23.36	54.90	3.17	4.75
157	28.95	7.35	23.72	54.90	3.17	5.00
159	29.41	7.56	24.07	54.90	3.17	5.00
160	29.63	7.77	24.78	54.90	3.17	5.00
164	29.86	7.98	25.49	54.90	3.17	5.25
167	30.31	7.98	26.19	54.90	3.33	5.25
170	30.76	7.98	26.90	54.90	3.33	5.25
171	30.99	7.98	27.61	54.90	3.33	5.25
173	31.22	7.98	27.96	54.90	3.50	5.25
174	31.22	8.40	28.32	54.90	3.50	5.25
182	31.22	8.40	29.03	55.80	3.50	5.25
183	31.22	8.40	29.73	56.70	3.50	5.25
185	31.22	8.82	30.44	58.50	3.50	5.25
187	31.22	8.82	31.15	59.40	3.50	5.25
188	31.31	8.82	31.50	59.40	3.58	5.25
190	31.44	8.82	31.86	60.30	3.67	5.25
192	31.67	8.82	32.04	60.30	3.67	5.25
194	31.89	8.82	32.12	60.30	3.67	5.38
195	31.89	8.82	32.39	60.30	3.67	5.50
197	31.89	8.82	32.48	60.30	3.67	5.50
200	31.89	8.82	32.57	60.30	3.75	5.50
201	31.89	8.82	33.10	60.30	3.75	5.63
202	31.89	8.82	33.63	60.30	3.75	5.63
204	31.89	8.82	34.07	61.20	3.83	5.63
207	31.89	8.82	34.51	61.20	3.83	5.75
208	31.89	9.03	34.69	62.10	3.83	5.75
210	31.89	9.24	34.78	62.10	3.83	5.75
213	31.89	9.24	34.87	63.00	3.83	5.75
216	31.89	9.24	35.04	63.00	3.92	5.75
220	31.89	9.24	35.04	63.00	3.92	5.75
223	31.89	9.24	35.13	63.00	4.00	5.75
225	31.89	9.45	35.22	63.00	4.00	5.75

Table M2.1 Total amount of hydrogen production results for the runs within Section 5.2.2 (cont'd)

H2 (mL)						
Time (h)	Raw OMW	Ozone	Zeolite 4-A	Clay	Fenton's Reagent	UV treatment
227	31.89	9.45	35.22	63.00	4.00	5.88
230	31.89	9.45	35.22	63.00	4.00	5.88
233	31.89	9.45	35.22	63.00	4.00	6.00
237	31.89	9.45	35.22	63.00	4.00	6.00
240	31.89	9.45	35.22	63.00	4.00	6.00
244	31.89	9.45	35.22	63.00	4.00	6.00
247	31.89	9.45	35.22	63.00	4.00	6.00
249	31.89	9.45	35.22	63.00	4.00	6.00
250	31.89	9.45	35.22	63.00	4.00	6.00

Table M2.2 Dry cell weight results for the runs within Section 5.2.2

Dry Cell Weight (g /L)						
Time (h)	Raw OMW	Ozone	Zeolite 4-A	Clay	Fenton's Reagent	UV treatment
0	0.082	0.091	0.086	0.112	0.084	0.070
8	0.091	0.091	0.098	0.098	0.085	0.071
17	0.201	0.101	0.106	0.090	0.081	0.080
27	0.321	0.106	0.133	0.101	0.072	0.084
40	0.317	0.097	0.145	0.114	0.084	0.088
50	0.322	0.100	0.124	0.105	0.086	0.094
64	0.336	0.109	0.165	0.117	0.091	0.092
74	0.344	0.118	0.214	0.131	0.101	0.102
88	0.317	0.121	0.221	0.170	0.104	0.105
98	0.298	0.124	0.219	0.187	0.103	0.119
113	0.309	0.135	0.223	0.199	0.105	0.115
123	0.296	0.154	0.218	0.207	0.103	0.124
137	0.298	0.166	0.223	0.222	0.105	0.134
161	0.307	0.160	0.216	0.217	0.101	0.130
169	0.311	0.161	0.221	0.225	0.104	0.128
185	0.304	0.163	0.222	0.215	0.104	0.127

Table M2.3 pH results for the runs within Section 5.2.2

pH						
Time (h)	Raw OMW	Ozone	Zeolite 4-A	Clay	Fenton's Reagent	UV treatment
0	6.87	6.65	6.94	6.68	6.57	6.66
8	7.08	6.6	7.03	6.89	6.55	6.71
17	7.54	6.54	7.13	6.95	6.59	6.65
27	7.62	6.78	7.23	6.91	6.51	6.77
40	7.95	6.84	7.16	6.97	6.54	6.84
50	8.04	6.83	7.28	7.08	6.61	6.94
64	7.3	7.03	7.41	7.12	6.64	6.91
74	7.34	7.02	7.43	7.2	6.67	6.85
88	7.16	7.24	7.55	7.34	6.73	6.84
98	7.36	7.38	7.68	7.32	6.79	6.86
113	7.42	7.55	7.58	7.35	6.89	6.81
123	7.48	7.68	7.45	7.41	6.92	6.84
137	7.51	7.58	7.51	7.48	6.85	6.82
161	7.59	7.49	7.49	7.45	6.84	6.78
169	7.51	7.32	7.52	7.42	6.81	6.79
185	7.55	7.27	7.46	7.39	6.78	6.75

M3. Experimental data for Section 5.2.3 (Photofermentative Hydrogen Production with the Effluents of Dark Fermentation)

Table M3.1 Experimental data for the runs within Section 5.2.3

Time (h)	100% fermentor effluent (acclimated sludge)			50% fermentor effluent (acclimated sludge			50% fermentor effluent (nonacclimated sludge)		
	H2 (mL)	Dry Cell Weight (g/L)	pH	H2 (mL)	Dry Cell Weight (g/L)	pH	H2 (mL)	Dry Cell Weight (g/L)	pH
0	0	0.080	6.71	0	0.085	6.85	0	0.070	6.75
8	0	0.081	6.75	0.5	0.088	6.92	0	0.071	6.71
17	0	0.088	6.71	0.8	0.092	6.99	0	0.073	6.68
27	0.5	0.092	6.81	1	0.101	7.01	0	0.076	6.72
40	1	0.098	6.82	1.5	0.124	7.02	0	0.080	6.75
50	1.2	0.100	6.94	1.8	0.188	7.01	0	0.100	6.84
64	1.5	0.115	6.91	2.5	0.201	7.12	0.5	0.135	6.83
74	2	0.136	6.85	2.9	0.286	7.3	0.8	0.148	7.03
88	2.5	0.155	6.84	3.2	0.315	7.38	1	0.185	7.02
98	3	0.175	6.86	3.5	0.325	7.35	1.2	0.198	7.24
113	3.4	0.181	6.81	4	0.338	7.3	1.4	0.235	7.38
123	3.8	0.192	6.84	5.2	0.350	7.45	1.6	0.268	7.3
137	4.2	0.205	6.82	6.8	0.381	7.52	1.75	0.289	7.38
161	4.5	0.238	6.78	8.5	0.402	7.71	2	0.292	7.35
169	4.7	0.235	6.79	10	0.402	7.62	2.4	0.295	7.3
185	5	0.240	6.75	11.8	0.395	7.66	3	0.288	7.35

Time (h)	4% fermentor effluent (nonacclimated sludge)			4% raw OMW		
	H2 (mL)	Dry Cell Weight (g/L)	pH	H2 (mL)	Dry Cell Weight (g/L)	pH
0	0	0.076	6.68	0	0.082	6.87
8	0	0.083	6.71	0	0.091	7.08
17	3	0.088	6.88	0.8	0.201	7.54
27	5	0.136	6.78	0.91	0.321	7.62
40	10	0.244	6.81	1	0.317	7.95
50	14	0.310	6.94	2	0.322	8.04
64	22	0.344	7.01	4.6	0.336	7.3
74	27	0.333	7.02	10.4	0.344	7.34
88	34	0.350	6.98	14	0.317	7.16
98	38	0.356	7.12	17.4	0.298	7.36
113	41	0.394	7.01	22	0.309	7.42
123	43	0.465	6.98	24	0.296	7.48
137	48	0.520	7.02	25.6	0.298	7.51
161	50	0.510	6.89	30	0.307	7.59
169	51	0.521	6.92	30.4	0.311	7.51
185	51	0.539	6.84	31	0.304	7.55

M4. Experimental data for Section 5.3.2 (Effect of Clay Quantity on the Pretreatment of OMW)

Table M4.1 Total amount of hydrogen production results for the runs within Section 5.3.2

H2 (mL)					
Time (h)	0.1 g/L clay	1 g/L clay	10 g/L clay	20 g/L clay	50 g/L clay
0	0.00	0.00	0.00	0.00	0.00
16	0.00	0.00	0.00	0.50	0.48
25	0.00	0.00	0.00	0.80	0.72
30	0.00	0.00	0.00	1.25	1.20
42	0.72	2.50	2.96	1.52	1.44
50	1.44	6.00	7.10	1.80	1.76
53	1.66	8.00	9.46	2.70	2.64
58	1.80	10.00	11.83	3.60	3.52
63	1.94	10.80	12.78	3.60	3.52
64	2.09	11.00	13.01	3.70	3.84
67	2.23	13.00	15.38	3.80	3.92
70	2.38	14.00	16.56	3.90	4.00
72	2.52	15.00	17.75	4.00	4.08
74	2.66	16.50	19.52	4.10	4.16
78	2.81	18.00	21.29	4.10	4.24
79	2.95	19.10	22.60	4.20	4.32
80	3.10	20.00	23.66	4.50	4.40
81	3.24	20.80	24.61	4.80	4.48
85	4.18	21.00	24.84	5.00	4.72
89	5.04	22.50	26.62	5.20	4.96
91	6.48	22.80	26.97	5.40	5.20
92	8.64	22.90	27.09	5.80	5.44
93	10.80	23.00	27.21	6.40	5.60
94	12.96	23.30	27.56	7.10	6.93
96	13.97	23.75	28.10	9.00	8.78
97	14.04	23.75	28.10	9.80	9.56
98	14.18	23.90	28.27	11.00	10.74
100	14.33	24.20	28.63	14.20	13.86
101	14.47	24.60	29.10	15.30	14.93
102	14.62	24.80	29.34	18.00	17.57
103	14.76	25.00	29.58	20.00	19.52
104	14.90	25.20	29.81	22.00	21.47
105	15.05	25.40	30.05	23.00	19.20
106	15.19	25.60	30.28	23.50	22.40
108	15.34	25.80	30.52	25.00	25.60
110	15.48	26.00	30.76	26.40	28.80
113	15.62	26.20	30.99	28.60	32.00
115	15.77	26.40	31.23	30.00	35.20
116	15.84	26.60	31.47	32.40	38.40
119	15.91	26.70	31.59	33.30	41.60
122	15.91	26.80	31.70	34.20	43.20
123	16.06	26.90	31.82	36.00	44.80

Table M4.1 Total amount of hydrogen production results for the runs within Section 5.3.2 (cont'd)

H2 (mL)					
Time (h)	0.1 g/L clay	1 g/L clay	10 g/L clay	20 g/L clay	50 g/L clay
126	16.20	27.00	31.94	37.80	44.80
128	16.34	27.10	32.06	39.60	44.80
131	16.49	27.20	32.18	41.40	44.80
133	16.63	27.30	32.30	43.20	44.88
137	16.78	27.40	32.41	44.00	44.96
140	16.92	27.60	32.65	44.80	45.44
143	16.92	28.00	33.12	45.60	45.92
146	17.28	28.50	33.72	46.40	46.40
148	17.42	29.00	34.02	47.20	47.20
151	17.57	29.50	34.30	48.00	47.36
153	17.71	30.00	35.49	48.80	47.63
155	17.86	30.40	35.96	49.00	47.82
157	18.00	30.60	36.20	49.10	47.92
159	18.14	30.80	36.44	49.10	47.92
160	18.29	31.00	36.67	49.10	47.92
164	18.36	31.20	36.91	49.10	47.92
167	18.43	31.40	37.15	49.10	47.92
170	18.50	31.60	37.38	49.10	47.92
171	18.58	31.80	37.62	49.10	47.92
173	18.65	32.00	37.86	49.10	47.92
174	18.72	32.20	38.09	49.20	48.02
182	18.79	32.40	38.33	49.20	48.02
183	18.94	32.60	38.57	49.20	48.02
185	19.08	32.80	38.80	49.20	48.02
187	19.22	33.40	39.51	49.20	48.02
188	19.37	33.60	39.75	49.30	48.12
190	19.51	33.90	40.10	49.30	48.12
192	19.66	34.20	40.46	49.30	48.12
194	19.80	34.50	40.81	49.30	48.12
195	19.94	34.80	41.17	49.30	48.12
197	20.09	35.40	41.88	49.30	48.12
200	20.23	36.00	42.59	49.50	48.31
201	20.38	36.30	42.94	49.50	48.31
202	20.55	36.60	43.30	49.50	48.31
204	20.78	37.00	43.77	49.50	48.31
207	20.89	37.20	44.01	49.50	48.31
208	21.06	37.50	44.36	49.50	48.31
210	21.23	37.80	44.72	49.50	48.31
211	21.38	38.10	45.07	49.70	48.51
213	21.60	38.40	45.43	49.80	48.60
215	21.82	38.70	45.78	49.90	48.70
217	22.03	38.80	45.90	50.00	48.70
219	22.25	38.90	46.02	50.50	48.70
221	22.46	39.00	46.14	51.00	48.70
223	22.68	39.10	46.26	51.50	48.70
225	22.90	39.20	46.37	52.00	48.70
226	23.11	39.30	46.49	52.50	48.96

Table M4.1 Total amount of hydrogen production results for the runs within Section 5.3.2 (cont'd)

H2 (mL)					
Time (h)	0.1 g/L clay	1 g/L clay	10 g/L clay	20 g/L clay	50 g/L clay
227	23.33	39.30	46.49	52.80	49.04
229	23.54	39.60	46.85	53.00	49.60
230	23.76	39.60	46.85	53.20	50.16
232	24.00	39.80	47.04	53.40	50.72
236	24.00	40.00	47.18	53.80	51.28
239	24.00	40.00	47.32	54.00	52.00
240	24.00	40.00	47.46	54.60	52.24
242	24.00	40.00	47.60	55.20	53.60
244	24.00	40.00	47.74	55.80	54.00
245	24.00	40.00	47.88	56.10	54.40
248	24.00	40.00	48.02	56.40	54.80
250	24.00	40.00	48.16	56.70	55.20
252	24.00	40.00	48.30	57.00	55.36
254	24.00	40.00	48.37	57.50	55.44
255	24.00	40.00	48.37	57.80	55.52
256	24.00	40.00	48.37	57.80	55.60
258	24.00	40.00	48.37	57.80	55.68
260	24.00	40.00	48.37	57.80	55.76
262	24.00	40.00	48.37	58.00	55.84
263	24.00	40.00	48.37	58.00	55.92
264	24.00	40.00	48.37	58.00	56.00
266	24.00	40.00	48.37	58.00	56.08
268	24.00	40.00	48.37	58.00	56.16
270	24.00	40.00	48.37	58.00	56.24
272	24.00	40.00	48.37	58.00	56.40
275	24.00	40.00	48.37	58.00	56.40
278	24.00	40.00	48.37	58.00	56.40
280	24.00	40.00	48.37	58.00	56.40
282	24.00	40.00	48.37	58.00	56.40
285	24.00	40.00	48.37	58.00	56.40
290	24.00	40.00	48.37	58.00	56.40

Table M4.2 Dry cell weight results for the runs within Section 5.3.2

Dry Cell Weight (g /L)					
Time (h)	0.1 g/L clay	1 g/L clay	10 g/L clay	20 g/L clay	50 g/L clay
0	0.112	0.103	0.103	0.086	0.083
8	0.145	0.136	0.136	0.098	0.101
15	0.165	0.189	0.189	0.106	0.115
22	0.203	0.191	0.191	0.133	0.160
28	0.233	0.203	0.203	0.145	0.169
42	0.263	0.226	0.226	0.155	0.173
60	0.282	0.250	0.250	0.165	0.189
74	0.287	0.276	0.276	0.214	0.204
95	0.324	0.309	0.309	0.221	0.210
106	0.310	0.345	0.345	0.219	0.215
120	0.350	0.378	0.378	0.223	0.245
134	0.330	0.382	0.396	0.218	0.267
146	0.345	0.383	0.383	0.223	0.288
166	0.356	0.374	0.374	0.216	0.277
178	0.376	0.370	0.370	0.221	0.279
198	0.356	0.374	0.352	0.222	0.283

Table M4.3 pH results for the runs within Section 5.3.2

pH					
Time (h)	0.1 g/L clay	1 g/L clay	10 g/L clay	20 g/L clay	50 g/L clay
0	6.67	6.65	6.69	6.87	6.7
8	6.87	6.9	6.91	7.08	6.94
15	6.98	7	6.97	7.3	6.98
22	7.3	6.78	7.08	7.1	7.08
28	7.1	6.84	7.2	7.12	6.98
42	7.14	6.83	7	7.17	7.01
60	7.24	7.03	6.85	7.24	7.2
74	7.15	7.02	6.94	7.23	7.33
95	7.09	7.24	7.05	7.48	7.47
106	7.01	7.38	7.14	7.53	7.38
120	7.25	7.55	7.16	7.66	7.45
134	7.38	7.68	7.29	7.65	7.38
146	7.43	7.58	7.55	7.68	7.55
166	7.55	7.49	7.68	7.64	7.68
178	7.68	7.32	7.58	7.66	7.58
198	7.58	7.27	7.49	7.66	7.49

M5. Experimental data for Section 5.3.3 (Effect of Dilution Rates on the Photofermentative Hydrogen Production)

Table M5.1 Total amount of hydrogen production results for the runs within Section 5.3.3

H2 (mL)							
Time (h)	Effluent of clay treatment (4%)	Effluent of clay treatment (50%)	Effluent of clay treatment (100%)	Time (h)	Raw OMW (4%)	Raw OMW (50%)	Raw OMW (100%)
0	0.00	0.00	0.00	0.00	0.00	0	0
16	0.50	0.00	0.00	19.00	0.80	0	0
25	0.80	0.90	0.00	28.00	0.90	0	0
30	1.25	1.20	0.14	33.00	1.40	0	0
42	1.52	1.80	0.20	54.00	2.04	0	0
50	1.80	2.00	0.22	56.00	2.71	0	0
53	2.70	2.10	0.24	57.00	3.17	0	0
58	3.60	2.40	0.26	61.00	4.30	0	0
63	3.60	2.60	0.28	63.00	4.75	0	0
64	3.70	2.80	0.30	64.00	5.88	0	0
67	3.80	3.00	0.32	66.00	6.79	0	0
70	3.90	3.40	0.34	67.00	7.24	0	0
72	4.00	3.90	0.36	68.00	8.60	0	0
74	4.10	4.30	0.38	70.00	9.05	0	0
78	4.10	4.80	0.40	73.00	10.41	0	0
79	4.20	5.40	0.44	77.00	11.31	0	0
80	4.50	5.80	0.48	79.00	12.21	0	0
81	4.80	6.10	0.52	81.00	12.67	0	0
85	5.00	6.60	0.56	85.00	13.57	0	0
89	5.20	6.90	0.60	89.00	14.02	0	0
91	5.40	7.00	0.64	91.00	14.48	0	0
92	5.80	7.20	0.68	92.00	14.93	0	0
93	6.40	7.50	0.72	93.00	15.38	0	0
94	7.10	7.60	0.76	94.00	16.06	0	0
96	9.00	7.80	0.80	96.00	16.51	0	0
97	9.80	8.10	0.84	97.00	16.97	0	0
98	11.00	8.40	0.88	98.00	17.42	0	0
100	14.20	8.70	1.00	100.00	17.64	0	0
101	15.30	9.00	1.12	101.00	18.10	0	0
102	18.00	9.60	1.24	102.00	18.55	0	0
103	20.00	10.20	1.36	103.00	19.23	0	0
104	22.00	10.50	1.48	104.00	19.68	0	0
105	23.00	10.80	1.56	105.00	20.13	0	0
106	23.50	11.10	1.60	106.00	20.58	0	0
108	25.00	11.40	1.66	108.00	21.04	0	0
110	26.40	12.00	1.80	110.00	21.49	0	0
113	28.60	12.60	1.94	113.00	21.94	0	0
115	30.00	13.20	2.08	115.00	22.17	0	0
116	32.40	13.80	2.22	116.00	22.62	0	0
119	33.30	14.40	2.36	119.00	23.07	0	0
122	34.20	15.00	2.50	122.00	23.52	0	0

Table M5.1 Total amount of hydrogen production results for the runs within Section 5.3.3 (cont'd)

H2 (mL)							
Time (h)	Effluent of clay treatment (4%)	Effluent of clay treatment (50%)	Effluent of clay treatment (100%)	Time (h)	Raw OMW (4%)	Raw OMW (50%)	Raw OMW (100%)
123	36.00	15.90	2.64	123.00	23.98	0	0
126	37.80	16.20	2.78	126.00	24.20	0	0
128	39.60	16.80	3.00	128.00	24.43	0	0
131	41.40	17.40	3.40	131.00	24.66	0	0
133	43.20	18.00	3.80	133.00	25.11	0	0
137	44.00	18.30	4.20	137.00	25.56	0	0
140	44.80	18.90	4.60	140.00	25.79	0	0
143	45.60	19.20	4.66	143.00	26.24	0	0
146	46.40	19.80	4.72	146.00	26.69	0	0
148	47.20	20.10	4.78	148.00	27.14	0	0
151	48.00	20.40	4.84	151.00	27.60	0	0
153	48.80	20.70	4.90	153.00	28.05	0	0
155	49.00	21.00	4.96	155.00	28.50	0	0
157	49.10	21.80	5.02	157.00	28.95	0	0
159	49.10	22.40	5.08	159.00	29.41	0	0
160	49.10	23.60	5.14	160.00	29.63	0	0
164	49.10	23.70	5.20	164.00	29.86	0	0
167	49.10	23.80	5.60	167.00	30.31	0	0
170	49.10	23.80	5.68	170.00	30.76	0	0
171	49.10	24.20	5.76	171.00	30.99	0	0
173	49.10	24.30	5.84	173.00	31.22	0	0
174	49.20	24.60	5.92	174.00	31.22	0	0
182	49.20	24.90	6.00	182.00	31.22	0	0
183	49.20	25.10	6.08	183.00	31.22	0	0
185	49.20	25.40	6.20	185.00	31.22	0	0
187	49.20	25.90	6.40	187.00	31.22	0	0
188	49.30	26.20	6.60	188.00	31.31	0	0
190	49.30	26.50	6.88	190.00	31.44	0	0
192	49.30	26.60	7.15	192.00	31.67	0	0
194	49.30	26.60	7.29	194.00	31.89	0	0
195	49.30	26.60	7.43	195.00	31.89	0	0
197	49.30	26.60	7.70	197.00	31.89	0	0
200	49.50	26.60	7.98	200.00	31.89	0	0
201	49.50	26.60	8.25	201.00	31.89	0	0
202	49.50	26.60	8.53	202.00	31.89	0	0
204	49.50	26.60	8.60	204.00	31.89	0	0
207	49.50	26.60	9.08	207.00	31.89	0	0
208	49.50	26.60	9.16	208.00	31.89	0	0
210	49.50	26.60	9.24	210.00	31.89	0	0
211	49.70	26.60	9.32	213.00	31.89	0	0
213	49.80	26.60	9.40	216.00	31.89	0	0
215	49.90	26.60	9.48	220.00	31.89	0	0
217	50.00	26.60	9.56	223.00	31.89	0	0
219	50.50	26.60	9.64	225.00	31.89	0	0

Table M5.1 Total amount of hydrogen production results for the runs within Section 5.3.3 (cont'd)

H2 (mL)							
Time (h)	Effluent of clay treatment (4%)	Effluent of clay treatment (50%)	Effluent of clay treatment (100%)	Time (h)	Raw OMW (4%)	Raw OMW (50%)	Raw OMW (100%)
221	51.00	26.60	9.72	227.00	31.89	0	0
223	51.50	26.60	9.80	230.00	31.89	0	0
225	52.00	26.60	10.20	233.00	31.89	0	0
226	52.50	26.60	10.60	237.00	31.89	0	0
227	52.80	26.60	10.84	240.00	31.89	0	0
229	53.00	26.60	11.00	244.00	31.89	0	0
230	53.20	26.60	11.16	247.00	31.89	0	0
232	53.40	26.60	11.32	249.00	31.89	0	0
236	53.80	26.60	11.48	250.00	31.89	0	0
239	54.00	26.60	11.64				
240	54.60	26.60	11.80				
242	55.20	26.60	11.96				
244	55.80	26.60	12.00				
245	56.10	26.60	12.16				
248	56.40	26.60	12.20				
250	56.70	26.60	12.26				
252	57.00	26.60	12.32				
254	57.50	26.60	12.38				
255	57.80	26.60	12.40				
256	57.80	26.60	12.44				
258	57.80	26.60	12.46				
260	57.80	26.60	12.52				
262	58.00	26.60	12.60				
263	58.00	26.60	12.64				
264	58.00	26.60	12.64				
266	58.00	26.60	12.64				
268	58.00	26.60	12.64				
270	58.00	26.60	12.64				
272	58.00	26.60	12.64				
275	58.00	26.60	12.64				
278	58.00	26.60	12.64				
280	58.00	26.60	12.64				
282	58.00	26.60	12.64				
285	58.00	26.60	12.64				
290	58.00	26.60	12.64				

Table M5.2 Dry cell weight results for the runs within Section 5.3.3

Dry Cell Weight (g /L)							
Time (h)	Effluent of clay treatment (4%)	Effluent of clay treatment (50%)	Effluent of clay treatment (100%)	Time (h)	Raw OMW (4%)	Raw OMW (50%)	Raw OMW (100%)
0	0.086	0.083	0.078	0	0.082	0.080	0.072
8	0.098	0.103	0.086	8	0.091	0.062	0.062
15	0.106	0.102	0.089	17	0.201	0.070	0.059
22	0.133	0.132	0.091	27	0.321	0.073	0.064
28	0.145	0.162	0.108	40	0.317	0.077	0.063
42	0.155	0.197	0.113	50	0.322	0.082	0.066
60	0.165	0.193	0.125	64	0.336	0.080	0.068
74	0.214	0.214	0.131	74	0.344	0.089	0.071
95	0.221	0.221	0.154	88	0.317	0.091	0.084
106	0.219	0.250	0.171	98	0.298	0.104	0.088
120	0.223	0.242	0.178	113	0.309	0.100	0.082
134	0.218	0.260	0.181	123	0.296	0.108	0.078
146	0.223	0.281	0.192	137	0.298	0.117	0.076
166	0.216	0.273	0.183	161	0.307	0.113	0.074
178	0.221	0.269	0.178	169	0.311	0.111	0.073
198	0.222	0.267	0.177	185	0.304	0.110	0.074

Table M5.3 pH results for the runs within Section 5.3.3

pH							
Time (h)	Effluent of clay treatment (4%)	Effluent of clay treatment (50%)	Effluent of clay treatment (100%)	Time (h)	Raw OMW (4%)	Raw OMW (50%)	Raw OMW (100%)
0	6.87	6.77	6.56	0	6.87	6.57	6.58
8	7.08	6.65	6.58	8	7.08	6.55	6.50
15	7.30	6.69	6.67	17	7.54	6.59	6.51
22	7.10	6.81	6.61	27	7.62	6.51	6.49
28	7.12	6.92	6.69	40	7.95	6.44	6.45
42	7.17	7.01	6.85	50	8.04	6.42	6.48
60	7.24	7.07	6.92	64	7.30	6.38	6.50
74	7.23	7.12	6.78	74	7.34	6.40	6.55
95	7.48	7.15	6.98	88	7.16	6.31	6.62
106	7.53	7.12	6.92	98	7.36	6.19	6.65
120	7.66	7.13	6.83	113	7.42	6.12	6.67
134	7.65	7.14	6.94	123	7.48	6.08	6.65
146	7.68	7.12	6.96	137	7.51	6.05	6.69
166	7.64	7.08	6.91	161	7.59	6.09	6.65
178	7.66	7.05	6.86	169	7.51	6.02	6.62
200	7.66	7.01	6.83	185	7.55	6.03	6.64

M6. Experimental data for Section 5.3.4 (Spent clay regeneration)

Table M6.1 Total amount of hydrogen production results for the runs within Section 5.3.4

H2(mL)				
Time (h)	Effluent of clay (fresh) pretreatment	Effluent of clay (regenerated) pretreatment	Time (h)	Raw OMW
0	0.00	0.00	0	0.00
16	0.50	0.00	19	0.80
25	0.80	0.00	28	0.90
30	1.25	0.75	33	1.40
42	1.52	0.94	54	2.04
50	1.80	2.40	56	2.71
53	2.70	3.15	57	3.17
58	3.60	3.42	61	4.30
63	3.60	3.69	63	4.75
64	3.70	3.97	64	5.88
67	3.80	4.24	66	6.79
70	3.90	4.51	67	7.24
72	4.00	4.79	68	8.60
74	4.10	5.06	70	9.05
78	4.10	5.34	73	10.41
79	4.20	5.61	77	11.31
80	4.50	5.88	79	12.21
81	4.80	6.16	81	12.67
85	5.00	7.93	85	13.57
89	5.20	9.58	89	14.02
91	5.40	12.31	91	14.48
92	5.80	16.42	92	14.93
93	6.40	20.52	93	15.38
94	7.10	24.62	94	16.06
96	9.00	26.54	96	16.51
97	9.80	26.68	97	16.97
98	11.00	26.95	98	17.42
100	14.20	27.22	100	17.64
101	15.30	27.50	101	18.10
102	18.00	27.77	102	18.55
103	20.00	28.04	103	19.23
104	22.00	28.32	104	19.68
105	23.00	28.59	105	20.13
106	23.50	28.86	106	20.58
108	25.00	29.14	108	21.04
110	26.40	29.41	110	21.49
113	28.60	29.69	113	21.94
115	30.00	29.96	115	22.17
116	32.40	30.10	116	22.62
119	33.30	30.23	119	23.07
122	34.20	30.23	122	23.52

Table M6.1 Total amount of hydrogen production results for the runs within Section 5.3.4 (cont'd)

H2 (mL)				
Time (h)	Effluent of clay (fresh) pretreatment	Effluent of clay (regenerated) pretreatment	Time (h)	Raw OMW
123	36.00	30.51	123	23.98
126	37.80	30.78	126	24.20
128	39.60	31.05	128	24.43
131	41.40	31.33	131	24.66
133	43.20	31.60	133	25.11
137	44.00	31.87	137	25.56
140	44.80	32.15	140	25.79
143	45.60	32.15	143	26.24
146	46.40	32.83	146	26.69
148	47.20	33.11	148	27.14
151	48.00	33.38	151	27.60
153	48.80	33.65	153	28.05
155	49.00	33.93	155	28.50
157	49.10	34.20	157	28.95
159	49.10	34.47	159	29.41
160	49.10	34.75	160	29.63
164	49.10	34.88	164	29.86
167	49.10	35.02	167	30.31
170	49.10	35.16	170	30.76
171	49.10	35.29	171	30.99
173	49.10	35.43	173	31.22
174	49.20	35.57	174	31.22
182	49.20	35.70	182	31.22
183	49.20	35.98	183	31.22
185	49.20	36.25	185	31.22
187	49.20	36.53	187	31.22
188	49.30	36.80	188	31.31
190	49.30	37.07	190	31.44
192	49.30	37.35	192	31.67
194	49.30	37.62	194	31.89
195	49.30	37.89	195	31.89
197	49.30	38.17	197	31.89
200	49.50	38.44	200	31.89
201	49.50	38.71	201	31.89
202	49.50	39.05	202	31.89
204	49.50	39.48	204	31.89
207	49.50	39.69	207	31.89
208	49.50	40.01	208	31.89
210	49.50	40.33	210	31.89
211	49.70	40.63	213	31.89
213	49.80	41.04	216	31.89
215	49.90	41.45	220	31.89
217	50.00	41.86	223	31.89
219	50.50	42.27	225	31.89

Table M6.1 Total amount of hydrogen production results for the runs within Section 5.3.4 (cont'd)

H2 (mL)				
Time (h)	Effluent of clay (fresh) pretreatment	Effluent of clay (regenerated) pretreatment	Time (h)	Raw OMW
221	51.00	42.68	227	31.89
223	51.50	43.09	230	31.89
225	52.00	43.50	233	31.89
226	52.50	43.91	237	31.89
227	52.80	44.32	240	31.89
229	53.00	44.73	244	31.89
230	53.20	45.14	247	31.89
232	53.40	45.60	249	31.89
236	53.80	45.60	250	31.89
239	54.00	45.60	-	-
240	54.60	45.60	-	-
242	55.20	45.60	-	-
244	55.80	45.60	-	-
245	56.10	45.60	-	-
248	56.40	45.60	-	-
250	56.70	45.60	-	-
252	57.00	45.60	-	-
254	57.50	45.60	-	-
255	57.80	45.60	-	-
256	57.80	45.60	-	-
258	57.80	45.60	-	-
260	57.80	45.60	-	-
262	58.00	45.60	-	-
263	58.00	45.60	-	-
264	58.00	45.60	-	-
266	58.00	45.60	-	-
268	58.00	45.60	-	-
270	58.00	45.60	-	-
272	58.00	45.60	-	-
275	58.00	45.60	-	-
278	58.00	45.60	-	-
280	58.00	45.60	-	-
282	58.00	45.60	-	-
285	58.00	45.60	-	-
290	58.00	45.60	-	-

Table M6.2 Dry cell weight and pH results for the runs within Section 5.3.4

Dry Cell Weight (g /L)					pH				
Time (h)	Effluent of clay (fresh) pret.	Effluent of clay (regenerated) pret.	Time (h)	Raw OMW	Time (h)	Effluent of clay (fresh) pret.	Effluent of clay (regenerated) pret.	Time (h)	Raw OMW
0	0.086	0.091	0	0.082	0	6.87	6.80	0	6.87
8	0.098	0.108	8	0.091	8	7.08	6.90	8	7.08
15	0.106	0.120	17	0.201	15	7.30	7.02	17	7.54
22	0.133	0.145	27	0.321	22	7.10	7.10	27	7.62
28	0.145	0.189	40	0.317	28	7.12	7.12	40	7.95
42	0.155	0.204	50	0.322	42	7.17	7.13	50	8.04
60	0.165	0.232	64	0.336	60	7.24	7.12	64	7.3
74	0.214	0.254	74	0.344	74	7.23	7.17	74	7.34
95	0.221	0.276	88	0.317	95	7.48	7.24	88	7.16
106	0.219	0.289	98	0.298	106	7.53	7.23	98	7.36
120	0.223	0.286	113	0.309	120	7.66	7.20	113	7.42
134	0.218	0.299	123	0.296	134	7.65	7.33	123	7.48
146	0.223	0.312	137	0.298	146	7.68	7.47	137	7.51
166	0.216	0.308	161	0.307	166	7.64	7.38	161	7.59
178	0.221	0.315	169	0.311	178	7.66	7.27	169	7.51
198	0.222	0.310	185	0.304	198	7.66	7.34	185	7.55

M7. Experimental data for Section 5.4.2 (Process Evaluation)

Table M7.1 Organic constituents present in the different streams of a two-stage hydrogen production process, given in Section 5.4.2

Stream Number		1	4	5	6	7	9
Stream Name		Raw OMW	Effluent of washing	Effluent of clay pret.	PBR Inlet	Bacterial Inoc.	Effluent of PBR
Sugars (mg/L)	Glucose	910	-0-	880	33	-0-	2.2
	Xylose	670	20	640	22.8	-0-	11.1
	Arabinose	130	-0-	120	4.2	-0-	2.9
	Mannitol	70	-0-	60	2	-0-	0.7
Alcohol (mg/L)	Methanol	2100	60	1990	62.8	-0-	50.5
	Ethanol	2320	50	2240	81.6	-0-	66.2
Organic Acids (mg/L)	Acetic Acid	9710	70	9620	311.6	16	61.8
	Formic acid	270	-0-	240	8.2	-0-	6.4
	Prop. acid	1800	40	1710	67.8	-0-	2.7
	Butyric Acid	1950	30	1890	69.6	-0-	8
	Lactic Acid	1140	90	1020	70.2	2	10.7
Phenol (mg/L)	Phenol	2340	890	1320	50	-0-	7.3
	p-cresol	1380	1090	120	-0-	-0-	-0-
	m-cresol	2560	990	1170	40	-0-	5.5
	o-cresol	970	580	140	-0-	-0-	-0-
Amino acid (mg/L)	Aspartic acid	7320	70	7230	84	0	40
	Glutamic acid	7650	110	7530	92	2	21.8
	Tyrosine	2450	280	2290	54	0	41.8
Total Sugar (g/L)		15.1	0.6	12	1.44	-0-	1.16
Total Phenol (g/L)		12.2	4.9	4	0.14	-0-	09
Total solids (g/L)		40.7	1.2	6	0.15	0.40	0.38

Table M7.2 Elemental constituents present in the different streams of a two-stage hydrogen production process, given in Section 5.4.2

Stream Number		1	4	5	6	7	9
Stream Name		Raw OMW	Effluent of washing	Effluent of clay pret.	PBR Inlet	Bacterial Inoc.	Effluent of PBR
ELEMENTS (mg/L)	K	3500	360	3000	10.4	1.64	2.18
	Na	140	92	700	19.6	0.62	2.18
	Fe	39	110	160	4.6	0.48	1.71
	Ca	100	8.9	85	2.2	-0-	0.26
	Mg	96	12	78	2.8	0.24	1.16
	B	4.7	-0-	4.2	0.22	-0-	0.13
	Pb	4.6	1.1	2.3	-0-	-0-	-0-
	Zn	1.8	-0-	1.2	-0-	-0-	-0-
	Mn	0.91	-0-	1.1	-0-	-0-	-0-
	Co	0.25	-0-	0.2	-0-	-0-	-0-
	Mo	0.04	-0-	0.03	-0-	-0-	-0-
	Ni	0.20	3.5	0.32	-0-	-0-	-0-
	Cr	0.23	-0-	0.11	-0-	-0-	-0-
	Cu	0.05	-0-	0.070	-0-	-0-	-0-

M8. Experimental data for Section 5.5 (Outdoor Hydrogen Production in a Solar Bioreactor for Scale-up)

Table M8.1 Experimental data for the runs within Section 5.5

Time (h)	H2 (mL)	Dry Cell Weight (g/L)	pH	Hour	Date
0	0	095	7.41	11:00	218.2003
8	0	0.142	8.12	19:00	218.2003
21	5	0.220	7.81	08:00	228.2003
31	20	0.238	8.34	18:00	228.2003
45	80	0.270	8.78	08:00	238.2003
55	180	0.349	9.85	18:00	238.2003
69	335	0.334	9.68	08:00	248.2003
80	440	0.368	9.84	19:00	248.2003
94	620	0.295	9.66	09:00	258.2003
97	700	0.297	9.65	12:00	258.2003
100	750	0.305	9.66	15:00	258.2003
104	780	0.315	9.69	19:00	258.2003
118	1230	0.294	8.80	09:00	268.2003
128	1350	0.307	9.50	19:00	268.2003
143	1700	0.314	9.40	10:00	278.2003
151	1800	0.326	9.51	18:00	278.2003
166	2100	0.309	9.44	09:00	288.2003
172	2300	0.307	9.50	15:00	288.2003
191	2550	0.302	9.41	10:00	298.2003
199	2555	0.297	9.49	18:00	298.2003
214	2560	0.301	9.56	09:00	308.2003
220	2560	0.300	9.59	15:00	308.2003

CURRICULUM VITAE

PERSONAL DETAILS

Name: Ela EROGLU

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EDUCATIONAL DEGREES

Field		Institution	Date
PhD	Chem. Eng.	Middle East Technical University (3.93 / 4.0)	2002 - Present
MSc	Chem. Eng.	Middle East Technical University (3.72 / 4.0)	2000 - 2002
BSc	Chem. Eng.	Hacettepe University (1st Rank) (3.32 / 4.0)	1996 - 2000

WORK EXPERIENCE

Research Assistant	METU-Dept. of Chemical Engineering	2000 - Present
Summer Practice (Engineering Intern)	Kırıkkale- TUPRAS Petroleum Refinery	1999
Summer Practice (Engineering Intern)	Adana- MarSA Margarine & Oil Factory	1998

THESIS

1. PhD Thesis

Date: July 2002 - Present
Thesis Title: Biological Hydrogen Production from Olive Mill Wastewater and its Applications to Bioremediation
Supervisor: Prof. Dr. Inci Eroglu
Co-supervisor: Prof. Dr. Ufuk Gunduz
Institution: Middle East Technical University, Ankara / TURKEY

2. MSc Thesis

Date: September 2000 - July 2002
Thesis Title: Hydrogen Production from Olive Mill Wastewater by *Rhodobacter sphaeroides* O.U01
Supervisor: Prof. Dr. Inci Eroglu
Co-supervisor: Prof. Dr. Ufuk Gunduz
Institution: Middle East Technical University, Ankara / TURKEY

PUBLICATIONS

a. Articles Published in International Journals

1. **Eroğlu E.**, Gündüz U, Yücel M, Türker L, and Eroğlu İ, "Photobiological hydrogen production by using olive mill wastewater as a sole substrate source", International Journal of Hydrogen Energy, 29/2: 163-171, 2004.
2. **Eroğlu E.**, Gündüz U, Yücel M, Türker L, and Eroğlu İ, "Biological Hydrogen Production from Olive Mill Wastewater with Two Stage Processes", accepted to be published in the International Journal of Hydrogen Energy (IJHE) - Special Biohydrogen Issue for International Hydrogen Energy Congress & Exhibition (IHEC-2005).

b. Articles Published in the Proceeding Books / CD of International Conferences

3. **Eroğlu E.**, Gündüz U, Yücel M, Türker L, and Eroğlu İ, "Biological Hydrogen Production Potential of Olive Mill Wastewater", International Hydrogen Energy Congress & Exhibition (IHEC-2005), article published in the conference CD, 13-15 July, Istanbul, TURKEY, 2005.
4. **Eroğlu E.**, Gündüz U, Yücel M, Türker L, and Eroğlu İ, "Effect of Illumination Period on Photobiological Hydrogen Production from Olive Mill Wastewater", EMCC-3 (East Mediterranean Chemical Engineering Conference), article published in the conference CD, 13-15 May, Thessaloniki, GREECE, 2003.
5. **Eroğlu E.**, Tabanoğlu A., Gündüz U, Yücel M, Türker L, and Eroğlu İ, "The Relationship Between Growth Kinetics and Hydrogen Production by *Rhodobacter sphaeroides"* , 1st European Hydrogen Energy Conference (EHEC), article published in the conference CD, 2-5 September, Grenoble, FRANCE, 2003.
6. **Eroğlu E.**, Eroğlu İ., Gündüz U., Yücel M. and Türker L., "Biological Hydrogen Production from Olive Mill Wastewater by *Rhodobacter sphaeroides* O.U01", International Conference on Environmental Problems of Mediterranean Region (EPMR-2002) in Near East University, article published in the conference CD, 15-18 April, Nicosa, NORTHERN CYPRUS, 2002.

c. Articles Published in the Proceeding Books / CD of National Conferences

7. **Eroğlu E.**, Gündüz U., Yücel M., Türker L., and Eroğlu İ., "Effect of Pretreatment Techniques on the Biological Hydrogen Production by Olive Mill Wastewater", 14th Biotechnology Congress, 31 August – 2 September, Osmangazi Univ. – Eskisehir, TURKEY, pg.172-175, 2005. (Turkish)

8. **Eroğlu E.**, Gündüz U., Yücel M., Türker L., Eroğlu İ., "Biological Hydrogen Production Behavior of Different Olive Mill Wastewater Samples", 6th National Chemical Engineering Congress, article published in the conference CD, 7-10 September, Ege Univ.- İzmir, TURKEY, 2004. (Turkish)
9. **Eroğlu E.**, Gündüz U., Yücel M., Türker L., and Eroğlu İ., "Photobiological Hydrogen Production Potential of Olive Wastes", Bioenergy (2004) Symposium, Proceeding Book: pg. 193-199, 20-22 October, Ege Univ.- İzmir, TURKEY, 2004. (Turkish)
10. **Eroğlu E.**, Eroğlu İ., Gündüz U., Yücel M, and Türker L., "Utilization of Waste Materials During the Production of Hydrogen by Biological Processes", Workshop on Biological Hydrogen Production and Its Industrial Applications, Turkish Scientific and Technical Research Council - Research Institute for Genetic Engineering and Biotechnology, article published in the conference CD, 16 April, Gebze- Kocaeli, TURKEY, 2004. (Turkish)
11. **Eroğlu E.**, Eroğlu İ., Gündüz U., Meral Y., and Türker L., "The effect of Illumination Pattern on the Photobiological Hydrogen Production by Olive Mill Wastewater", 4th National Clean Energy Symposium, Proceeding Book, Volume 2: pg. 761-769, 16-18 October, Istanbul, TURKEY, 2002. (Turkish)
12. **Eroğlu E.**, Eroğlu İ., Gündüz U., Meral Y., and Türker L., "Biological Hydrogen Production from Olive Mill Wastewater", 5. National Chemical Engineering Congress, article published in the conference CD, 2-5 September, Ankara, TURKEY, 2002. (Turkish)

<u>d. Abstracts Published in the Proceeding Books / CD of International Conferences</u>

13. **Eroğlu E.**, Gündüz U, Yücel M, Türker L, and Eroğlu İ, "Improvement of Photobiological Hydrogen Production from Olive Mill Wastewater by Pre-treatment", COST-841 Working Group-3

Workshop on Biosynthesis and Regulation of Hydrogenases, Book of Abstracts, 1-3 May, Porto, PORTUGAL, 2005.

14. **Eroğlu E.**, Eroğlu İ., Gündüz U., Türker L., and Yücel M., "Photobiological Hydrogen Production from Different Olive Mill Wastewater Samples", 7th International Hydrogenases Conference, Book of Abstracts, 24-29 August, The Univ. of Reading-ENGLAND, 2004.
15. **Eroğlu E.**, Gündüz U, Yücel M, Türker L, and Eroğlu İ, "Effect of Fe and Mo Addition on Biological Hydrogen Production from Olive Mill Wastewater by *Rhodobacter sphaeroides* O.U01", COST-841 Working Group-3 Workshop on Biosynthesis and Regulation of Hydrogenases, Book of Abstracts: p. 49., 5-8 October, 2002, Cercedilla-Madrid, SPAIN, 2002.

e. Others

16. Eroğlu İ., Oztürk T., Ozbayoğlu G., Sahin M., Bilici U., and **Eroğlu E.**, "Utilization Potential of Hydrogen Energy and Boron Compounds", World Energy Council - Turkish National Committee, Scientific Report of Hydrogen and Boron Working Group, December, 2004. (Turkish)
17. **Eroğlu E.**, "Biological Hydrogen Production Studies in Middle East Technical University", Journal of Science and Technic, Turkish Scientific and Technological Research Council of Turkey (TUBITAK), pg.46-47, April, 2006. (Turkish)

RESEARCH PROJECTS

1. "HYVOLUTION: Non- thermal production of pure hydrogen from biomass", EU Sixth Framework Project, 2006-2010, Researcher. (International)

2. “Hydrogen Production, Storage and Electricity Production by Fuel Cells”, National & International Research Projects of Turkish State Planning Agency, 2005 - 2008, Researcher. (International)
3. “Improvement of Biological Hydrogen Production Efficiency and Investigation of a Process for its Industrial Applications”, METU Research Fund Project, Project No: BAP-2003-07-02-02, 2003 - 2006, Researcher. (National)
4. “Genetic and Biochemical Studies for the Improvement of Photobiological Hydrogen Production Efficiencies of Hydrogenase Enzyme in Rhodobacter species”, European Union – COST Action:841 Research Project, 2002 - 2005, Researcher. (International)
5. “Biological Hydrogen Production by using Oil Wastes”, METU Research Fund Project, Project No: AFP 2001-07-02-00-26, 2001 – 2002, Researcher. (National)
6. “Investigation of Hydrogen Production via Biotechnological Methods, Its Storage and Conversion into Electricity by Fuel Cells”, METU Research Fund Project, Project No: AFP 99-06-02-13, 1999 – 2002, Researcher. (National)

AWARDS

1. Hasan Orbey Award in Graduate Studies, Department of Chemical Engineering, METU, September, 2005.
2. Ihsan Dogramacı Superior Success Award, Hacettepe University, 2000.
3. 1st Rank Student Award, Dept. of Chemical Engineering, Hacettepe University, 2000.

INTERNATIONAL CONFERENCE ORGANIZATION

1. Scientific Committee Member of International Hydrogen Energy Congress & Exhibition (IHEC-2005), Istanbul – TURKEY, 13-15 July, 2005.
2. Organization Committee Member of Annual Working Groups Meeting of COST Action-841, Istanbul – TURKEY, 13-16 July, 2005.

COURSES ASSISTED

1. Undergraduate Courses:
   a. Thermodynamics (I)
   b. Introduction to Chemical Engineering
2. Graduate Courses:
   a. Advanced Chemical Reaction Engineering
   b. Multiphase Reactors

COMPUTER SKILLS

MATLAB 6 Series, MATHCAD, MATHEMATICA, Microsoft Office

LANGUAGE SKILLS

1. Turkish: Native Language
2. English: Fluent
3. German: Beginner

INTERESTS

Traveling, Literature, Ethnical and classical music, Documentaries, Archeology, Collecting old magazines and music records, Photography