PHOTOTROPHIC HYDROGEN PRODUCTION BY AGAR-IMMOBILIZED RHODOBACTER CAPSULATUS

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KAMAL E. M. ELKAHLOUT

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IMPROVEMENT OF PHOTOTROPHIC HYDROGEN PRODUCTION BY AGAR-IMMOBILIZED *RHODOBACTER CAPSULATUS*

Submitted by **KAMAL E. M. ELKAHLOUT** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Biotechnology Department, Middle East Technical University** by,

Prof. Dr. Canan ÖZGEN
Dean, Graduate School of **Natural and Applied Sciences** _____

Prof. Dr. İnci EROĞLU
Head of Department, **Biotechnology** _____

Prof. Dr. Meral YÜCEL
Supervisor, **Biological Sciences Dept., METU** _____

Prof. Dr. İnci EROĞLU
Co-Supervisor, **Chemical Engineering Dept., METU** _____

Examining Committee Members

Prof. Dr. Ufuk GÜNDÜZ
Biological Sciences Dept., METU _____

Prof. Dr. Meral YÜCEL
Biological Sciences Dept., METU _____

Prof. Dr. Hüseyin Avni ÖKTEM
Biological Sciences Dept., METU _____

Assoc. Prof. Füsun İNCİ EYİDOĞAN
Eğitim Fak., Başkent Üniversitesi _____

Dr. Yavuz ÖZTÜRK
TÜBİTAK MAM _____

Date: 11/02/2011

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

Name, Last name: Kamal E. M. Elkahlout

Signature:

ABSTRACT

PHOTOTROPHIC HYDROGEN PRODUCTION BY AGAR-IMMOBILIZED *RHODOBACTER CAPSULATUS*

Kamal E. M. Elkahlout
Ph.D. Department of Biotechnology
Supervisor : Prof. Dr. Meral Yucel
Co-Supervisor: Prof. Dr. Inci Eroğlu

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Hydrogen production by photosynthetic bacteria is attractive field as production is fueled by solar energy. Hydrogen production potential of two photosynthetic bacteria *R.capsulatus* (DSM1710 wild type and *R.capsulatus* YO3 Hup^- uptake hydrogenase deleted mutant strain) were examined in agar immobilized systems. In the present work agar and glutamate concentrations were optimized for immobilization of bacteria while feeding bacteria with 40/2-4 mM acetate/ glutamate. Immobilized bacteria produced hydrogen for 420-1428 hours covering 5-7 rounds. Optimizing of acetate concentration indicated that 60 mM produced the highest observed yield around 90-95%.

Results shown that 2.5 mg dry cell weight/mL is the optimum cell concentration for wild type strain while 5 mg dry cell weight/mL was optimum for YO3 strain. Using either glycerol or sodium dithionite caused decrease in hydrogen production capacity of immobilized bacteria. It was observed that agar provided protection against inhibition effect of ammonium. Co-

immobilization of bacteria with packed cells of *H. salinarium* increased total hydrogen production capacity by about 1.14-1.41 folds.

Hydrogen production by immobilized bacteria in panel photobioreactor was achieved by a novel system which allowed long term hydrogen production. Immobilized *R. capsulatus* DSM 1710 in panel reactor worked for about 67-82 days covering 4-5 rounds while immobilized *R. capsulatus* YO3 worked for 69-72 days covering seven rounds.

Keywords: *Rhodobacter capsulatus*, agar immobilization, hydrogen production, co-immobilization, *Halobacterium salinarium.*

ÖZ

AGARA İMMOBILIZE *RHODOBACTER CAPSULATUS* ILE FOTOSENTETIK HIDROJEN ÜRETIMI

Kamal E. M. Elkahlout
Ph.D. Biyoteknoloji Bölümü
Tez Yöneticisi: Prof. Dr. Meral Yucel
Ortak Tez Yöneticisi: Prof. Dr. Inci Eroğlu

Şubat 2011, 273 sayfa

Fotosentetik bakteriler ile hidrojen üretimi güneş enerjisini yakıt olarak kullanabilen cazip bir alandır. *R.capsulatus* (DSM1710 yabani suş) ve *R.capsulatus*(YO3 Hup^- uptake hydrogenase geni silinmiş mutant suş)'ün hidrojen üretim potansiyeli agar jele immobilize edilerek test edilmiştir. Çalışmanın bu kısmında 40/2-4 mM asetat/glutamat besleyici olarak kullanılmış ve farklı agar ile glutamat konsantrasyonlarında immobilizasyon optimize edilmiştir. Immobilize bakteri 5-7 tur 420-1428 saat boyunca hidrojen üretmiştir. 60 mM asetat kullanımında %90-95 verim ile en iyi sonuc gözlemlenmiştir.

Sonuçlara göre yabani suş için 2.5 mg , YO3 suşu için ise 5 mg kuru hücre ağırlığı/mL optimum olarak bulunmuştur. Gliserol veya sodium ditionat kullanımı bakterinin hidrojen üretim kapasitesini düşürmesine sebep olmuştur. Agarın amonyumun inhibisyonuna karşı koruma

sağladığı gözlemlenmiştir. Bakterilerin *H. salinarium* ile birlikte immobilizasyonu hidrojen üretimini 1.14-1.41 kat artırmıştır.

Immobilize bakterilerin panel fotobioreaktörlerde yeni bir sistem kullanılarak uzun süreli hidrojen üretimi sağlanmıştır. Immobilize *R. capsulatus* DSM 1710 panel reaktörlerde 4-5 tur 67-82 gün çalışırken *R. capsulatus* YO3 7 tur 69-72 gün çalışmıştır.

Anahtar kelimeler: *Rhodobacter capsulatus*, agar immobilizasyonu, hidrojen üretimi, farklı asetat konsnatrasyonları, ko-immobilizasyon, *Halobacterium salinarium*.

To my wife and my children for their patience and love

To the memory of my parents

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GC	**Gas Chromatography**
HPLC	**High Performance Liquid Chromatography**
Ni	**Nickel**
Fe	**Iron**
nif	**Nitrogenase-Nitrogen fixing gene**
anif	**Alternative nitrogenase gene**
NAD/NADH	**Nicotinamide adenine dinucleotide (oxidized and reduced for)**
ATP	**Adenosine Triphosphate**
Hup^-	**Deleted hydrogase-uptake mutant**
Mo	**Molybdenum**
A.D.	**Anno Domini (Christian calendar)**
B.C.	**Before Christ**
PSI	**Photosystem I**
PSII	**Photosystem II**
bchl	**Bacteriochlorophyl**
TCA	**Tricarboxylic Acid Cycle**
PNS	**Photosynthetic Non-Sulfur Bacteria**
Wet wt	**Wet weight**
Dry wt	**Dry weight**
DCW	**Dry Cell Weight**
BP medium	**Biebl and Pfennig medium**
SDT	**Sodium dithionite**
hupSLC	**Hydrogenase operon**

CHAPTER 1

INTRODUCTION

Sun was the first energy source known by early humans. Sunset and the need for energy during cold times pushed humans to search for energy source for warming and cooking. Once the early humans discover how to create a spark and build a fire, their lives changed. Creating fire was just the first steps of humankind for ongoing quest of earth energy resources to improve their lives. Through major eras of humankind history, wood was the mainstay of life for shelter and for transportation on land and water and as a source of energy for heat and light. During the past and even now days people enjoyed the advantage of energy sources including, sun, wind, running water and even animals to provide energy and to do work for fulfilling different life requirements.

Through the history eras it was clear that civilization requirements lead to improve and advancing the use of energy resources. Around 3500 B. C. in ancient Egypt the earliest known sailboats, harnessing the power of the wind to travel faster and further, while increasing trade with neighboring lands. By 500 B.C. Greeks were building what we now call "passive solar" homes to take better advantage of the sun's light and warmth. And by 85 B.C. Romans were enjoying baths heated with water from geothermal hot springs.

Around the same time, the Greeks made advances in use of running water. They developed waterwheels to grind grain, a task previously done by hand or with animal power. And by 640 A.D. Persians had developed a method to harness wind energy for grinding grain. Europeans modified this method to new versions of windmills throughout medieval times. Wood remained the main-used energy resource. In 1300s Germans built the first blast furnaces to burn wood at extremely high temperatures, allowing them to produce large quantities of iron Coal as

energy source was known and used at least as early as the first century A.D. it took more than a thousand years for coal to become a dominant source of energy. By the late1600s coal had become more popular than wood in England (www.energyforkeeps.org/book_chapters/eforkeeps_pre_ch1.pdf, 2011).

By rising of the industrial revolution coal occupied central position as energy source putting the fossil fuel as the main and principle source of energy until coming up the oil age. Industrial revolution and modern civilization devoted the fossil fuels as the mainstay of modern economy in addition to nuclear power which is restricted for counties having nuclear power technology. In fact oil and natural gas is not only energy source but they present the main raw materials of petrochemical industries and related manufacturing processes. But on the other hand consumption of fossil energy sources consistent of consortium of complicated problems related mainly to deleterious environmental risks threatened stability of ecological systems. Poisonous gases are released to atmosphere like carbon monoxide, sulfur oxides and nitrogenous oxides.

Carbon dioxide as the main global warm agent is released in millions of tons per year making this problem much more extreme one. Other industries and energy production processes are involved in releasing dangerous chemical compounds that contribute mainly in devoting ozone layer depletion problem.

The above stated issues in addition to depletion of fossil fuels (coal, natural gas and petroleum supply are carbon base fuels) sources, regional and international conflictions and wars to control traditional energy sources strongly directed many researches for seeking new clean, renewable and cosmopolitan distributed energy source that devoid profound problems caused by fossil fuel consumption.

As logic consequences of this intensive argument, feasibility of future energy economy based on hydrogen as energy source and carrier has been widely discussed (Getoff, 1990; Dostrovsky, 1991). Hydrogen based energy can satisfy requirements for environmentally friend energy source in addition to ability of local production. Conversion of hydrogen will not produce environmentally harmful wastes; in contrast major end product of conversion of hydrogen is water which is valuable commodity in many countries.

Hydrogen is abundant and renewable source as it is estimated to constitute up to more than 90% of all atoms or three quarters of the universe mass. Hydrogen has highest energy to weight ratio of fuels. One kilogram of hydrogen has energy as 2.1 kilograms of natural gas and 2.8 kilograms as gasoline. Energy to volume ratio is equivalent to ¼ of that of petroleum and 1/3 of natural gas (Elkahlout, 2002).

1.1 Hydrogen production methods

The efficient and sustainable production of hydrogen is necessary for the development of sustainable hydrogen economy.

Different methods are employed in hydrogen production including reforming of oil or natural gas, fermentation of biomass, gasification of coal, electrolysis, thermochemical, photo- and biocatalysis. The least cost method is a chemical methods particularly that one involving thermal reforming of steam and methane and other hydrocarbons in addition to gasification of coal. Electrochemical methods including electrolysis of water produce 10% of commercial hydrogen (Shrev, 1984) while about 90% is produced by reforming of hydrocarbons (Markove *et al*., 1995). Hydrogen production by thermal conversion of steam and hydrocarbons release large amounts of carbon dioxide and such type of production methods cause pollution for the environment even they are feasible from economical view.

The great advantages, especially the environmental ones, gained from consumption of hydrogen as energy resource would be true if hydrogen production processes involved nonpolluting methods. Hydrogen production by electrolysis is an option as non-polluting method but it needs large amount of electricity, produced by fossil fuel, and mostly consumed expensive materials for building electrolyzing instruments.

Major attempts for production of chemical fuels through involving solar-energy driven processes focused on hydrogen production. Solar energy which was used by humans since the old history reacquired its prestige. It is clean, renewable energy and attracted attention as alternative to fossil and nuclear power for future power. It can be converted to other useful and storable energy forms like hydrogen. Unlimited supply of solar energy has drawn attention for using it for hydrogen production. Solar energy conversion includes photoelectrolysis, photovoltaic, thermoelectric, solar radiation concentration and photobiological (Bicelli, 1986 and Markov *et al*., 1995).

1.2 Biological hydrogen production methods

Biological hydrogen production methods are environment friendly and consume less energy comparing to thermochemical and electrochemical processes (Das and Veziroglu 2001 and Türker *et al*., 2008). Rapid advancement and development in biotechnology fields in last decades attracted intensive research work for biohydrogen production technology.

Biological hydrogen production methods can be classified into three classes including the following:

1- Hydrogen production by dark fermentation methods

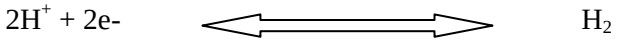
2- Photobiological hydrogen production methods which can be sub-classed into two types

a) Photoautotrophic hydrogen production

b) Photohetertrophic hydrogen production

3- Hydrogen production by coupled systems and mixed cultures

Biological hydrogen production processes were performed either by whole cells or their enzymes. Accordingly, it is believed that overall efficiency of the process depends primarily on the activity and amount of these enzymes (Hallenbeck and Menemann, 2002). Studies showed that in addition to the activity and amount of enzymes there are other factors are participating the overall efficiency of the process. Those factors involving metabolic events and pathways such as electron flow to the enzymes (Jeong and Jouanneau, 2000). Hydrogen production by biological methods follows a simple general reaction as illustrated below:



The simplicity of this reaction did not reflect the complexity of hydrogen evolving enzymes. They contain highly organized complex of metal-clusters as active sites. At the same rout synthesis of those enzymes involves highly controlled and organized process employing accessory enzymes and protein maturation steps (Leach and Zamble, 2007). Two major types of enzymes are involved in hydrogen production process including hydrogenase and nitrogenase. Those enzymes will be discussed and explained later in the context of this chapter.

It is estimated that sunlight is the source of ultimate energy resource in photobiohydrogen production process. Microorganism can utilize biomass, carbon dioxide and light energy to drive hydrogen production reactions. Discussion about such reactions and processes will be introduced in next sections.

1.2.1 Hydrogen production by dark fermentation

Several types of microorganisms have the ability to produce hydrogen under anoxic environments. Those microorganisms have the capacity to degrade organic matter and then reduce them to short chain organic acids during energy production metabolism.

However the reduction of organic substrates by those organisms is not completed to carbon dioxide formation and short chain organic acids like lactate, butyrate and acetate are produced (Akkerman *et al*., 2003). Strict and facultative and anaerobes are in need to regulate electron flow in the metabolism by using different mechanisms. Some of the employed regulatory mechanisms are reflected by the ability of some microorganisms to dispose the excess electrons in the form of molecular hydrogen by the virtue of hydrogenase enzyme activity.

However not all of hydrogenases have hydrogen production ability. Some types of hydrogenases are hydrogen uptake enzymes (NiFe hydrogenases) that convert hydrogen into protons and electrons. Electrons are used to reduce NAD^+ to $NADH + H^+$ via quinone pool in the membrane. The resulted reducing equivalents are used to drive cellular activities (Akkerman *et al*., 2003).

Employing dark fermentation processes for hydrogen production was investigated by many workers. *Enterobacter aerogenes* HU-101 was evaluated in packed-bed reactor for hydrogen production by utilizing glycerol-containing wastes discharged after biodiesel manufacturing with maximum hydrogen production rate reached to 63 mmol.H_2/l/h (Ito *et al*., 2005).

Fermentative hydrogen production by *Clostridium butyricum* CWBI1009 and *Citrobacter freundii* CWBI952 was examined in pure and mixed cultures by using different carbohydrates including starch, glucose, lactose and sucrose. The highest yield was obtained from mixed culture with starch giving 0.73 mol H2/mol hexose/l (Beckers *et al*., 2010).

Hydrogen production from glucose by *Citrobacter freundii* by using synthetic medium containing glucose was tested. From 1 mole of glucose 1.286 mole of hydrogen was produced. The rate of gas production and hydrogen production was 0.71 and 0.45 1/h respectively (Kumar& Vatsala 1989).

Biohydrogen production by *Enterobacter cloacae* IIT-BT 08 improvement was conducted by controlling pH at 6.5. Bacterial were fed with glucose and higher hydrogen yield was 3.1mol Hydrogen per mol glucose (Khanna N., *et al*., 2010).

Hydrogen production by mixed fermentative cultures was conducted by several workers using glucose, mixed biomass substrates and from waste materials. Enhancement effect of nitrogen gas bubbling on hydrogen production from glucose by mixed culture was examined in continuous-stirred tank reactor. Hydrogen production rate increased from 1.446 ml/min to 3.131 ml/min after bubbling nitrogen gas with a rate 15 times the hydrogen production rate (Mizuno *et al*., 2000).

Biohydrogen production by fermentative process examined by (Wangtant *et al*., 2007) using glucose as carbon source in continuous stirred tank reactor at pH 5.5. They got hydrogen ratio as 38.5-73.9% in the produced biogas. The highest achieved yield was about 1.24 mol.H_2/mol glucose. The workers concluded that Clostridia and Bacilli were predominant population of bacteria in the reactor.

Recently, some obligate aerobes were shown to be able to evolve H_2, immobilized aerobic Bacillus licheniformis produces H_2 up to 0.7mol H_2/mol glucose. Aerobic H_2 producers occur as species able to assimilate molecular N_2: *Rhzobium* spp., *Azotobacter* spp., *Azospirillum brasilense*, and some others. *Synechococcus* spp. has a high potential for H_2 production in fomenters and outdoor cultures (Nandi and Sengupta, 1998).

Nearly, in major cases within anaerobic microorganisms, H_2 production is catalyzed by hydrogenases. In contrast, photosynthetic bacteria produce H_2 by the aid of nitrogenase, i.e. by the enzyme providing the capacity for N_2 fixation. The facultative anaerobic bacterium *Klebsielle pneumonia* has the ability to produce H_2 with the participation of both hydrogenase and nitrogenase. Other organisms have displayed evidence for H2 production with participation of both hydrogenase and nitrogenase like *Azospirillum brazilens* (Kondratieva and Gogotove; 1983).

The main advantage in dark fermentation of hydrogen production is that the microorganism can produce hydrogen from organic matters without being affected by day and night cycle. In addition to that, there are some hydrogenases tolerant to oxygen and are not affected by the high hydrogen pressure (Vincent *et al*., 2007).

1.2.2 Photobiological hydrogen production

Photobiological hydrogen production is carried out either by photoautotrophic or photoheterotrphic organisms. Microalgae and cyanobacteria considered as photoautotrophic

hydrogen producers. They can use light as energy source to mediate fixation of carbon dioxide as sole carbon source into organic compounds. Hydrogen production by photoheterotrophic hydrogen producers is mediated by nitrogen fixing enzyme in the absence of nitrogen. Both types of hydrogen production mechanisms will be discussed in the following sections.

Photoautotrophic hydrogen production

During oxygenic photosynthesis microalgae and cyanobacteria utilize sunlight energy to mediate fixation of carbon dioxide into energy-rich organic compounds $[C_n(H_nO)_n]$. Under anaerobic conditions, these organisms have ability to produce hydrogen by photolysis of water employing light as energy source. Hydrogen production by this way is catalyzed by oxygen sensitive hydrogenase enzyme.

$$4H_2O + \text{light energy} \xrightarrow{===} 2O_2 + 4H_2 \text{ , } \Delta G^\circ = +1498 \text{ kJ} \quad \text{(Kars 2008)}$$

Such process is called direct photolysis since light energy is used directly for splitting of water molecule into hydrogen and oxygen. The produced gas is a mixture of hydrogen and oxygen. The drawback of this process is the need for separation of the two gases. The indirect photolysis occurred when there is separation of oxygen and hydrogen production either in time or place.

Indirect photolysis process was cleared by studies done with the green algae *Chlamydomonas reinhardtii*. Phase of oxygen production and carbon accumulation was separated from phase of hydrogen production and metabolites consumption through sulfur deprivation (Melis *et al.*, 2000 and Melis 2002). Heterocystous cyanobacteria are examples on process separation by place. Heterocysts are special type of cell compartments where nitrogenase is protected away from oxygen inhibition effect while substrates are provided by the vegetative cells. Hydrogen production by indirect photolysis has limitations due to oxygen sensitivity of hydrogenases and low light conversion efficiency.

Green algae were considered as being light-dependent water-splitting catalysts, but hydrogen production using green algae was not feasible. Their hydrogenase enzyme is too oxygen-labile for sustainable H_2 production (Asada and Miyake., 1999). The indirect photolysis by green algae is still attracting studies and improvements (Hallenbeck and Benemann, 2002). Hydrogen production by *Chlamydomonas reinhardtii* immobilized on different glass types of

silica beads and fibers under sulfur deprivation has been examined (Laurinavichene *et al.*, 2006; Laurinavichene *et al*., 2008 and Hahn *et al.*, 2007).

Photoheterotrophic hydrogen production

Microalgae and plants have two photosystems PSI and PSII while purple bacteria have only one photosystem which is localized in the intracellular membrane (Akkerman *et al.*, 2003). Photosynthetic bacteria carry out anoxygenic photosynthesis using organic compounds and reduced sulfur compounds as electron donors, which are categorized as non-sulfur and sulfur photosynthetic bacteria, respectively.

Some non-sulfur bacteria are potent hydrogen producers utilizing organic acids such as lactic, succinic, butyric, and malic acids or alcohols. Purple bacteria can produce hydrogen under anaerobic conditions in the absence of nitrogen. They have the ability to utilize simple organic acids as electron donors. Hydrogen formation is catalyzed by nitrogenase which received electrons from ferrodoxin (Fd). Under ammonia-limited conditions, those electrons are used by ATP-dependent nitrogenase to reduce molecular nitrogen into ammonium.

In the absence of nitrogen, nitrogenases utilize the electrons provided by organic acids to reduce the protons into molecular hydrogen in an ATP-dependent reaction (Kars *et al*., 2008; Thorneley and Lowe, 1983; Lianng and Burris 1988). Passing one electron from nitrogenase consumes 2 ATP equivalents. And since two electrons are needed to produce one hydrogen molecule (H_2) so four ATP equivalents are consumed by nitrogenase to give one molecule of molecular nitrogen;

$2H^+ + 2e- + 4ATP ===\rightarrow H_2 + 4\ ADP + 4Pi$

Since the reaction is not producing oxygen (anoxygenic photosynthesis) nitrogenase will not suffer inhibition effect of oxygen. This reaction indicating that hydrogen production by nitrogenase enzyme is an inherent property.

The main drawback of nitrogenase based hydrogen production compared to hydrogenase based hydrogen production is the need for energy in the form of ATP while hydrogenase did not require any extra energy. Despite of such disadvantage, the required energy is captured from the sun and electrons are provided by feed stocks or waste streams. This condition made photoheterotrophic hydrogen production process more efficient.

Since light energy is not used for water oxidation, the efficiency of light energy for the production of hydrogen by photosynthetic bacteria is theoretically much higher than that by cyanobacteria. From the practical point of view, photosynthetic bacteria are important since they can be used for dual purposes of wastewater treatment and hydrogen production (Sasikala *et al*., 1992; Yigit *et al*., 1999; Zhu *et al*., 1995).

Agricultural wastes and biomass have been demonstrated for hydrogen production. Algal biomass was examined for hydrogen production. The biomass might be subjected to chemical or biological digestion and the products could be supplied to photosynthetic bacteria for hydrogen production (Ike *et al*., 1996). Polysaccharides like starch are major constituent of algal biomass. The algal biomass was fermented by a lactic acid bacterium and the enriched-lactic acid effluent supplied to photosynthetic bacteria (Ike *et al*., 1996).

Lactic acid is an excellent carbon source for H_2 production by photosynthetic bacteria, and a maximum H_2 yield of 8 mol/mol starch-glucose from algal biomass was observed. It was shown that supplying photosynthetic bacteria *Rhodobacter* sp with lactic acid as electron donor for hydrogen production has brought 6 to 8% of the incident light energy to be converted to H_2 gas (combustion energy) in laboratory experiments (Miyake and Kawamura 1987).

As mentioned above, nitrogenase is in charge of hydrogen production in photosynthetic bacteria whereas hydrogenases may be active for hydrogen uptake in many photosynthetic bacteria (Miyake *et al*. 1989).

Employing of photosynthetic bacteria has several advantages over cyanobacteria as H2-producers organisms:

They generally have high rates of H_2 production. They have ability to degrade a wide variety of organic substrates to H_2 and CO_2 with a more or less high efficiency.

Since they use anoxygenic photosynthesis, the H_2 produced is free of contaminating oxygen.

Photosynthetic bacteria can trap light energy over a wide spectral range and can withstand high light intensities. In principle, they can utilize organic wastes as nutrients. Using of these wastes may encourage the economical aspects, with H_2 as a useful by-product. The photosynthetic bacteria are versatile in terms of their metabolism, which enable them to remain functional under many different environmental conditions.

They are much more amenable to genetic manipulation. They can also produce hydrogen as a by-product of processes such as water purification or biomass production (Vignais, *et al*., 1985).

The photo-evolution of H_2 occurs in the absence of N_2 and of high concentrations of ammonium ions. This process requires excess production of ATP from photophosphorylation

and reducing equivalents from organic substrates (Hillmer and Gest, 1977 a,b , and Vignais, *et al.*, 1985). Photosynthetic microorganisms use the same process found in plant and algal photosynthesis, but end product is hydrogen gas instead of carbon containing biomass.

Photobiological systems have the distinct advantage that the collector system self assembles. Thus as long as the cells can be kept alive and viable for extended periods of time, the capital costs of such systems are potentially quite low. However, there may be considerable costs involved in the fabrication of reactor systems necessary to maintain the organisms under optimum conditions (Bolton, 1996).

1.3 The photosynthetic bacteria

Anoxygenic phototrophic bacteria including purple and green ones are prokaryotic and photosynthetic organisms lacking photosystem II. Those bacteria perform anoxygenic photosynthesis for gaining energy. They are aquatic gram-negative organisms that inhabit wide spectrum of environments, including marine and fresh water systems. The bacteria have ability to mediate nitrogen and carbon dioxide by the aid of solar energy. Bacteriochlorophylls are the main characteristics pigments found in bacteria in addition to various carotenoids giving them different colors ranging between red, purple, brown and orange (Pfennig & Truper, 1974).

1.3.1 Classification

Taxonomical situation of photosynthetic bacteria within Kingdom “Prokaryota” is illustrated in Table 1.1 (Gibbon & Muray, 1978). They name Anoxyphotobacteria aroused from the fact that they do not produce hydrogen during photosynthesis reaction as was observed in cyanobacteria.

Two different groups, purple bacteria and green bacteria, comprise anoxygenic photosynthetic bacteria. Those two groups are different in their cytological structure. Differentiation between orders within the classes Rhodospirillales (purple bacteria) and Chlorobiales is carried out on the basis of variations in structure and pigment content of the photosynthetic apparatus.

The photosynthetic apparatus is formed of reaction center and antennae chlorophyll units. In Rhodospirillales, this reaction center form intracytoplasmic invagination structure located entirely in the cytoplasm. In Chlorobiales, the bulk of antenna bacteriochlorophyll,

consisting of bchl c, d, or e, is contained in distinct organelles known as chlorosomes (Trüper & Pfennig, 1981). On the base of physiological and ecological observations photosynthetic bacteria have been classified into four families (Table 1.1).

Two families are comprising the order Rhodospirillales; Rhodospirillaceae (purple non-sulfur bacteria) and Chromatiaceae (purple sulfur bacteria). Two more families constitute the order Chlorobiales; the Chlorobiaceae (green sulfur bacteria) and Chloroflexaceae. It was concluded that photosynthetic phenotypes are extremely ancient. The oldest bacteria were anaerobic including the purple photosynthetic bacteria and the cyanobacteria together with the clostridia (Gibson *et al.* 1979) and (Fox *et al*. 1980).

1.3.2 Growth properties

Purple non sulfur bacteria are versatile organisms enjoy wide spectrum of growth mode. They can lead photoautotrophic metabolism with H_2 + CO_2 (Hallenbeck *et al*., 1990a), chemoautotrophic growth with H_2 + O_2 + CO_2 (Madigan and Gest, 1978). They can enjoy photoheterotrophic, chemoheterotrophic (Sasikala and Ramana, 1990a), mixotrophic, and by fermentative metabolism (Madigan and Gest, 1978). Such a wide spectrum of growth modes is not found in green sulfur bacteria which committed obligate autotrophy and anaerobiosis.

Metabolic flexibility of PNS bacteria enabled them to switch from one mode to another depends on the available conditions such as, light, C source, degree of anaerobiosis, and available organic compounds. Such ability enabled bacteria to grow and utilize wide range of organic acids and simple carbon sources. Hydrogen sulfide metabolism in PNS bacteria is restricted to low concentration of the material while at high concentrations H_2S became toxic to PNS bacteria (Lindquist, 1999).

Under anaerobic conditions in the presence of light source, PNS bacteria grow normally as photoorganoheterotrophs; they trap light energy and use organic acid molecules as electron and carbon source. In the absence of light, most purple non-sulfur bacteria can grow aerobically as chemoorganoheterotrophs, but some species carry out fermentations and grow anaerobically. Synthesis of bacteriochlorophyll and carotenoids is inhibited by oxygen and because of that cultures growing aerobically in the dark are colorless (Presscott *et al*., 1993). PNS bacteria require B-group vitamins for growth and most of them unable to use inorganic electron donors other than H_2 for growth (Vignais *et al*., 1985).

PNS bacteria show large variation in morphology (Table-1.1). They may take spiral (Rhodospirillum), rod (Rhodopseudomonas), half-circle or circle-shape (Rhodocyclus). Some of them may even form prosthecae and buds (Rhodomicrobium). The versatile metabolism modes exhibited by PNS bacteria enabled most of them to be prevalent in the mud and water of lakes and ponds with abundant organic matter and low sulfide levels. In addition to that, marine species were registered and studied (Presscott *et al.*, 1993).

1.4 Enzymes catalyzing hydrogen metabolism

1.4.1 Nitrogenase

By the virtue of ATP-dependent nitrogenase enzyme complex, anoxygenic phototrophic bacteria have ability of fixing N_2. Nitrogen fixation ability has been elucidated for the photosynthetic cyanobacteria, archaebacteria, aerobes, anaerobes, facultative anaerobes, microaerobic bacteria, actinomycetes in association with non-leguminous plants, and root nodule bacteria in symbiosis with leguminous plants (Burris, 1991). Energy supply for N_2 fixation by N_2-fixing organisms is provided by photosynthesis reactions of microorganisms themselves or by photosynthesis of green plants. Figure 1.1 shows cylindrical representation of nitrogenase enzyme.

Table 1.1 Subdivision of the Kingdom "Prokaryotae" (Taken from Vignais *et all*, 1985)

Kingdom Prokaryotae		Characteristics features
Divisions:	I. Gracilicutes II. Firmacutes III. Mollicutes IV. Mendocutes	
Division I	Gracilicutes	Have Gram-negative type of cell wall.
Class I	Photobacteria	Prokaryotes able to carry out photosynthesis either oxygenic or anoxygenic. Photopigments may be chlorophyll a or b or bacteriochlorophyll a, b, c, d, or e
Subclass I	Oxyphotobacteriae	Bacteria that produce oxygen during photosynthesis
Order I	Cyanobacteriales	Cells contain chlorophyll a and phycobiliproteins
Order II	Prochlorales	Cells contain chlorophyll a and b but no phycobiliproteins
Subclass II	Anoxyphotobacteriae	Carry out a phototrophic metabolism under anaerobic conditions. Do not produce oxygen during photosynthesis
Order I	Rhodospirillales	Cell membranes contain bacteriochlorophyll a or b, the pigments being located on internal membrane systems continuous with the cytoplasmic membrane
Order II	Chlorobiales	Cells contain bacteriochlorophyll c, d, or e and small amounts of a. Pigments are located in the cytoplasmic membrane and in "chlorobium vesicles"

Table 1.2 Recognized Genera of Anoxygenic Phototrophic Bacteria

Taxonomic group	Morphology
Purple bacteria	
Purple sulfur bacteria (chromatiaceae and Ectothiorhodospiraceae)	
Amoebobacter	Cocci embedded in slime; contain gas vesicles
Chromatium	Large or small rods
Lamprocystis	Large cocci or ovoids with gas vesicles
Lamprobacter	Large ovals with gas vesicles
Thiocapsa	Small cocci
Thiocystis	Large cocci or ovoids
Thiodictyon	Large rods with gas vesicles
Thiosprillum	Large spirilla
Thiopedia	Small cocci with gas vesicles; cells arranged in flat sheets
Ectothiorhodospira	Small spirilla; do not store sulfur inside the cell
Purple nonsulfur bacteria (Rhodospirillaceae)	
Rhodocyclus	Half-circle or circle
Rhodomicrobium	Ovoid with stalked budding morphology
Rhodopseudomonas	Rods, dividing by budding
Rhodobacter	Rods and cocci
Rhodopila	Cocci
Rhodosprillum	Large or small sprilla
Green bacteria	
Green sulfur bacteria (Chlorobiaceae)	
Anacalochloris	Prosthecate spheres with gas vesicles
Chlorobium	Small rods or vibrios
Pledictyon	Rods or vibrios, some form 3-D net; contain gas vesicles
Prosthecochloris	Spheres with prosthecae
Green gliding bacteria (Chloroflexaceae)	
Chloroflexus	Narrow filaments (multicellular), up to 100 ❍m long
Chloroherpeton	Short filaments (unicellular)
Chloronema	Large filaments (multicellular), up to 250 ❍m long; contain gas vesicles
Oscillochloris	Very large filaments, up to 2,500 ❍m long; contain gas vesicles

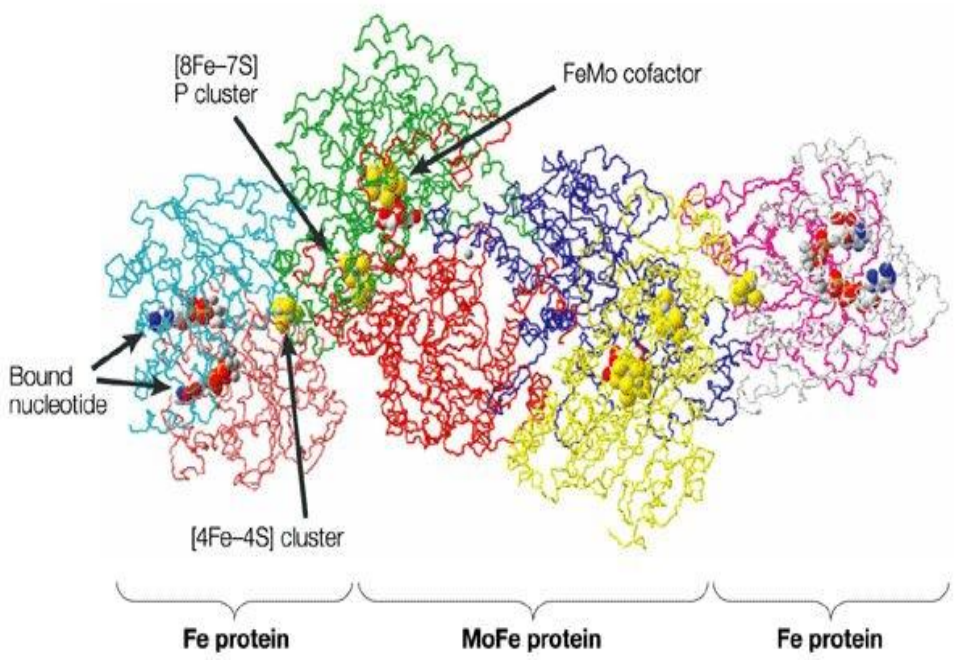


Figure 1.1Representation of nitrogenase enzyme complex (Horton *et al*., 2006)

Structure and molecular properties

Nitrogenase complex has been isolated for the first time by (Bulen and Lecomte 1966), from *Azitobacter vinelandii.* Later studies proved existence of three genetically distinct types including nif, vnf, and anf (Scneider *et al*., 1997). Mo-containing nitrogenase (nif system), which is found in all diazotrophs, is the most wide spread and intensively characterized system. Later on nitrogenases from different microorganisms were isolated by several workers. Active form of nitrogenase was isolated from *Rhodopseudomonas capsulatus* (Hallenbeck *et al*., 1982 a)

Nitrogenase complex contains two oxygen labile protein subunits, the molybdenum-iron protein (MoFe-protein, component I or dinitrogenase) and the iron protein (Fe-protein, component II or dinitrogenase reductase). Nitrogenases isolated from anoxygenic phototrophic bacteria (Wilison *et al*, 1983) were found to be similar in size, structure including amino acids composition, and gene coding. While Fe-proteins were found to have identical structures in all nitrogenase systems, MoFe component of the alternative nitrogenases differs from the

conventional tetrameric structure of MoFe proteins. Alternative Fe-only nitrogenase was found and characterized in *R. capsulatus* (Schneider *et al*., 1991). Its MoFe protein has a small 14 kda, subunit giving the Fe-nitrogenase a hexameric structure in contrast to tetrameric MoFe nitrogenase (Schneider *et al*., 1997).

The MoFe is 200.000-250.000 molecular weight tetramer protein formed of two α and two ß subunits. It binds and reduces N_2 or other substrates. MoFe protein has two molybdenum atoms, about 30 Fe atoms and 30 inorganic sulfur atoms in the form of sulfide (S_2^-). Sixteen of the Fe atoms associated with S_2^- in four cubic [4Fe-4S] (Vignais *et al*., 1985). The remained metal atoms are arranged in two copies of a cofactor named FeMo cofactor (Nelson *et al*., 1983). The FeMo protein was isolated and thought to the site of substrate binding (Shah and Brill, 1977)

The Fe protein is a dimmer in structure with molecular weight around 60.000. Each of the subunits of the dimmer protein is folded in a single α/ß type domain. A cluster of [4Fe-4S] bridges the subunits at one end of the molecule (Eady, 1991). Each subunit of the dimmer is provided with two nucleotide-binding sites for binding of MgATP and MgADP. The second function site, [4Fe-4S] cluster, undergoes a one-electron redox cycle (Howard and Rees, 1994). The conformational change of the protein upon binding of MgATP, enables the MgATP-Fe protein complex to reduce the MoFe protein during nitrogenase mechanism. (Vignais *et al*., 1985).

Catalytic mechanism and regulation

Molecular nitrogen is the ultimate substrate used by nitrogenase enzyme. It is reduced by nitrogenase complex in accordance to the following equation reaction:

$$N_2 + 8H^+ + 8e^- + 16MgATP \longrightarrow 2NH_3 + 16MgADP + 16Pi + H_2$$

According to the above equation it is clear that H_2 production is an intimate part of the mechanism (Madigan *et al*., 1997). Nitrogenase has the ability to reduce other substances including cyanide, acetylene, azide, hydrazine, alkyl isocynanide, and nitrous oxide (Burris, 1991). Absence of any other substrate, nitrogenase forward its reaction power to reduce protons into molecular hydrogen (Bulen *et al*., 1965) according to the following reaction.

$$4H^+ + 2e^- + 4ATP \longrightarrow H_2 + 4ADP + 4Pi$$

Reaction mechanism of nitrogenase is divided into two parts, the redox cycle between the Fe-protein and the MoFe-protein, and the substrate reaction cycle. Events of electron transfer process in the nitrogenase system have been elucidated by following EPR (electron paramagnetic resonance) signals of the nitrogenase components at low temperature (Burris, 1991).

Substrate reduction reaction by nitrogenase can be summarized in four steps as following:

An electron carrier like ferredoxin transfers one electron to reduce Fe-protein with ATP binding.

- Fe-protein and MoFe-protein bind together to form a complex.
- The electron is passed from Fe-protein to MoFe protein with ATP hydrolysis.
- Fe-protein MoFe-protein complex is then dissociated.

According to previous described steps it is possible to summarized electron transfer events as following;

Fe-protein ⟹ Mo-Fe protein ⟹ Substrate

Figure 1.2 shows the overall mechanism.

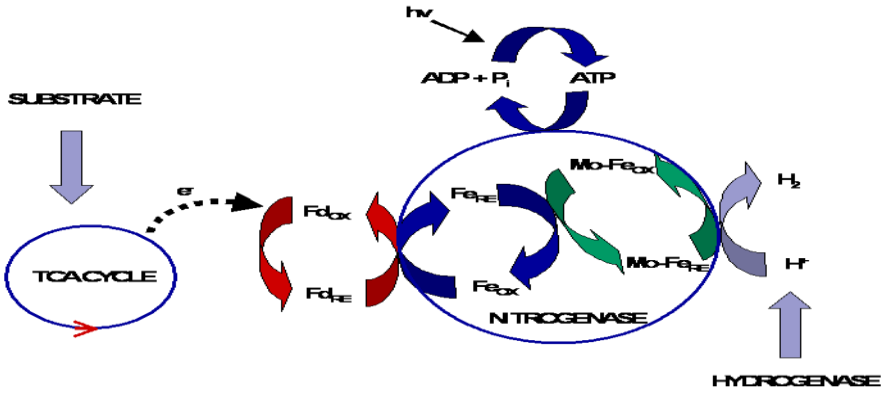


Figure 1.2 Mechanisms of Nitrogenase and Functional Association With TCA Cycle and Hydrogenase.

Physiological effectors of nitrogenase

Nitrogenase genes are highly regulated at the transcriptional level by sophisticated regulatory networks that respond to multiple environmental stimuli (Dixon and Kahn, 2004). Reversible posttranslational modification of nitrogenases known as switch-off/switch-on was observed in several alphaproteobacteria including *Rhodobacter capsulatus*. The main environmental factors affecting nitrogenase enzyme activity are oxygen, amount of ammonium (fixed nitrogen), light, presence of molecular nitrogen and amount of metal ions (iron and molybdenum). In addition to that, redox conditions of the cells are intrinsic factors that influence the activity of nitrogenase.

Mo-nitrogenase is extremely sensitive to oxygen. It can lead to irreversible damage to the enzyme. Fe-protein was shown to be more sensitive to oxygen inhibition than Mo-Fe protein. Obligated anaerobes fix nitrogen in the absence of oxygen while facultative aerobes shown nitrogenase repression under aerobic conditions (Robert and Brill, 1981). Oxygen also might cause reversible inhibition (switch-off) of nitrogenase similar to ammonium inhibition effect. As a conclusion, nitrogen fixation or hydrogen production has to be performed under anaerobic conditions in anoxygenic photosynthetic bacteria.

Ammonium inhibits nitrogenase activity by the aid of feedback mechanism. Inhibition mechanism involves inactivation of Fe-protein by covalent modification. This feedback inhibition is a property found in PNS bacteria. The modifying group interfere the interaction with MoFe-protein causing catalytic inactivation (Vignais *et al*., 1985).

Levels of nitrogenase control in *R. capsulatus* in response to ammonium are shown in Figure 1.3. Presence of fixed nitrogen source like ammonium in growth medium of the bacteria stimulates reversible inactivation of the nitrogenase. Inhibition started by attachment of ADP-ribose group from NAD+ to an arginine residue in one subunit of homodomeric NifH protein causing inactivation of that protein (switch-off).

The process is catalyzed by an enzyme called dinitrogenase-reductase ADP-ribosyltransferase (DraT). The inactivation effect is reversible and when ammonium is exhausted by cellular metabolism the ADP-ribose group is removed by dinitrogenase-reductase activating glycohydrolase enzyme (DraG). Removing of the ADP-ribose group leads to activation of the NifH (dinitrogenase reductase) enzyme (switch-on) (Huergo *et al*., 2006; Drepper *et al*., 2003).

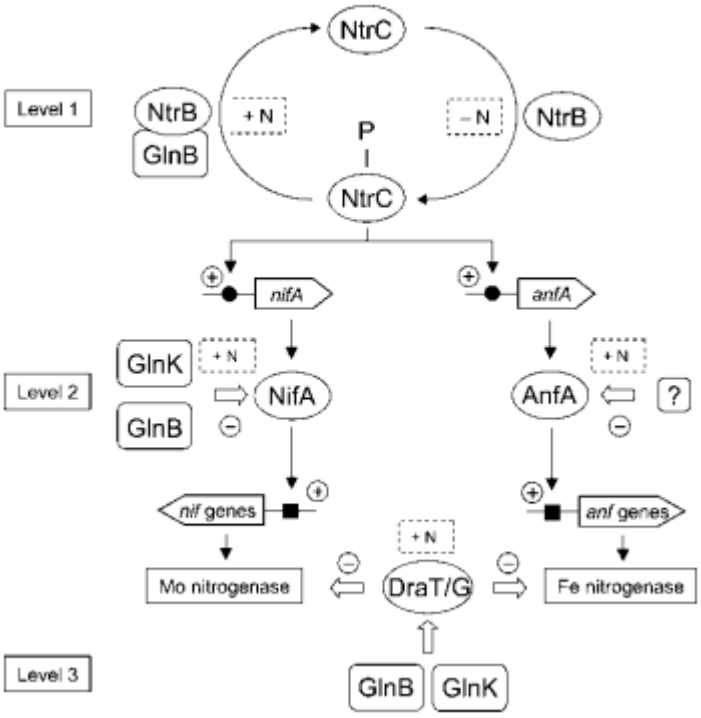


Figure 1.3 Levels of nitrogenase control in response to the availability of ammonium (Drepper *et al*., 2003)

It was shown that double mutation in glnB-glnK resulted in synthesis of active Mo-nitrogenase even in the presence of repressive concentrations of ammonium in *R. capsulatus* (Drepper *et al*., 2003).

Synthesis of nitrogenase enzyme does not require light. It might be carried out in the dark under non-inhibitory oxygen conditions. Studies indicated that light strongly stimulates nitrogenase activity of whole cells. Since nitrogenase synthesis is related to the bacterial photophosphorylation activity then it is expected that bacteria with higher phosphorylation capacity will synthesize more active nitrogenase (Reidl *et al*., 1983; Jouanneau *et al*., 1985).

The photophosphorylation capacity of photosynthetic bacteria cells is greater in cells grown under high light intensity than in cells grown under low light intensities (Steinborn and Oelze, 1989). As a result high ATP production rate under good illumination conditions might lead to better hydrogen production activity.

1.4.2 Hydrogenases

Hydrogenases are the second group of key enzymes concerning hydrogen metabolism. They are found in the majority of microorganism having ability to produce molecular hydrogen. In anoxygenic photosynthetic bacteria hydrogenase enzyme catalyzes the reversible oxidation of hydrogen hence it gained the name hydrogen-uptake hydrogenase.

In general, all known hydrogenases (EC 1.12) are Fe-S-containing proteins, and the majority contained nickel. Hydrogenases catalyze the reversible reaction:

$$H_2 \quad \Longleftrightarrow \quad 2H^+ + 2e^-$$

Hydrogenases in various organisms differ in their properties including molecular weight, electron donor-acceptor they react with, cellular organization and other characters. In *R. sphaeroides* and in most anoxygenic photosynthetic bacteria, hydrogenase is mainly in charge of hydrogen consumption but under certain conditions including its Km values and the bacterial preferences they can produce hydrogen (Van Haaster *et al*., 2005).

Several types of hydrogenases were studied and classified according to their metal ions content including [NiFe]-hydrogenases (Figure 1.4), [FeFe]-hydrogenases (Figure 1.3) and Fe-only Hydrogenase. [NiFe]-hydrogenases which have been declared as the most numerous and best studied class of hydrogenases in bacteria (Vignais and Billoud, 2007). It is a type of αβ heterodimer consists of a core with large α-subunit hosting a bimetallic active site [NiFe] and small β-subunit housing [Fe-S] clusters. The small β-subunit transfers the electrons via its [Fe-S] clusters and the large α-subunit catalyze the reaction via its active site which is housing the heterobinuclear [NiFe] metallocenter (Frey, 2002).

Other subgroups of [NiFe] hydrogenases were recognized depending on the sequence analysis of the small and large subunits. They include membrane bound uptake hydrogenases, hydrogen sensors, NADP-reducing, bidirectional NADP/NAD-reducing and energy converting membrane associated hydrogen evolving hydrogenase (Vignais and Billoud. 2007; Kovacs *et al*., 2004; Vignais *et al*., 2001).

Membrane bound respiratory hydrogenases carried out oxidation of hydrogen and then the reduction of quinine occurs. During anaerobic respiration several electron acceptors are in use including NO_3^-, SO_4^{2-}, fumarate or carbon dioxide. During aerobic respiration oxygen is being as the final electron acceptor. Energy is then recovered in the form of proton motive force

after electron passage to the final acceptors. By this way it seems that uptake hydrogenases help to maintain the energy balance of the cell.

The ultimate physiological effectors of uptake-hydrogenase are oxygen, hydrogen and availability of metal ions (iron and nickel). Analysis of transcriptional events was carried out to explore effects of oxygen, hydrogen and nickel on expression levels of [NiFe]-hydrogenases (Axelsson *et al*., 2002; Boison *et al*., 2000). These studies illustrated that increasing level of both hydrogen and nickel caused increase the activity of hydrogen-uptake hydrogenase. It was found that reducing level of oxygen influenced the uptake-hydrogenase activity and the presence of hydrogen triggered expression of some hydrogenases by the virtue of hydrogen sensing regulatory hydrogenase and two-component system. Events of hydrogen effect on hydrogenase expression were examined in detail in *R. capsulatus* (Dischert *et al*., 1999).

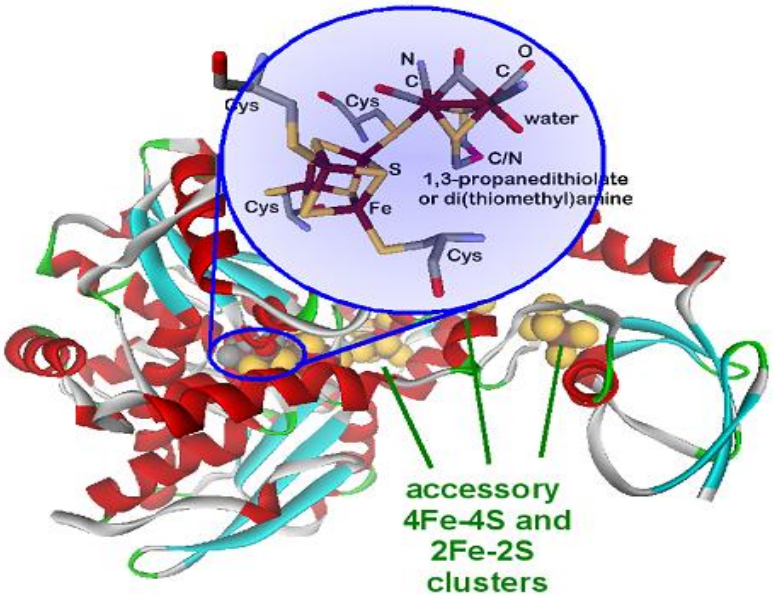


Figure 1.4 Molecular structure of FeFe-hydrogenase from *Chlostridium pasteurianum* I with H-cluster (catalytically active site) and accessory clusters (Peters *et al*., 1998)

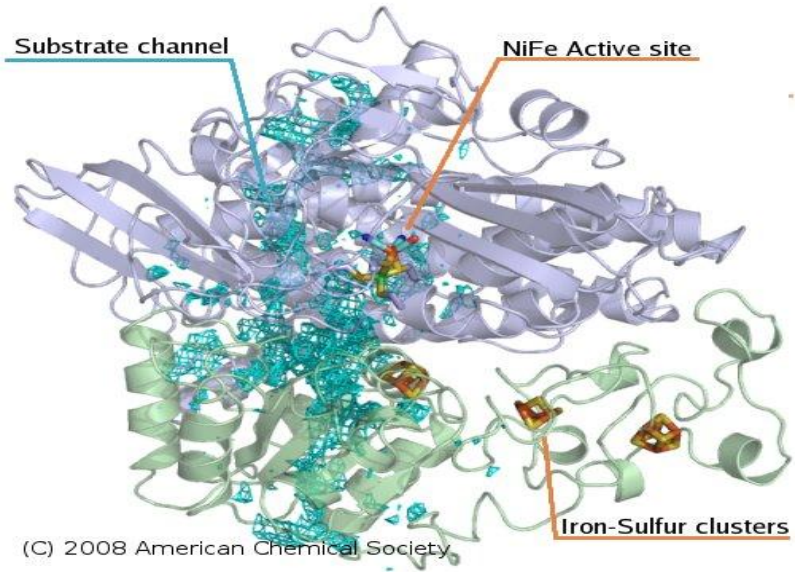


Figure 1.5 Molecular structure of NiFe-hydrogenase from *Desulfovibrio gigas* with Fe-S cluster and heterobinuclear metallocenter (catalytically active site)

1.5 Cell immobilization technology

Long time before immobilization terminology is built up and before this technology was admitted as a topic for planned research, immobilization of whole cell microorganisms was being employed industrially in the microbial production of vinegar using wood shavings overgrown with bacteria, and in the trickling filter or percolating process for wastewater treatment.

A biocatalyst whether it was intact whole cells or chemical compounds extracted from living cells (as enzymes) is described as immobilized if its motility has been restricted by chemical or physical means. So, whole cell immobilization was defined as "the physical confinement or localization of intact cells to a certain region of space with preservation of some desired catalytic activity" (Karel *et al*., 1985). According to that, immobilization process often mimics what occurs naturally when cells grow on surfaces or within natural structures to form natural biofilms.

Interest with immobilization techniques started at early times of the 20th century when yeast invertase enzyme contacted with active charcoal. The contacted charcoal with the invertase enzyme was found to break down sucrose (Nelson & Griffin 1916). This work was the first

known published report about enzyme immobilization. No further attention was paid for such work till 1948 when a report was published about immobilization of urease by treatment with alcohol and common salt (Sumner, 1948).

During 1950's (Grubhofer & Schlieth) as well as Manecke and others demonstrated that specifically acting synthetic polymers could be used to bind active proteins. 1960's showed dramatic increase in publications reflected the world-wide interest in immobilization technology. The first industrial application was in 1969 by using immobilized aminocyclase enzyme in a continuous process for optical resolution of DL-amino acids (Chibata *et al*., 1972).

The first enzyme engineering conference held in USA at 1971 focused on immobilized enzymes. This aroused interest continued till today. Up to the 1970's single immobilized enzymes were in concern and then interest was further extended for more complex systems as organelles and whole living cells Around the end of 1970's not only microorganisms were involved in that technology but also cells from plant- and animal tissue cultures were immobilized. Table 1.3 illustrates the most important events in the history of immobilization technology.

Immobilization of microbial cells in biological processes can occur either as natural phenomenon or through artificial process. The attached cells in natural habitat exhibit significant growth. The artificially immobilized cells are allowed restricted growth. Various immobilization protocols and numerous carrier materials were tried.

The cell immobilization process has also triggered the interest in bioreactor design. Immobilized whole microbial cells and/or organelles eliminates the often tedious, time consuming, and expensive steps involved in isolation and purification of extracellular products. It also tends to enhance the stability of the enzymes and the whole living cells.

Table 1.3 Most important events in the history of immobilization technology

Step	Date	Description	Typical process
I	1815	Empirical use without knowledge of details of immobilization.	Trickling processes of acetic acid & waste-water treatment
II	1969	Simple one-enzyme reactions without cofactor regeneration	Production of L-a.a, isomerization of glu.
III	1985	Two-enzyme reactions including cofactor regeneration	Production of L-a.a in membrane reactors

Immobilization technology applied for microbial cells has advantages over suspended cultures and in the same time it has limitations. The most encountered advantages of immobilization techniques over suspended cultures are summarized as the following:

1) Immobilization provides high cell concentration in the process.

2) Immobilization provides cell reuse and eliminates the costly processes of cell recovery and cell recycling.

3) Immobilization eliminates cell washout problems at high dilution rates occurred during continuous suspended cultures

4) Combination of high cell concentrations and high flow rates allows high volumetric productivities.

5) Provides favorable micro-environmental conditions for the immobilized cells

6) Improves genetic stability

7) Protects against shear damage which may caused during mixing or aeration of the reactor.

Limitations for applying immobilization techniques could be encountered in the following items:

1) Often the product of interest has to be excreted from the cell

2) Complications with diffusion limitations

3) Control of microenvironment conditions is difficult due to heterogeneity in the system

4) Growth and gas evolution can lead to disruption of the immobilized matrix

1.6 Methods applied for immobilization technology

The most widely methods applied for immobilization technology includes the following:

- Adsorption
- Covalent bonding
- Cross-linking
- Entrapment
- Encapsulation

Adsorption was the oldest reported immobilization technique as invertase enzyme was adsorbed to active charcoal (Nelson & Griffin 1916). The first known applied immobilization of whole cells by adsorption is the adsorption of bacterial cells on wood shavings during industrial production of vinegar (Hartmeier 1988). As an example of adsorption in medical applications is

binding of Escherichia coli cells an ion exchange resin (Ruggieri, *et al*., 1986). Microbial cells were immobilized by adsorption on different supports like kieselguhr, wood, glass ceramic, plastic materials (Hartmeier 1988).

The adsorption phenomenon is based on electrostatic interactions (van der Walls forces) between the charged support and microbial cell. The actual charge on support surfaces is still unknown and this limits the proper choice for microbial attachment. The charge on the cell surface and the composition of cell wall carrier composition will also play a predominant role (Hartmeier 1988).

Carrier properties greatly influence cell-support interaction. All glasses or ceramic supports are comprised of varying proportions of oxides of alumina, silica, magnesium, zirconium, etc. which result in bond formation between the cell and the support. Several procedures of cell adsorption based on pH dependence are reported (Hartmeier 1988).

The second immobilization method is applying covalent bonding techniques. Covalent bond is built between activated inorganic support and cell in the presence of a binding (crosslinking) agent. For covalent linking, chemical modification of the surface is necessary (Hartmeier 1988).

During cross-linking methods microbial cells are immobilized by cross-linking each other with bi- or multifunctional reagents such as glutaraldehyde, toluenediisocyanate (Hu 1986). The toxicity of the chemicals used for cross-linking obviously imposes limitations for the general applicability of these procedures (Bucke and Brown 1983).

The most extensively studied and applied method in cell immobilization for industrial purposes is the entrapment of microbial cells in polymer matrices and absorption to a surface (Bucke and Brown 1983). Entrapment in gel and absorption to surfaces provide the most approximate circumstances in which cells might find themselves in the nature. It was concluded that entrapment within gel is proven to be probably the most successful means of immobilizing cells (Bucke and Brown 1983).

1.7 Hydrogen production by immobilized bacteria

Whole cell immobilization was defined as "the physical confinement or localization of intact cells to a certain region of space with preservation of some desired catalytic activity" (Karel *et al.*, 1985), and is a process that often mimics what occurs naturally when cells grow on surfaces or within natural structures.

Matrices used for cell entrapment for hydrogen production purposes include agar, alginate, carrageenan, cellulose and its derivatives, collagen, gelatin, epoxy resin, photo cross-linkable resins, polyacrylamide, polyester, polystyrene and polyurethane; see Table 1.4. Entrapment was the most widely used method for immobilization and agar was the most used gel for entrapment while acrylamide gel was used successfully for the chemotrophs (Sasikala *et al*., 1996).

All reports point to the long term stabilization of immobilization process. Many data are available on the enhancement in rates of hydrogen evolution by immobilization, with a 2 to 10-folds increase in the hydrogen evolution rate reported (Vincenzini *et al*., 1982; Felten *et al*., 1985 ; Aredelean *et al*., 1989 ; Sasikala *et al*., 1990a ; Singh *et al*., 1990).

Hirayama *et al*.,(1986) indicated that both carrageennan and alginate became fragile in about a week when used as cell immobilization matrices. They also concluded that acrylamide gel and photo-cross-linkable resin considerably inhibited hydrogen production in spite of stability of the gel. In this study the entrapment technique by using agar matrix has been applied for immobilization of photosynthetic bacteria for hydrogen production.

Throughout the coming texts bio-hydrogen production by different immobilization method including immobilization within agar matrix will be reviewed. In all the studied cases, hydrogen evolution rates by free cells started to decline and completely ceased after some time while immobilized cells continued to produce hydrogen at the same rates for a longer time.

Continuous photoproduction of hydrogen was demonstrated by using *R. capsulatus* immobilized in carrageenan of at a rate 3 ml/h for 16 days (Francou and Vignas, 1984), agar-entrapped cells *Rhodopseudomonas* sp Miami PBE 2271 at the same rate over 10 days (Mutsunaga and Mitsui, 1982), agar-immobilized *R. rubrum* for 3000 hours with a loss of 60% of activity (Felten *et al*., 1985), agar-immobilized *Chromatium* over a period of more than 300 hours (Ikemoto and Mitsui, 1984), agar-cellulose fiber immobilized *R. rubrum* B-9 for 60 days (Hirayama *et al*., 1986).

Alginate-immobilized cells of *Rhodopseudomonas* sp. BHU 1 (Singh *et al*., 1990) and *R. sphaeroides* O.U.001 (Sasikala *et al*., 1990) showed a 4-fold increase in hydrogen evolution rate over free cells, whereas a 2- to 10-fold increase was observed in agar (beads)-immobilized *R. rubrum* (Felten *et al*., 1985).

Table 1.4 Production of hydrogen by various bacteria in different immobilization materials (Sasikala *et al*., 1996)

Substrate	Organism	Support	Production rate µmol/h/mg
Water	*Anabaena cylindrical*	Glass beads	0.135 (dry wt.)
	Anabaena	Agar	0.003 (dry wt.)
	Anabaena	k-carrageenan	3.24 (mmol/h/g dry gel)
	Mastigocladus sp.	PVA	0.1 (µA/µg chl/cm^2)
	Oscillatoria sp.	Agar	13 (µl/mg dry wt.)
	Chlorela vulgaris	Agar	0.98 (chl.)
	Chloroplast + *Clostridium butyricum*	Agar	6.9 (chl.)
Organic carbon	*Clostridium butyricum*		
Alcohol from waste		Polyacrylamide	0.083 ((dry wt.)
		Agar	0.014 (dry wt.)
		Collagen	0.172 (dry wt.)
Glucose	*Klebsiella pneumonia*	Collagen	0.032 (dry wt.)
		Dialysis membrane	2.1 (protein)
	Azotobacter vinelandi	Ion-exchange resin	60 (wt resin)
		Agar	0.26 (wet wt)
		Cotton gauze	0.48 (wet wt)

Immobilization of *oscillatoria* sp. Miami BG7 within agar caused significantly improved hydrogen production capacity of the bacteria. Rate and longevity of H_2 production compared to free cell suspension. H_2 production was sustained for three weeks with an observed rate of 13 µl H_2 mg dry wt.-1 h-1 (Philips and Mitsui, 1986).

Cells of *Anabaena azollae* were immobilized in synthetic polymer foams and cellulosic hollow fibers. Immobilization enabled continuous H_2 and ammonia production for one month (Park *et al*.,1991).

Anabaena varibilis were immobilized in a hollow-fiber photobioreactor for studying H_2 production and CO_2 uptake. It was observed that increasing rate of CO_2 concentration in the gas phase led to decrease H_2 photoproduction and increased CO_2 uptake by cells, which was accompanied by O_2 evolution (Markove *et al*., 1995).

Several photosynthetic bacteria isolated from enrichment cultures made from the water waste of a water pond of a cool-drink refilling station. The isolated bacteria were investigated for hydrogen production and it was observed that sp, gave the best performance. Immobilized cells of *rhodopseudomonas* isolate were applied for hydrogen production of H_2 from sewage and wastewater (Sunita and Mitra 1993).

Immobilized *Rhodobacter sphaeroides* RV cells on porous glass within a photobioreactor continued producing hydrogen for about 40 days. The rate of H_2 evolution by one ml of porous glass was about 1.4 ml/h (Tsyganov *et al*,.1993).

Substrate consumption and hydrogen production from wastewater by immobilized cells of photosynthetic bacteria was examined by Xu, *et al*., (1995). They use *Rhopseudomonas capsulata* 386 and *Rhodopseudomonas* sp. The workers examined agar and alginate matrices for immobilizing the bacteria. They found that H_2 producing capacity of immobilized cells with agar was higher than that of alginate immobilized cells.

Polyurethane foam was used to immobilize *Rhodobacter sphaeroides* GL-1 in a continuous flow photobioreactor. Rate of H_2 production was observed as 0.21 ml /h ml/foam-matrix under optimal conditions. Efficiency of conversion of lactic acid to H_2 was recorded as 86% (Fedorov *et al*., 1998).

Establishing immobilized system for simultaneous hydrogen production and wastewater treatment was carried out by immobilizing *Rhodospirillum fulvum* and *Rhodobacter sphaeroides* on porous glass. Long-term work time (up to 900 hours) was observed under light induction. They observed a maximum rate of H_2 production (3.6 ml/h/ml porous glass) was recorded by using immobilized *Rhodobacter sphaeroides* GL1. It was observed that 60-80% decrease in the lactate of wastewater concentration was achieved. According to the obtained results, workers concluded that, simultaneous H_2 production and wastewater treatment is a feasible process (Tsygankov, *et al*., 1998 b).

Improving agar immobilization systems for hydrogen production by photosynthetic bacteria in the presence of ammonia has been examined by corporation of cationic polymer with agar matrix. *Rhodobacter sphaeroides* RV was immobilized in agar/chitosan matrix in the presence of ammonium. Presence of chitosan has improved hydrogen production compared to that in control containing only agar. It was concluded that chitosan increased to some extent the resistance to diffusion of positively charged NH_4^+, but had no effect on negatively charged lactate used as carbon source during the work (Zhu *et al*., 1999 a).

Hydrogen production by *Rhodobacter sphaeroides* immobilized in agar gels from the wastewater of tofu factory has been examined. It was found that immobilization protected photosynthetic bacteria from the inhibitory effect of ammonium (Zhu, *et al*.,1999 b).

Clostridium tyrobutyricum JM1, was packed with polyurethane foam as support matrix in a fixed-bed bioreactor and was operated at different hydraulic retention time (HRT) to evaluate its performance for hydrogen production. The reactor achieved the maximal hydrogen production rate of 7.2 l H_2/L/ d at 2 h HRT, where hydrogen content in biogas was 50.0%, and substrate conversion efficiency was 97.4%. (Jo *et al*., 2008).

It has been observed that when whole cells of photosynthetic bacteria (*Rhodopseudomonas sphaeroides* or *Rhodobacter sphaeroides* are entrapped inside the reverse micelles, the H_2 production enhanced from 25 to 35 folds. (Pandey and Pandey., 2007).

Photosynthetic bacteria *Rhodopseudomonas palustris* DSM 131 were immobilized indifferent gel matrices including agar, agarose, k-carrageenan and sodium alginate. Immobilized bacteria were fed with different concentrations of various types of aromatic compounds for examining hydrogen production capacity. Hydrogen yields on the bases of maximum theoretical values were 60%, 57%, 86% and 88% from mandelate, benzoylformate cinnamate and benzoate respectively. It was found that benzoate concentration above 16.5 mM was inhibitory for bacteria. The system carried out hydrogen production for 55 days with the immobilized bacteria. The process repeated five cycles during operation time with slowly decreasing efficiency (Fißler *et al*., 1995).

Hydrogen production capacity of five strains of photosynthetic bacteria has been evaluated by immobilizing bacteria in 4% agar in Roux bottles under continuous illumination conditions. Immobilized bacteria were fed with 88 mM lactate and acetate different concentrations of acetate (21, 42, 84 and 168 mM). The workers observed that *R. sphaeroides* IL106 gave the highest yield of 3.03 mol of hydrogen per mol of acetate when acetate concentration was low (21 mM) and *R. sphaeroides* RV strain given same rate of hydrogen production with acetate as the case of lactate, and the yield was 2.65-2.81 mol of hydrogen per mol of acetate consumed. It was observed that higher concentration of acetate was inhibitory to hydrogen production (Asada *et al*., 2008).

Hydrogen production photosynthetic bacteria *Rhodopseudomonas palustris* CQK 01 immobilized in polyvinyl alcohol-boric acid gel granule was conducted under continuous illumination. In this work particular attention was paid to examine effect of illumination wavelength and light intensity.

Other factors were in focus including substrate concentration, flow rate, pH and temperature effects on hydrogen production rate. Duration of each parameter examination cycle was 24 hours. It was found that immobilized bacteria gained maximum hydrogen production rate of 3.6 mmol/g cell dry weight/h. The optimum illumination wavelength was 590 nm and pH around 7.0 while optimum temperature for gaining maximum hydrogen production rate was 30°C. It was observed that photo-inhibition of the gel granules occurred at 7000 lux (Tian *et al*., 2009).

One of the new fields in hydrogen production by immobilized photosynthetic bacteria is encapsulation bacterial cells within sol-gel matrix. Photobiological hydrogen production by *Synechocystis* sp. PCC 6803 encapsulated in silica sol-gel was examined and monitored for up to five days. Hydrogen production capacity by encapsulated cells was at levels comparable or more than suspended cultures (Dickson *et al*., 2009). Workers concluded their work proofed hydrogen production capacity by sol-gel encapsulated photosynthetic bacteria, but the process is currently in need for more improvements and elucidations including hydrogen production activity for long time (longevity), hydrogen production rates at short periods of time, determining the interactions between gel matrix and encapsulated cells in addition to determining specific responses of these cells to encapsulation process.

Hydrogen production by *Rhodopseudomonas faecalis* RLD-53 immobilized in agar matrix was conducted by using soluble metabolites from ethanol fermentation bacteria *E. harbinense* B49. The maximum yield of hydrogen obtained from glucose fermentation by *E. harabinense* B49 was 1.83 mol.H_2/mol-glucose when glucose concentration was optimized at 9 g/l. It was found that acetate/ethanol ratio was increased by increasing phosphate buffer concentration which is favorable for photo-hydrogen production. The whole process including dark- and photo-fermentation resulted in total maximum value around 6.32 mol-H_2/mol-glucose (Liu *et al*., 2009).

Other applications for immobilized photosynthetic bacteria and microalgae were conducted by many workers. *Chlorella sorokiniana* UTEX 2805 strain co-immobilized with *Azospirillum brasilense* in alginate beads for removing ammonium from wastewater under extreme conditions of light and temperature (Bashan *et al*., 2008). Photosynthetic bacteria *Rhodobacter sphaeroides* S was immobilized in polyvinyl alcohol beads. The immobilized bacteria were employed for treatment of aquarium water by the aid of their denitrifying capacity (Nagadomi *et al*., 1999).

The microalga *Scenedesmus quadricauda* was immobilized in sodium alginate beads and examined for long-term storage and for application in water quality control in fish cultures. The immobilized microalgae maintained their physiological activities after three years of storage. Then they were applied for controlling and removing ammonium from Tilabia fish cultures (Chen 2001).

Alginate-entrapped *Chlamydomonas reinhartii* has been examined for sustaining nitrate consumption process. The workers proofed calcium and barium as gel beads stabilizing agents for nitrate consumption by the immobilized microalgae (Vílchez *et al*., 2001). *Chlamydomonas reinhartii* were also immobilized within alginate beads and applied for bio-sorption of heavy metal ions including mercury (II), cadmium (II) and lead (II) (Bayramoğlu *et al*., 2006).

Two strains of cyanobacteria including *Chroococcus* sp. HH-11 and *Nostoc calcicola* HH-12 were immobilized in separate within alginate matrix beads. The immobilized cyanobacteria were examined for bio-sorption and removal of chromium ions, Cr (VI) from synthetic stock solution. Workers concluded that *Chroococcus* sp. HH-11 cyanobacteria were more efficient in removing chromium ions from the medium (Anjana *et al*., 2007).

Several marine microalgae species were immobilized within calcium alginate beads and applied for removal of copper and cadmium metal ions from synthetic stock medium (Garrido *et al*., 2005).

1.8 Immobilization of mixed cultures and coupled co-cultured bacterial cells for hydrogen production

Significant improvement in biological hydrogen production is achieved by the use of coupled bacterial cells in reverse micelle systems. Two coupled systems (a) *Rhodopseudomonas palustris* CGA009/*Citrobacter* Y19, and (b) *Rhodobacter sphaeroides* 2.4.1/*Citrobacter* Y19 bacteria have been immobilized separately in aqueous pool of the reverse micelles. All reverse micellar systems of coupled bacterial cultures gave encouraging hydrogen production (rate as well as yield) compared to uncoupled bacterial culture (Singh and Krishna., 2008).

Rhodobacter sphaeroides O.U.001cultures were coupled with different amounts of packed cells of *Halobacterium salinarum* S9 or isolated purple membrane fragments. Comparing coupled systems prepared by *Halobacterium salinarum* packed cells or purple membrane fragments with *Rhodobacter sphaeroides* O.U.001 single systems, 4-6 fold of enhancement of hydrogen gas production have been observed in coupled ones. (Elkahlout 2002 and Zabut *et al*., 2006).

The hydrogen production from the organic fraction of municipal solid waste (OFMSW) by anaerobic mixed culture fermentation was investigated using batch experiments at 37°C. Seven varieties of typical individual components of OFMSW including rice, potato, lettuce, lean meat, oil, fat and banyan leaves were selected to estimate the hydrogen production potential. The hydrogen production potentials of rice, potato and lett1uce were 134 ml/ g-VS, 106 ml/g-VS, and 50 ml/g-VS respectively. The hydrogen percentages of the total gas produced from rice, potato and lettuce were 57–70%, 41–55% and 37–67% (Dong *et al*., 2009).

The halophilic bacteria *Halobacterium salinarium* MM22 co-immobilized with *Escherichia coli* in reverse micelles. The ultimate yield of H_2 production was over five times as large for immobilized cells as compared to the free cells (Khan and Bhat., 1990).

Continuous and stable H_2 production by co-immobilized system of *Phormidium valderianum*, *Halobacterium salinarium*, and *Escherichia coli* in a PVA alginate film has been maintained for more than 4 months. The intermittent supply of nitrogen was found to be essential to retain cellular activities (Bagai and Madamwar 1998).

Combination system of *Phormidium valderianum*, *Halobacterium salinarioum*, and *Escherichia coli* co-immobilized in PVA for carried H_2 production for over 60 days under ON/OFF light-dark cycles, 6 hours light and 18 hours dark (Bagai and Madamwar 1999).

Coupled system of *Halobacterium salinarium* and chloroplasts entrapped within reverse micelles recorded five folds enhancement in the rate of H_2 production compared to the aqueous suspension combination where no detectable H_2 was produced (Singh *et al*., 1999).

Hydrogen production by hydrogenase negative photosynthetic bacteria co-immobilized with fermentative bacteria in reverse micelles has been examined. More than two folds increase in hydrogen production was obtained by the use of Hup- mutants instead of wild-type photosynthetic bacteria together with *Citrobacter* Y19. It was concluded that all reverse micellar systems of coupled bacteria cultures gave encouragement for hydrogen production (rate as well as yield) compared to uncoupled bacterial culture (Singh.and Misra 2008).

Co-immobilizing of photosynthetic bacteria *Rhodobacter sphaeroides* RV and Lactobacillus in agar gel matrix has been conducted for hydrogen production by using glucose containing medium. Glucose was converted molecular hydrogen in a yield 7.1 mol of hydrogen per mol of glucose under illumination conditions (Asada *et al*., 2006).

1.9 Contributions of Bio-hydrogen group of METU

Biohydrogen group in Middle East Technical University (METU) has many contributions in Biohydrogen production research work since more than fifteen years. Other works concerning production, utilization and storage of hydrogen are in focus but this statement concerned with efforts and works about biohydrogen production feild.

The photoelectrochemical hydrogen production method was nearly a pioneering work in METU. This method was performed by studying coupling of *H. salinarium* with electrocatalytic system. It was found that the performance of electrocatalytic system was increased by many folds after coupling with *H. salinarium* (Sediroğlu *et al.,* 1999 & Yücel *et al.*, 2000).

A coupling system including *H. salinarium* and *Rhodobacter sphaeroides* O.U.001 has improved hydrogen production capacity of *Rhodobacter sphaeroides* more than 2.5 folds compared to non-coupled single cultures (Elkahlout 2002 & Zabut *et al.*, 2006).

A kinetic model of substrate consumption rates by *Rhodobacter sphaeroides* O.U.001 was developed by using L-malic acid as carbon source and sodium glutamate as nitrogen source. It was concluded that L-malic acid consumption is first order while sodium glutamate consumption rate followed the second order. The developed kinetic model related H_2 production with the amounts of L-malic acid and sodium glutamate (Eroğlu *et al.*, 1999).

Researching efforts in bio-hydrogen production methods continued with *Rhodobacter sphaeroides* O.U.001 by introducing a general scheme of hydrogen production through studying aspects of hydrogen production metabolism and kinetics of hydrogen production by *Rhodobacter sphaeroides* O.U.001 (Koku *et al.,* 2002 & Koku *et al.,* 2003).

Hydrogen production capacity of *Rhodobacter sphaeroides* O.U.001 was examined under different light intensities and different wavelength illumination protocols (Uyar *et al.*, 2007). It was found that lacking of infrared (750-950 nm wavelength) caused 39% decrease in hydrogen production capacity of bacteria while increasing of light intensity enhanced hydrogen production capacity of bacteria until saturation of light intensity at 270 W/m^2.

Genetic and molecular studies were conducted by bio-hydrogen group in METU aimed to examine hydrogen production capacities of genetically modified *Rhodobacter capsulatus* strains (Öztürk *et al*., 2006). Transcriptional analysis of *nif*D, *nif*K and hupS genes were carried out by using *Rhodobacter sphaeroides* O.U.001 which was fed with media containing different concentrations of molybdenum & iron (Kars *et al.*, 2006).

Improving hydrogen production capacity of *Rhodobacter sphaeroides* O.U.001was conducted by genetic deletion of uptake hydrogenase gene throghou site directed mutagenesis. The mutated bacteria had shown 20% increase of hydrogen production capacity compared to wild strain (Kars *et al.*, 2008). Evaluation of hydrogen production capacity by *Rhodobacter sphaeroides* O.U.001 wild type and its mutated (hupSL$^-$ mutant) was conducted by using L-malate and acetate. The mutated strain proofed its priority in hydrogen production but acetate does not seem to be an efficient carbon source for hydrogen production by *Rhodobacter sphaeroides* O.U.001 (Kars *et al.*, 2009).

Outdoor hydrogen production systems by photosynthetic bacteria has been carried out by METU biohydrogen group. Hydrogen production by *Rhodobacter sphaeroides* O.U.001 by using different organic acids in addition to olive mill wastewater was performed in 8 liters flat solar bioreactor under outdoor conditions (Eroğlu *et al.*, 2008).

Hydrogen production under outdoor conditions by *R. capsulatus* has been done by employing 80 liters solar tubular photobioreactor which was operated in fed-batch mode for thirty days while feeding bacteria with acetate as carbon source. The system gave 0.6 mol H_2/mol acetic acid (Boran *et al.*, 2010). Outdoor hydrogen production by using four liters panel photobioreactor and two strains of *Rhodobacter capsulatus* mutated (YO3 hup$^-$ and DSM 1710 wild type) was carried out for 75 days by using the YO3 strain and 55 days by using DSM 1710 strain. The maximum yield at 78% produced by YO3 strain (Avcioğlu *et al.*, 2011).

Biohydrogen production from biomass and waste material was studied by METU biohydrogen group. Hydrogen production by *Rhodobacter sphaeriodes* O.U.001 from olive mill wastewater was carried out (Eroğlu *et al.*, 2004) in addition to optimizing conditions of hydrogen production by using different physical pretreatment methods including pretreatment with clay (Eroğlu *et al.*, 2006; Eroğlu *et al.*, 2008 & Eroğlu *et al.*, 2009) and optimization of light conditions where diurnal lighting cycles were claimed as the best illumination regime for hydrogen production from olive mill wastewater by *Rhodobacter sphaeriodes* O.U.001 (Eroğlu *et al.*, 2010).

Two-stages hydrogen production systems for hydrogen production by photosynthetic bacteria biomass materials and wastewaters have been invistigated. Dark fermentation stage was carried out by activated sludge culture allowed to produce hydrogen from olive mill wastewater and the produced effluent used to feed *Rhodobacter capsulatus* O.U.001 for hydrogen production (Eroğlu *et al.*, 2006).

Thermophilic dark fermentation effluents (DFE) have been evaluated for hydrogen production by *R. capsulatus* DSM 1710. DFEs from glucose, potato steam peels hydrolysate and mollasses were supplied to the photosynthetic bacteria. It was found that the overall yield of the two-step fermentation was higher than the yield of single step dark fermentation (Özgür *et al.*, 2010 a). It was claimed that two –step process caused increasing of hydrogen yield of sucrose from 4.2 mol.H_2/mol sucrose in dark fermentation to 13.7 mol.H_2/mol sucrose by sequential dark and photofermentation (Özgür *et al.*, 2010 b). Supplying DFE of potato steam peels to different strains of photosynthtic bacteraia indicated that yield was depending on the composition of the DFE and on the bacterial strain where *Rhodobacter capsulatsu* YO3 exhibited the highest performance activity (Afsar *et al.*, 2011).

Long-term stability of biomass during hydrogen production by *Rhodobacter capsulatus* YO3 supplied with acetate medium was investigated in outdoor conditions by using panel photobioreactor operated in fed-batch process under outdoor conditions. Stable biomass concentration was obtained under high light intensity and high temperature while the obtained hydrogen yield was around 53% during 60 days operation time. Under low temperature and low light intensity hydrogen yield decreased to 44% during 20 days operation period (Androga *et al.* 2011). It was found that day/light cycles together with temperature flactuation significantly decreased hydrogen production capacity of *Rhodobacter capsulatus* (DSM 1710 and YO3 strains) taking in account that YO3 strain performed better than wild type DSM 1710 strain in outdoor conditions (Özgür *et al.*, 2010 c).

1.10 Scope of the study

This work aims fundamentally to design agar immobilizing system for photosynthetic bacteria and examine the effect of this immobilizing system on hydrogen production capacity of the bacteria (hydrogen production rate, total hydrogen produced, substrate conversion efficiency). Experiments were conducted by using two types of photobioreactor. The first type was (150 and 250) ml cell culture bottles which were used to conduct all the optimization and test experiments and the second type was a scaled up one liter working volume panel photobioreactor designed for the purpose of scaling up experiment.

During the course of this work two different strains of the photosynthetic bacteria *Rhodobacter capsulatus* were employed. The two strains include wild type strain DSM 1710 and a mutated strain named YO3 strain.

Effect of different concentrations of agar (3, 4, 5 & 6%) on hydrogen production by the photosynthetic bacteria was examined for selection of the most appropriate agar concentration to be used during subsequent experiments in this work. During this part of study, stability throughout working period longevity of the immobilizing system was examined. To increase working capacity of the reactor, different concentrations of acetate (40, 60, 80, 100 mM) were tested for optimization of acetate concentration for hydrogen production by immobilized bacteria.

Effect of different types of chemicals on hydrogen production capacity was done including, glycerol (2.5 and 5%), and reducing agent sodium dithionite (0.5, 1, 1.5 mM).

Protecting effect that might be offered by agar to the immobilized bacteria was investigated by using different concentration of ammonium chloride (2.5, 5 and 7.5 mM).

Co-immobilization of the packed cells of the halophilic bacterium *Halobacterium salinarium* with *Rhodobacter capsulatus* has been examined to explore the effect of co-immobilization on hydrogen production capacity of the photosynthetic bacteria *Rhodobacter capsulatus*.

The final part of this work aimed to build an adapted immobilizing system to be used for agar immobilizing bacteria in the panel reactor. Such a system has to reply for the requirements of long time stability and free gas elevation in addition to the possibility of illuminating the reactor from both surfaces.

All experiments during this work have been conducted as indoor experiments under continuous illumination and by using defined media for bacterial growth and hydrogen production.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacterial strains

Two strains of *Rhodobacter capsulatus* including Hup mutant strain named YO3 and a wild type one designed DSM1710 were used in the study. In the YO3 strain the uptake hydrogenase enzyme has been deleted by inactivation of hup SLC operon (Öztürk *et al*., 2006).

2.1.2 Chemicals

The used chemicals included acetate as carbon source and glutamate as nitrogen source for both activation and growing bacteria. Other chemicals included ferric citrate as iron source, potassium chloride, potassium dihydrogen phosphate as buffer and phosphate source, magnesium sulfate as magnesium ion source and sulfur source. Trace elements were added as SL7 solution. Added vitamins included thiamine, niacin, and biotin. Glycerol and sodium dithionite (SDT) were used to test their effect on hydrogen production. Used chemicals are listed in Appendix C, Table C7.

2.2 Methods

2.2.1 Growth of bacteria

Bacteria were taken from the deep freezer (-80°C) and activated by streaking method on nutrient BP agar medium containing 20 mM acetate and 10 mM glutamate (C/N ratio 20/10). Medium prescription is described in Appendix C, Table C1-C4.

The activated bacteria on agar plates then inoculated in the same nutrient medium free of agar but in a suspended culture. Activation period was 48 hours every time. During activation bacteria were illuminated with Tungsten lamps providing 4000 lux and the incubation temperature was 30 °C.

2.2.2 Preparation of the bacterial culture for immobilization

About 50 ml of the activated culture (OD_{660} = 2.00) were transferred to 500 ml transparent screwed bottle containing 450 ml of 20/10 growth medium, Table C.1. Growth temperature was 30 ^{0}C and 4000 lux illumination. After 2-3 days bacteria were collected by centrifugation at 10000 g for 20 min. The pellets were collected and re-suspend again in 10 ml of basal medium.

2.2.3 Immobilization procedure

Agar solutions of appropriate concentration was prepared either with distilled water or with nutrient medium and then autoclaved at 121°C for 20 minutes. The gelled agar was kept at 45° C within water-bath.

Bacterial cell suspension was added to the molded agar solution. The volume of the cell suspension was around 25% of the total amount of the mixture. The final concentration of immobilized bacterial cells was kept as 2.5 mg DCW/ml agar-gel (Zhu *et al*., 1999) or 5 mg DCW/ml agar-gel.

When using 150 ml cell culture bottle, 30 ml of molded agar-bacteria mixture was poured into the bottle and when the used cell culture bottle was 250 ml, 50 ml of molded agar-bacteria mixture was poured into the bottle. Each bottle was kept horizontally at 4°C for 10 minutes. Then gels will be formed and the entrapped bacteria will develop.

For 150 ml cell culture bottles, 120 ml of hydrogen production nutrient medium was added to each bottle and for 250 ml bottle 200 ml of hydrogen production medium was added to each bottle. At starting of each round, agar gelled with the bacteria was washed with basal medium (hydrogen production nutrient medium free of acetate and glutamate). After adding hydrogen production nutrient medium each time, culture was flushed with argon.

Cultures were kept at 30-32° C degrees and illuminated with tungsten lamps. Light intensity was around 4000 lux. Hydrogen gas was collected over water within glass bottles, see Figure 2.1. For gas analysis GC (Gas Chromatography) with TCD (Thermal Conductivity Detector) was used for detection of hydrogen concentration.

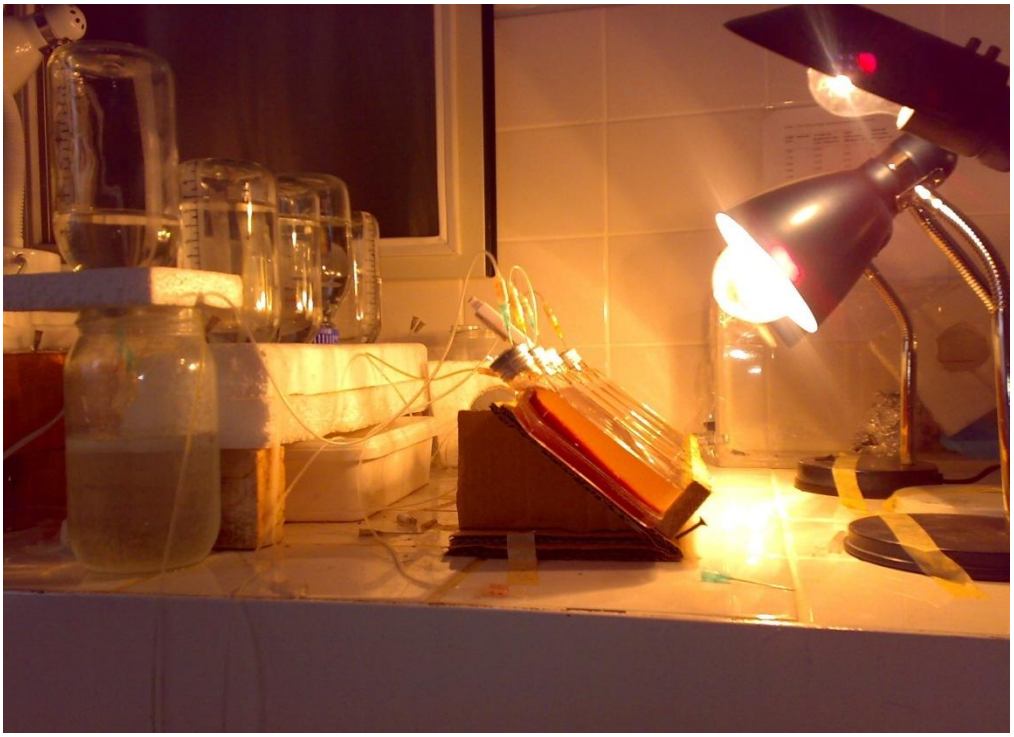


Figure 2.1 Experiment setup for hydrogen production by immobilized *R. capsulatus* in 250 ml cell culture bottles

2.3 Immobilization of bacteria in different concentrations of agar

Agar was used to entrap anoxygenic phototrophic bacterium *Rhodobacter capsulatus* YO3 (Hup mutant) & wild type DSM1710 strains for hydrogen production. Different concentrations of agar (3%, 4%, 5%, & 6%) were used to immobilize bacteria for hydrogen production. The best results were obtained when agar concentration was 3% and 4%.

Agar was gelled by using distilled water with YO3 strain. Agar gelled by distilled water was not successful with wild strain DSM1710, and so experiment switched to agar gelled with nutrient medium (20mM acetate/10 mM glutamate). Growing bacteria and immobilization, illumination and incubation procedures were applied as described in sections 2.2, 2.3 and 2.4. Experimental setup is shown in Figure 2.1.

Doubling of glutamate concentration was applied to examine whether increasing of glutamate will enhance hydrogen production capacity of the bacteria. Increasing of glutamate from 2 mM up to 4 mM was applied with *Rhodobacter capsulatus* DSM1710 (wild strain) and mutated YO3 (Hup- strain).

2.4 Effect of different concentrations of acetate on hydrogen production

To examine the effect of increasing acetate concentration on hydrogen production, all protocols explained in sections 2.2 were applied to grow, harvest and entrap the microorganisms within agar.

To each cell culture bottle, nutrient medium designed for hydrogen production containing 4 mM glutamate was added while the acetate concentration was varied as 60, 80 and 100 mM. The cell culture bottles were divided into three duplicate groups. One type of medium with definitive acetate concentration was used with each doubled group for three rounds R1, R2 and R3.

At the start of each round the cell culture bottle was rinsed with basal medium (nutrient medium without nutrients) two times, filled with the nutrient medium and purged with argon gas for 3-5 minutes to create anaerobic conditions.

Each culture was illuminated and incubated as described in sections 2.2 and 2.4. Gas was collected by water displacement in scaled glass bottles and analyzed by gas chromatography. Organic acids were analyzed by HPLC (High Performance Liquid Chromatography).

2.5 Effect of increasing bacterial concentration immobilized in agar on hydrogen production

Culture preparation and immobilization methods were applied as in sections 2.2, 2.3, and 2.4. Glutamate concentration was demanded as 4 mM and the methods which were used in section 2.6 have been applied. During entrapment of bacteria within agar gel, bacterial concentration was increased up to 5 mg DCW/ml agar gel. Different concentrations of acetate were used as described in section 2.3.

2.6 Effect of glycerol on hydrogen production

Culture preparation methods were applied as described in previous sections. 1.25 ml and 2.5 ml of sterilized glycerol was added to the bacterial suspension and mixed with agar to create final 2.5% and 5% glycerol within the bacteria-agar gel. Bacterial concentration was used as 5 mg DCW for each strain.

For this study the nutrient medium used was containing 60 mM acetate and 4 mM glutamate. The cell culture bottles containing the bacteria-agar gel was provided with 200 ml of the described nutrient medium and divided into doubled groups for each glycerol concentration for both strains. Incubation and illumination were achieved as described in section 2.2.3. Gas collection and analysis as described in previous sections.

2.7 Effect of sodium dithionite on hydrogen production

The effect of reducing agents on hydrogen production was investigated by two strains of photosynthetic bacteria which were grown and immobilized as described in the previous sections. Nutrient medium 60/4 was used as hydrogen production medium for this study. Various

concentrations of sodium dithionite were added to bacteria-gel complex and to the nutrient medium (60/4).

Scanning experiments were conducted with *Rhodobacter capsulatus* YO3 by using 1.5, 3.0 and 4.5 mM of sodium dithionite added to the bacteria-gel complex and to the medium. Depending on the results from this scanning trail the concentrations of sodium dithionite were scanned as 0.5, 1, and 1.5 mM of sodium dithionite. Sodium dithionite was added as described above in this section to the gel and the nutrient hydrogen production medium 60/4.

Cell culture bottles with the immobilized bacteria were divided into doubled groups for each concentration of sodium dithionite for both strains and illumination was applied as described in section 2.2.3. Gas was collected as described in section 2.2 and gas analysis was carried out by using GC.

2.8 Effect of using different concentrations of ammonium on hydrogen production

This part of work was applied to examine effect of ammonium on hydrogen production by the immobilized bacteria and to explore the protection capacity of agar against ammonium concentration in the medium. Bacteria were grown and immobilized as described in the previous sections. Nutrient medium was provided with 60 mM acetate while glutamate was replaced with different concentrations of ammonium chloride (2.5, 5 & 7.5 mM) as sole nitrogen source for hydrogen production during this study.

2.9 Effect of Co-immobilizing packed cells of *Halobacterium salinarium* S-9 on hydrogen production by *R. capsulatus*

The halophilic bacteria *Halobacterium salinarium* were used as packed cells to examine its effect on hydrogen production by photosynthetic bacteria *Rhodobacter capsulatus* (DSM 1710 & YO3 strains).

2.9.1 Growth of *Halobacterium salinarium* S-9

Halobacterium salinarium was grown by Large-scale method, which has been previously developed by Sediroglu (1997) and El-Bashiti (1998) in BioFlo1000 Adaptable Bioreactor having 2 liters capacity (New Brunswik Scientific). The bacteria have been taken from agar slants (preserved stock), inoculated into 5 ml of the growth medium and incubated overnight at 39ºC in shaker incubator. The culture was shaken at 120 rpm and illuminated with 100-watt lamp from 30-40 cm of distance. The 5 ml of the overnight grown samples have been inoculated into 250 ml flask containing 100 ml of growth medium and incubated for 3-4 days inside incubator. Mixing of the culture was achieved by magnetic stirrer. The growth medium in the fermentor, 2L volume, was inoculated with 60 ml of the activated culture.

The optimum growth and BR production was achieved according to (El-Bashiti, 1998) at 300 rpm stirring rate and 0.3 L/min, aeration. The temperature was adjusted at 39ºC. BioFlo 1000 Adaptable fermentor contains three modules consisting of vessel assembly, temperature measurement and control module and pH measurement and control module. Vessel assembly includes a 2 littered borosilicate water-jacket glass vessel, a quick release headplate, a drive shaft assembly with stainless steel ball bearings and Viton PTFE lip seals, one 6-blade Rushton impeller, heater blanket, Pt-100 temperature probe, pH probe, flow meter glass tube and stainless steel headplate with 7 x 12 mm and 5 x 6.35 mm.

The temperature measurement and control module measures and controls the temperature over the range of 0°C to 50°C. A digital display indicates the temperature of the medium to within 0.1°C. The temperature is adjusted by pressing the set button on the front panel and the control knob is used to set the required temperature, which is shown on the display. Outputs from the temperature and control module supply the heater for heating the vessel and operate a solenoid to allow cooling liquid through the cold finger to cool the unit.

The pH monitoring and control module records the pH of the vessel contents with a pH probe. The pH is displayed on a digital display. The module is fitted with two peristaltic pumps, which are connected for an alkali and acid sources. Controls are provided on the front panel to select the upper pH and lower pH set point. The module compares the preset value with the pH measured and if the fermentation is acidic the alkali pump is activated to correct the pH or if alkali, the acid pump operates.

The bacterial growth was followed by measuring the optical density at 660 nm using Spectrophotometer (Hitachi U-2000 Double Beam, Japan). The steady increase in biosynthesis

of bacteriorhodopsin was observed by following the change in absorbance at 570 nm. The formation of purple membrane reached to a maximum at the end of exponential growth phase as indicated by Oesterhelt and Stoeckenius (1974).

After 120-140 hours of growth, the cells were collected by centrifugation at 13000 g for 30 min in Sigma 4K15 centrifuge (12256-H rotor). The supernatant was discarded and the cell pellets were suspended in minimum amount of 4 M NaCl solution and kept in the deep freezer (-20°C) until use.

2.9.2 Preparation of co-immobilized packed cells of *H. salinarium* S-9 and *Rhodobacter capsulatus*

Packed cells suspension was taken from the freezed stock. The hydrogen production medium was mixed with the packed cells (Pc.) and shacked well until making the homogenous solution. Determination of BR amount was carried out by UV-visible spectrophotometer at 570 nm wavelength. Appropriate amount of BR in the packed cells (2.4 μmol/bottle) was mixed thoroughly with 40 ml molten agar (4%) and then *Rhodobacter capsulatus* concentrate was added to the mixture and allowed to be mixed properly. The new mixture which has 50 ml final volume was poured into 250 ml cell culture bottles. The bottles then passed to refrigerator in a horizontal position and allowed to be solidified at 4°C for 10 minutes. For preparing bottles for hydrogen production they were treated as described in section 2.2.3.

2.10 Immobilization of photosynthetic Bacteria in Agar in Panel Photobioreactor

Using panel reactor for hydrogen production by immobilized bacteria required new design modifications to adapt the design of panel reactor for such application. The employed panel reactor is made of two plexiglass panels which sandwiched a frame made from PVC, Figure 2.2. The frame and the two panels are fitted together as one piece by metal screws which were positioned in from each other by regular distances, see Figure 2.2. The reactor has 1 liter working volume.

Two different designs were tried to immobilize bacteria during hydrogen production by panel reactor.

The first design was a type of four wells which were made on the inner side of one of the two plexiglass panels. The agar-bacteria mixture was poured inside those wells; see Figure 2.2. This design was applied by using 5 mg DCW of *Rhodobacter capsulatus* YO3 strain only. Bacteria were fed with hydrogen production medium containing 40 mM of acetate and 4 mM of glutamate.

The second design (Figure 2.3) which was employed for the purpose of bacterial immobilization was a form of double frame made of glass. The frame parts were sandwiching a network cloth (Tulle) made of nylon. The frame parts and the cloth network were assembled together by using thermal silicon glue (Figure 2.4). The aim of using the cloth network was to provide a supporting medium for agar gel.

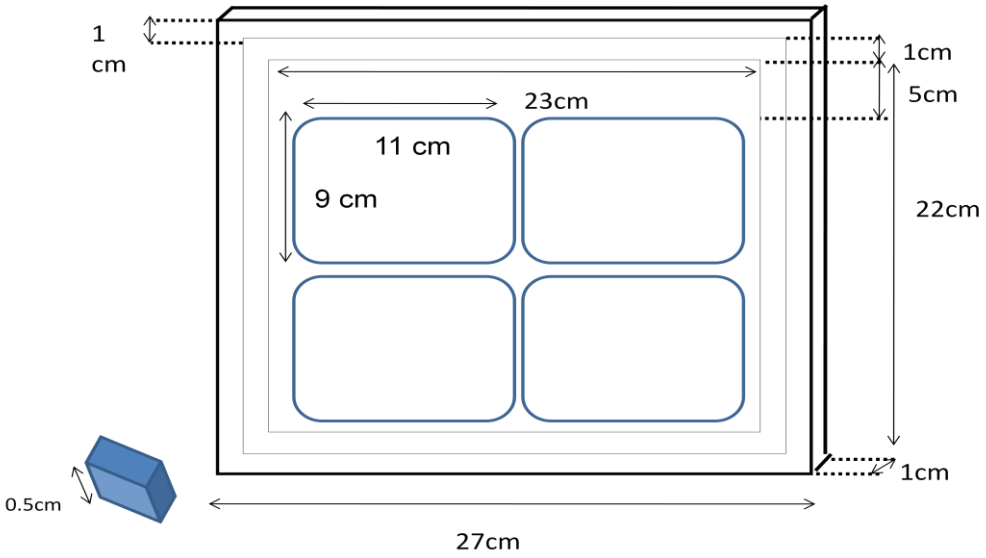


Figure 2.2 A drawing represents the structure of panel photobioreactor used for hydrogen production by immobilized bacteria (first design)

The second design was applied with both strains of bacteria employed in this work, YO3 strain and DSM 1710 strains. In case of YO3 strain 5 mg DCW/ml agar was applied while in case of DSM 1710 strain 2.5 mg DCW/ml agar was used.

The same steps were applied in preparation of entrapped bacteria in agar gel for panel photoreactor. In case of the first design, bacteria-agar complex was poured in the four wells located in one face of the panel reactor and then allowed to be gelled in the refrigerator for 10 minutes as described when using Roux bottles. The four wells together were filled with about

160 ml of the gel containing bacteria. The reactors then closed and filled with hydrogen production medium described in previous section and then the reactor flushed with argon for 15 minutes.

In case of using the second design, the frame was put horizontally on a glass panel and then 200 ml of bacteria-agar complex was poured until the inner part of the frame is completely filled with the complex. Another glass panel is used to cover the molten agar inside the frame to prevent flooding out while passing the frame for cooling in the refrigerator. The reactors then closed and filled with hydrogen production medium described in previous section and then the reactor flushed with argon for 15 minutes.

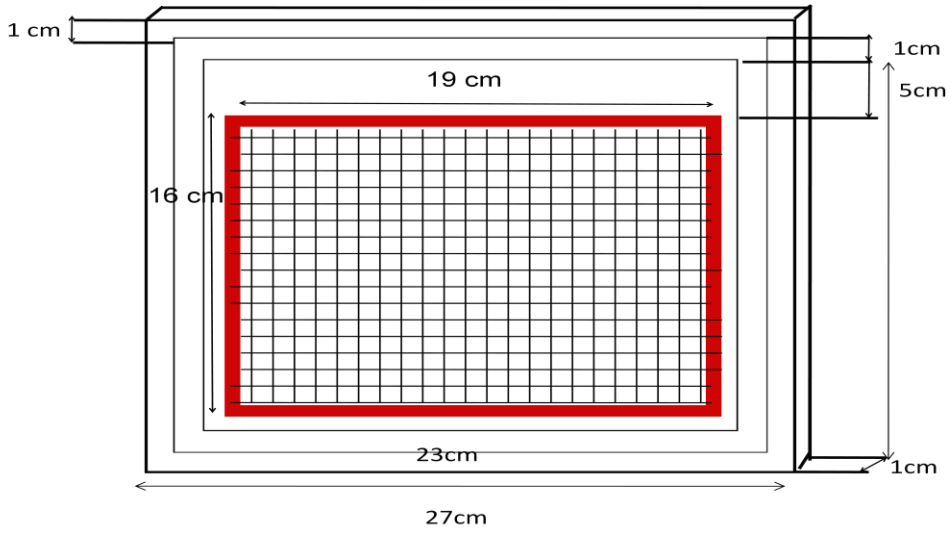


Figure 2.3 A drawing represents the structure of panel photobioreactor used for hydrogen production by immobilized bacteria (second design)

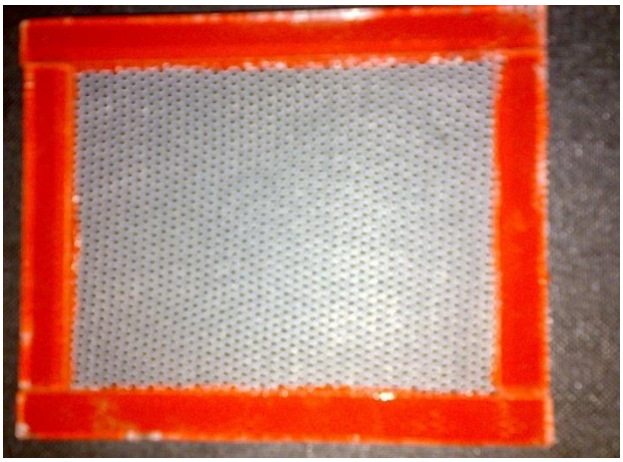


Figure 2.4 Structure of the frame used for immobilizing bacteria in the panel photobioreactor (second design)

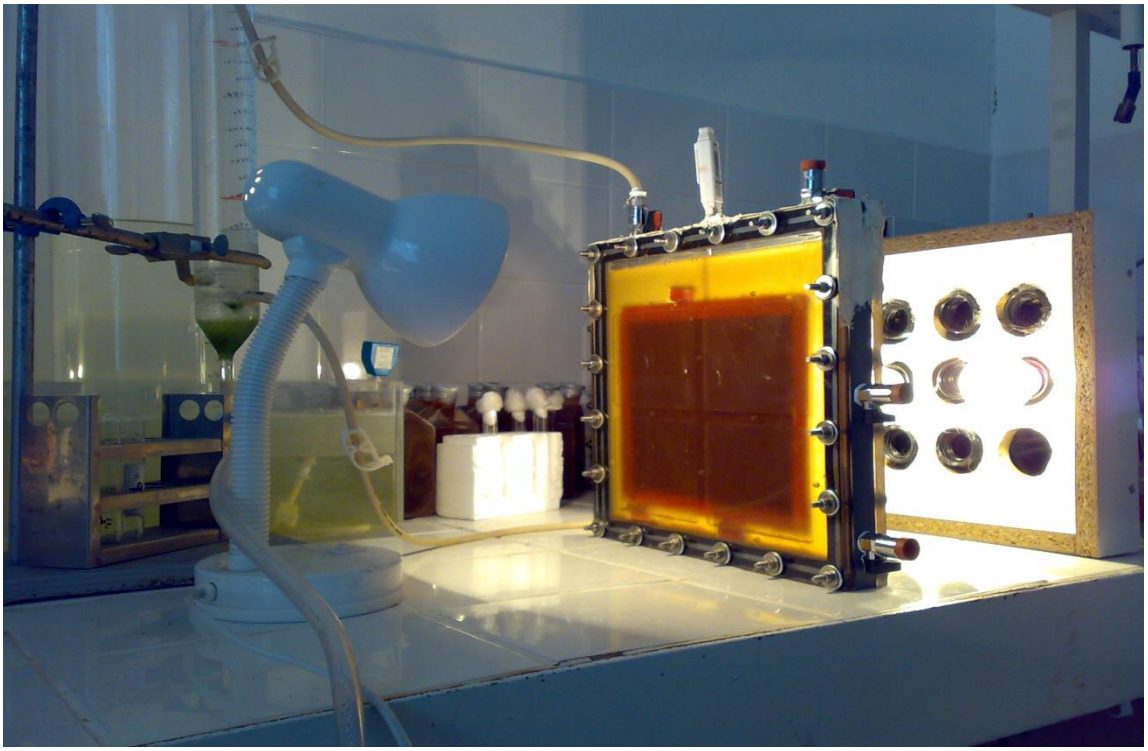


Figure 2.5 Experiment setup for hydrogen production by immobilized *R. capsulatus* panel photobioreactor

Reactors were illuminated either from one or two sides with tungsten lamps providing an average light intensity 4000 lux and incubated at 30°C. Hydrogen collection was achieved by water displacement in one liter bottles (Figure 2.5).

2.11 Analysis and sampling

Organic acids analysis was conducted to check acetic acid remains and formic acid formed during hydrogen production. Around one to two mililiters of hydrogen production medium were taken periodically (24 hours or 48 hours) from the reactots by using sterile syringes. The samples then centrifuged at 10000 g by using Sigma 4K15 centrifuge The supernatant is taken for filteration by using one milliliter syringe and 0.25 µm Millipore filters. The filtered aliquots then subjected for HPLC analysis. The HPLC instrument is Varian Prostar typeprovided with computerized system using prostar workstation software. For analysis of acetate remains and formate 100µl of the sample was injected automatically to the injection port. Analysis process was operated at 0.3ml/min flow rate by using 0.008N sulfuric acid as mobile phase. Run time was adjusted at 25 minutes while temperature was 35°C. The system used MetaCarb 87H coloumn , 300x7.8 mm. For detection PDA detector was employed at 210 nm wavelength.

Gas composition analysis was applied by using Agilent type GC system, 6890N version N.05.06100 provided with computerizing system and network analysis software. For analysis of gas composition 100 µl was aspirated from the the gas collection bottles and then injected into the injection port specified for gas analysis. The instrument was operated at 140°C oven temperature for 9 minutes run time and flow rate at 22.3 ml/min. The GC system is provided with Packed coloumn of the type SUPELCO 12390U, CARBOXEN 1000. The inert gas argon was employed as carrier gas.

CHAPTER 3

RESULTS

3.1 Optimization of immobilization conditions of photosynthetic bacteria in different concentrations of Agar

In this study, different concentrations of agar were used to optimize the most acceptable concentration for immobilization of photosynthetic bacteria in agar. Four agar concentrations (3%, 4%, 5% and 6%) were tested with the two bacterial strains (DSM 1710 and YO3).

3.1.1 Immobilization of *Rhodobacter capsulatus* DSM1710 in different concentrations of agar and effect of doubling glutamate concentration from 2mM to 4 mM.

In this part of study the four applied concentrations of agar were used with *Rhodobacter capsulatus* DSM1710 strain (wild type). This part of study continued for about 1191-1428 hours (approximately 50-60 days) and covered seven rounds. During the first four rounds of the experiment acetate concentration was 40 mM and glutamate concentration was 2 mM. From fifth to seventh round, glutamate concentration was increased up to 4 mM. Figure 3.1 to Figure 3.8 showing the results of total hydrogen produced for each concentration of agar and pH change during each process.

3.1.1.1 Hydrogen production by *Rhodobacter capsulatus* DSM1710 immobilized in 3% agar

In this experiment 3% of agar was used to immobilize the bacteria. The concentration of bacteria used to form the bacteria-agar complex was 2.5 mg DCW/ml of agar.

The volume of the cell culture bottle was 150 ml and the agar volume was 30 ml. The working volume of the bottle was 120 ml filled with hydrogen production nutrient medium with 40 mM acetate concentration and 2 mM glutamate. As mentioned above glutamate concentration was increased to 4 mM during fifth, sixth and seventh rounds. Incubation and illumination conditions were applied as described in materials and methods.

Operation of the reactor continued for about 1428 hours (approximately 60 days) and covered seven rounds. At the beginning of each round, the bottle was washed with basal medium and after filling with hydrogen production nutrient medium, it has been flashed by argon gas as described in material and methods.

Results of total hydrogen produced by immobilized *R. capsulatus* DSM 1710 in 3% agar are shown in Figure 3.1 while mode of pH change during the experiment is illustrated in Figure 3.2. Figure 3.1 shows that total hydrogen produced at the end of round R1-R4 ranged from 180-220 ml of hydrogen per bottle where glutamate concentration was 2 mM; Table A.1

After increasing glutamate concentration to 4 mM, total hydrogen produced during rounds R5-R7 ranged from 260-340 ml of hydrogen per bottle; Table A.2. Mode of pH change during all rounds of the experiment has shown stability trends between 6.8, the initial pH value, and 7.5 which was the highest pH value registered during round R7; see A. 3.

3.1.1.2 Hydrogen production by *Rhodobacter capsulatus* DSM1710 immobilized in 4% agar

In this experiment 4% of agar was used to immobilize the bacteria. The concentration of bacteria used to form the bacteria-agar complex was 2.5 mg DCW/ml of agar. The operation conditions were same as explained in section 3.1.1.1. As mentioned above glutamate concentration was graded up to 4 mM during fifth, sixth and seventh rounds. Incubation and illumination conditions were applied as described in materials and methods.

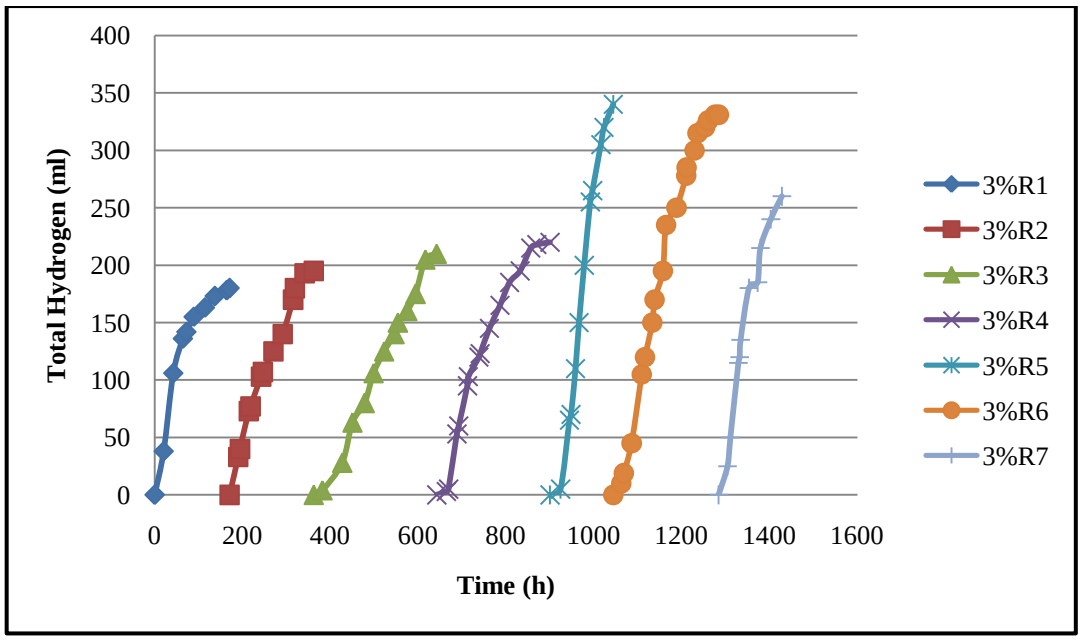


Figure 3.1 Total Hydrogen Production by *R. capsulatus* DSM1710 immobilized in 3% agar during all rounds

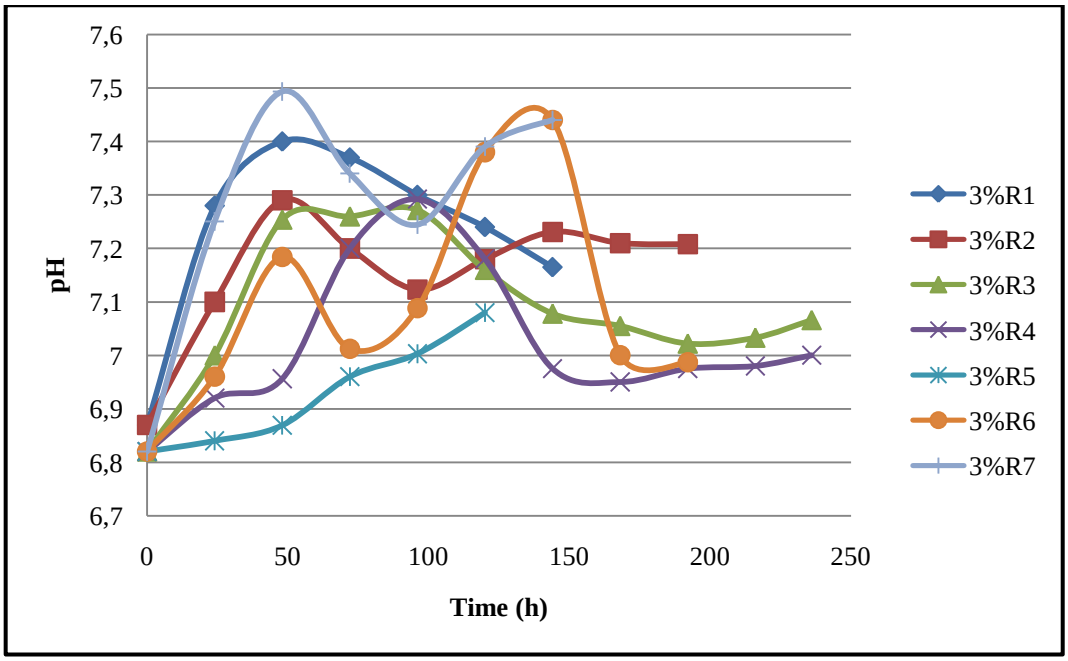


Figure 3.2 Mode of pH change during Hydrogen Production by *R. capsulatus* DSM1710 immobilized in 3% agar during all rounds

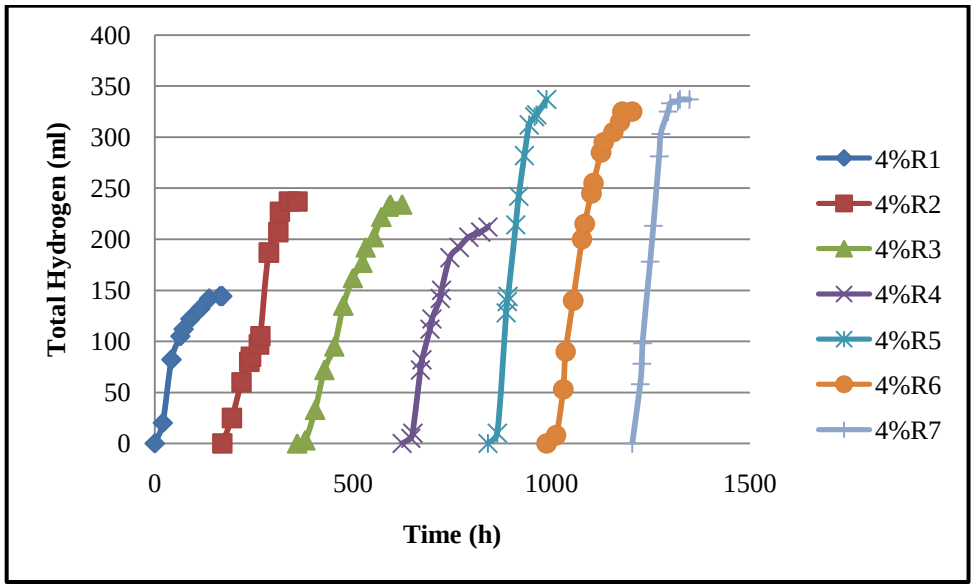


Figure 3.3 Total Hydrogen Production by *R. capsulatus* DSM1710 immobilized in 4% agar during all rounds

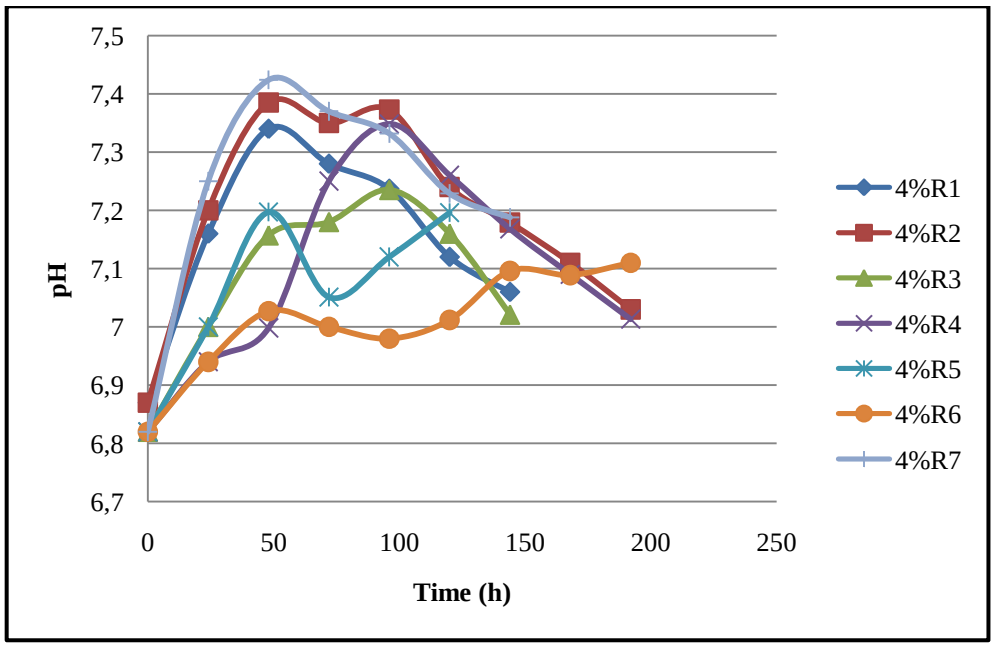


Figure 3.4 Mode of pH change during Hydrogen Production by *R. capsulatus* DSM1710 immobilized in 4% agar during all rounds

Operation of this experiment continued for about 1220 hours (approximately 51 days) and covered seven rounds. At the beginning of each round, the bottle was washed with basal medium and after filling with hydrogen production nutrient medium, it has been flashed by argon gas as described in material and methods.

Total hydrogen produced at the end of round R1-R4 ranged from 144-237 ml of hydrogen per bottle where glutamate concentration was 2 mM; Table A. 4 and Figure 3.3. After increasing glutamate concentration to 4 mM, total hydrogen produced during rounds R5-R7 ranged from 325-337 ml of hydrogen per bottle; Table A. 5 and Figure 3.3. Mode of pH change during all rounds of the experiment has shown stability trends between 6.8, the initial pH value, and 7.424 which was the highest pH value registered during round R5, Figure 3.4 and Table A. 6.

3.1.1.3 Hydrogen production by *Rhodobacter capsulatus* DSM1710 immobilized in 5% agar

In this experiment 5% of agar was used to immobilize the bacteria. The operation conditions were same as explained in section 3.1.1.1. As mentioned in sections 3.1.1.1 and 3.1.1.1 glutamate concentration was increased from 2 mM to 4 mM during fifth, sixth and seventh rounds. Incubation and illumination conditions were applied as described in materials and methods.

Operation of this experiment continued for about 1347 hours (approximately 56 days) and covered seven rounds. At the beginning of each round, the bottle was rinsed with basal medium and after filling with hydrogen production nutrient medium, it has been flashed by argon gas as described in material and methods.

Total hydrogen produced at the end of round R1-R4 ranged from 130-265 ml of hydrogen per bottle where glutamate concentration was 2 mM; Table A. 7 and Figure 3.5. After increasing glutamate concentration to 4 mM, total hydrogen produced during rounds R5-R7 ranged from 295-312 ml of hydrogen per bottle; Table A. 8 and Figure 3.5. Mode of pH change during all rounds of the experiment has shown stability trends between 6.8, the initial pH value, and 7.38 which was the highest pH value registered during round R1, Figure 3.6 and Table A. 9.

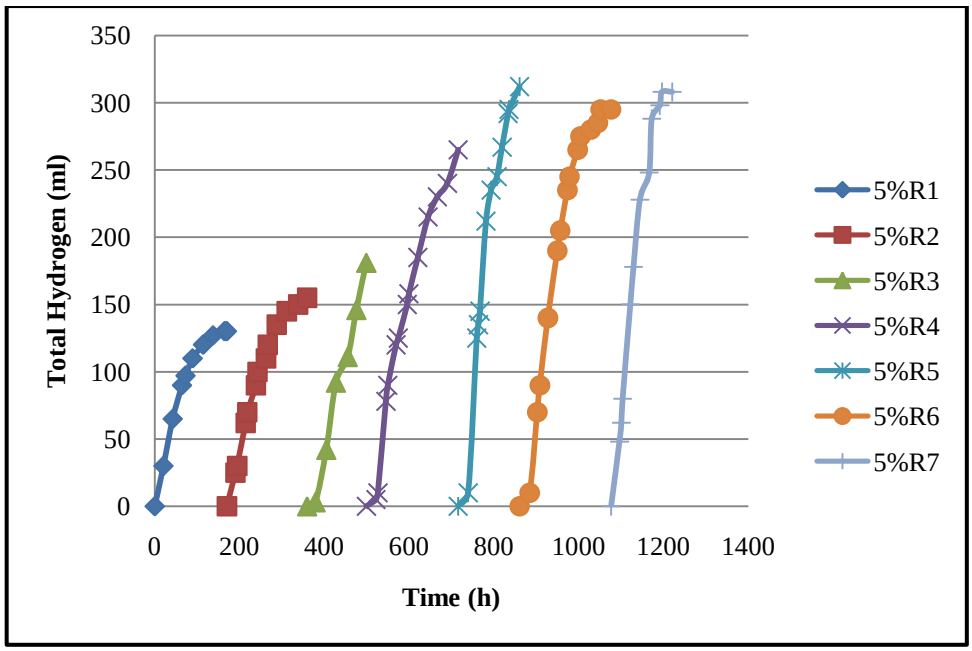


Figure 3.5 Total Hydrogen Production by *R. capsulatus* DSM1710 immobilized in 5% agar during all rounds

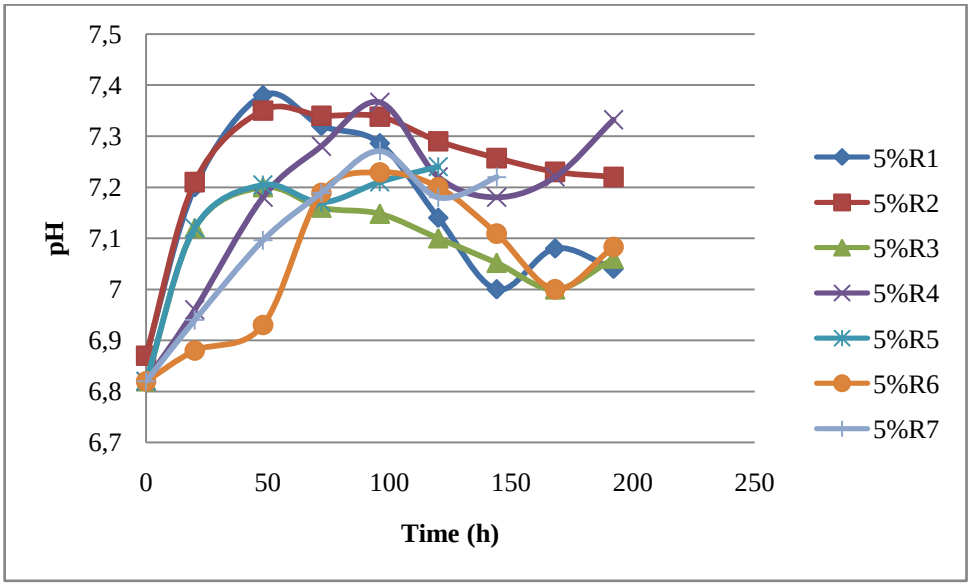


Figure 3.6 Mode of pH change during Hydrogen Production by *R. capsulatus* DSM1710 immobilized in 5% agar during all rounds

3.1.1.4 Hydrogen production by *Rhodobacter capsulatus* DSM1710 immobilized in 6% agar

In this experiment 6% of agar was used to immobilize the bacteria. The operation conditions were same as described in sections 3.1.1.1 and 3.1.1.2. As mentioned above glutamate concentration was graded up to 4 mM during fifth, sixth and seventh rounds. Incubation and illumination conditions were applied as described in materials and methods.

Operation of this experiment continued for about 1420 hours (approximately 60 days) and covered seven rounds. At the beginning of each round, the bottle was washed with basal medium and after filling with hydrogen production nutrient medium, it has been flashed by argon gas as described in material and methods.

Total hydrogen produced at the end of rounds R1-R4 ranged from 143-253 ml of hydrogen per bottle where glutamate concentration was 2 mM; Table A. 10 and Figure 3.7. After increasing glutamate concentration to 4 mM, total hydrogen produced during rounds R5-R7 ranged from 175-288 ml of hydrogen per bottle; Table A. 11 and Figure 3.7.

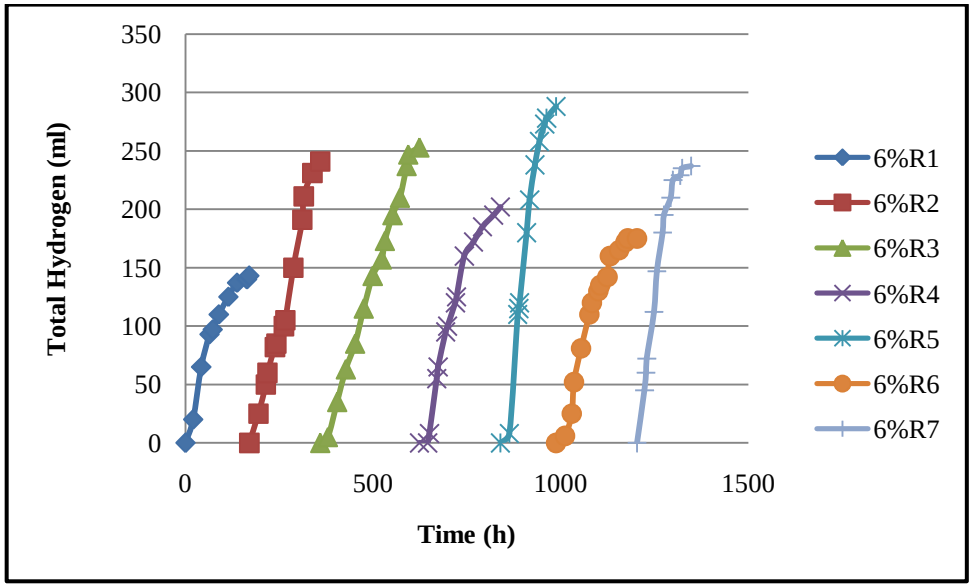


Figure 3.7 Total Hydrogen Production by *R. capsulatus* DSM1710 immobilized in 6% agar during all rounds

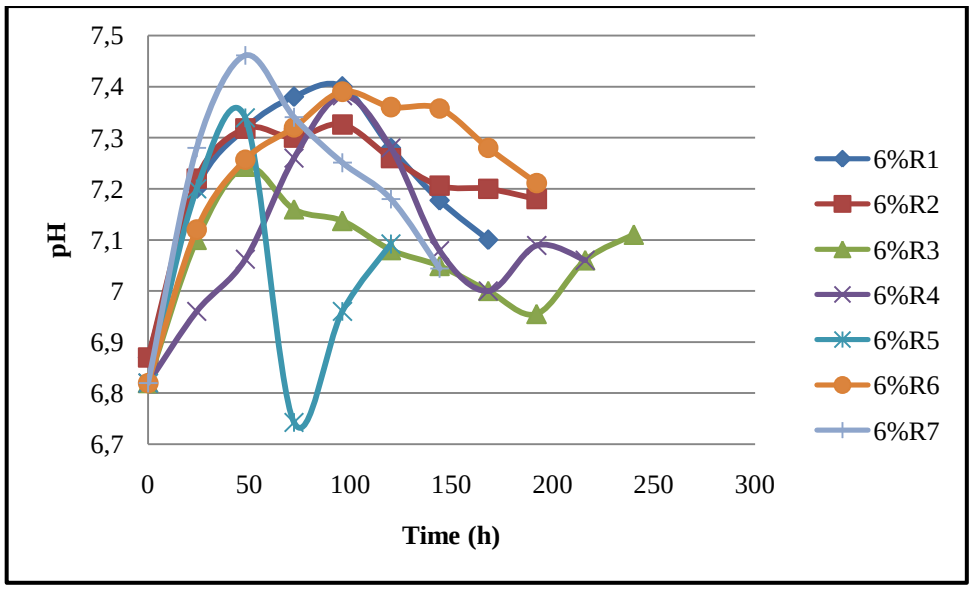


Figure 3.8 pH change during Hydrogen Production by *R. capsulatus* DSM1710 immobilized by 6% agar during all rounds

Mode of pH change during all rounds of the experiment has shown stability trends between 6.8, the initial pH value, and 7.461 which was the highest pH value registered during round R7, Figure 3.8 and Table A. 12.

3.1.2 Hydrogen production by *Rhodobacter capsulatus* YO3 immobilized in different concentrations of Agar and effect of doubling glutamate concentration from 2mM to 4 mM

In this part of study the four applied concentrations of agar were used with *Rhodobacter capsulatus* YO3 strain. This strain is a mutated strain with that has no hydrogen-uptake hydrogenase enzyme.

This part of study continued for about 302-737 hours (approximately 13-31 days). During the first four rounds of the experiment acetate concentration was 40 mM and glutamate concentration was 2 mM. After fourth round and during fifth round, glutamate concentration was increased up to 4 mM.

3.1.2.1 Hydrogen production by *Rhodobacter capsulatus* YO3 immobilized in 3% agar

Bacteria were immobilized in 3% agar. The operation conditions was same as explained in section 3.1.1.1 and 3.1.1.2 The working volume of the bottle was 120 ml filled with hydrogen production nutrient medium with 40 mM acetate concentration and 2 mM glutamate.

Operation of this experiment continued for about 690 hours (approximately 29 days) and covered five rounds. At the beginning of each round, the bottle was washed with basal medium and after filling with hydrogen production nutrient medium, it has been flashed by argon gas as described in material and methods.

At the end of rounds R1-R4 total hydrogen produced was ranging from 160-255 ml of hydrogen per bottle where glutamate concentration was 2 mM; Table A. 13 and Figure 3.9. After increasing glutamate concentration to 4 mM, total hydrogen produced at the end of round R5 reached to 455 ml of hydrogen per bottle. Mode of pH change during all rounds of the experiment has shown stability trends between 6.8, the initial pH value, and 7.48 which was the highest pH value registered during round R5, Figure 3.10 and Table A. 14.

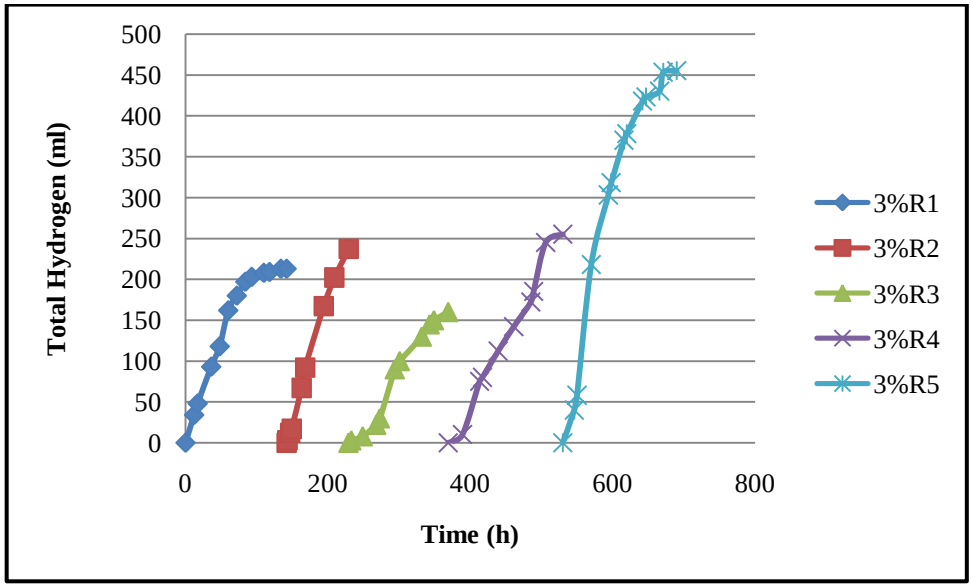


Figure 3.9 Total Hydrogen Production by *R. capsulatus* YO3 immobilized in 3% agar during all rounds

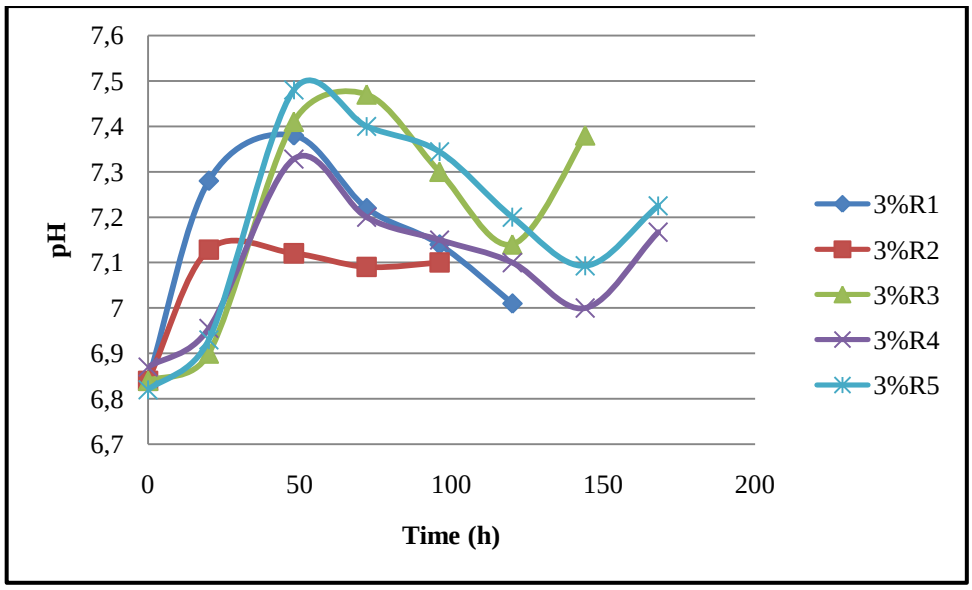


Figure 3.10 Mode of pH change during Hydrogen Production by *R. capsulatus* YO3 immobilized in 3% agar during all rounds

3.1.2.2 Hydrogen production by *Rhodobacter capsulatus* YO3 immobilized in 4% agar

In this experiment 4% of agar was used to immobilize the bacteria. The operation conditions were same as explained in sections 3.1.1.1 and 3.1.1.2. The working volume of the bottle was 120 ml filled with hydrogen production nutrient medium with 40 mM acetate concentration and 2 mM glutamate. As mentioned above glutamate concentration was graded up to 4 mM during fifth round. Incubation and illumination conditions were applied as described in materials and methods.

Operation of this experiment continued for about 713 hours (approximately 30 days) and covered five rounds. At the beginning of each round, the bottle was washed with basal medium then filled with hydrogen production nutrient medium and flashed by argon gas for anaerobic conditions.

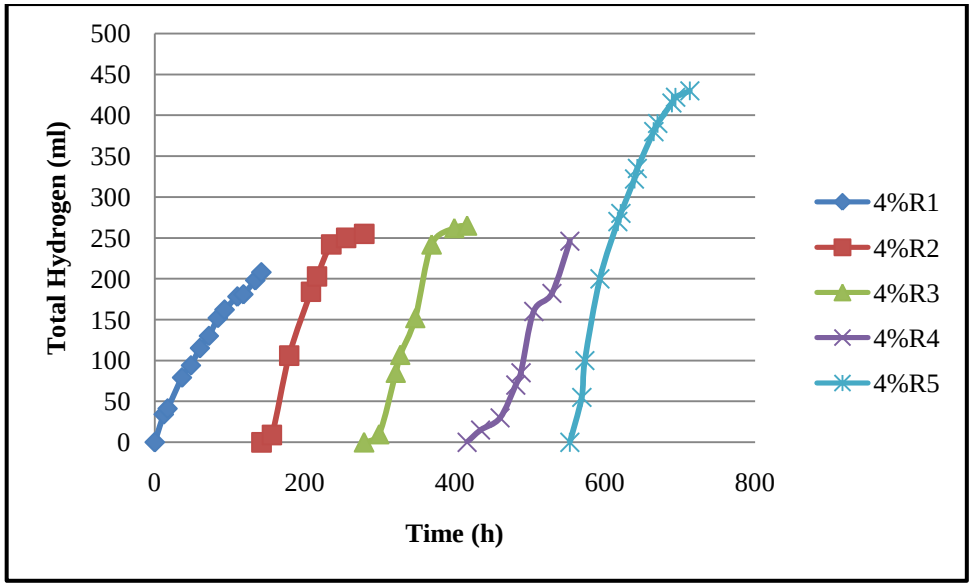


Figure 3.11 Total Hydrogen Production by *R. capsulatus* YO3 immobilized in 4% agar during all rounds

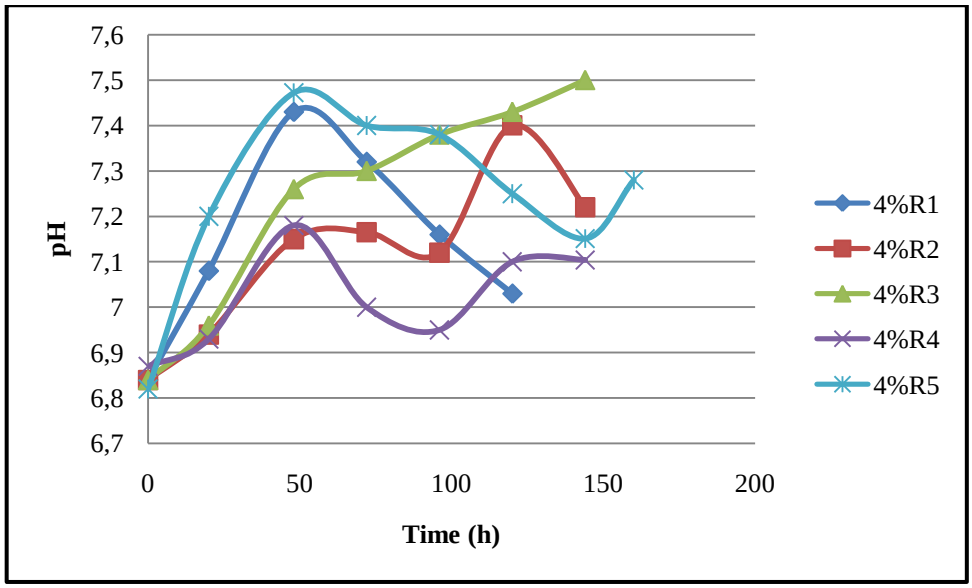


Figure 3.12 Changes in pH during Production by *R. capsulatus* YO3 immobilized in 4% agar during all rounds

At the end of rounds R1-R4 total hydrogen produced was ranging from 208-265 ml of hydrogen per bottle where glutamate concentration was 2 mM; Table A. 15 and Figure 3.11.

After increasing glutamate concentration to 4 mM, total hydrogen produced at the end of round R5 reached to 430 ml of hydrogen per bottle. Mode of pH change during all rounds of the experiment has shown stability trends between 6.8, the initial pH value, and 7.47 which was the highest pH value registered during round R5 (Figure 3.12 and Table A. 16).

3.1.2.3 Hydrogen production by *Rhodobacter capsulatus* YO3 immobilized in 5% agar

In this experiment 5% of agar was used to immobilize the bacteria. The operation conditions were same as explained in sections 3.1.1.1 and 3.1.1.2. In this part of study glutamate concentration was increased to 4 mM during fifth round. Incubation and illumination conditions were applied as described in materials and methods.

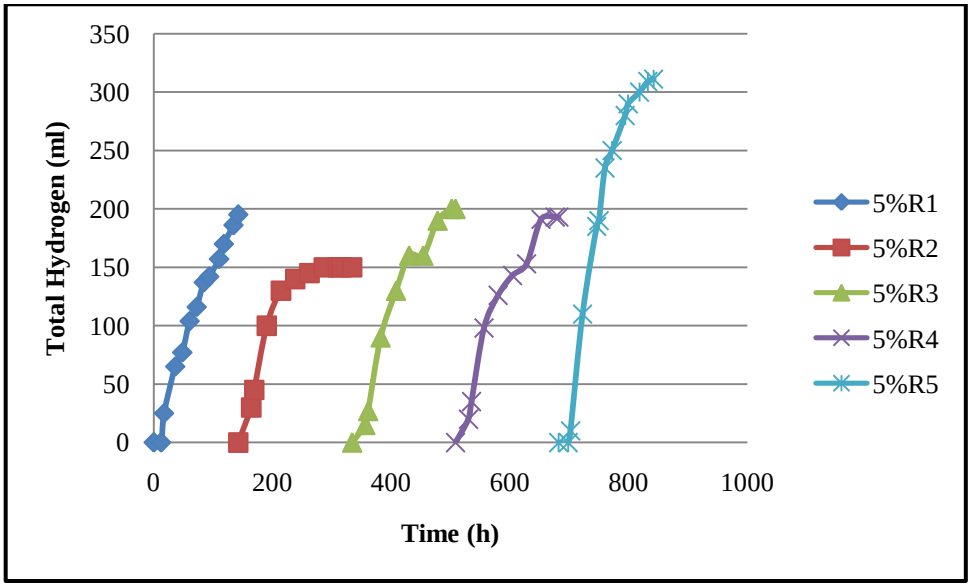


Figure 3.13 Total Hydrogen Production by *R. capsulatus* YO3 immobilized in 5% agar during all rounds

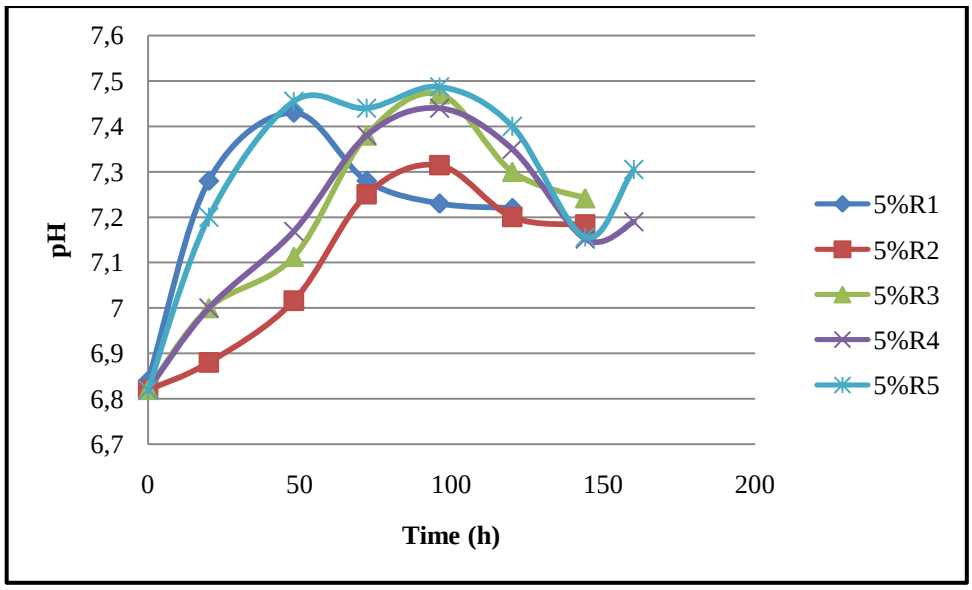


Figure 3.14 Mode of pH change during Hydrogen Production by *R. capsulatus* YO3 immobilized in 5% agar during all rounds

Operation of this experiment continued for about 842 hours (approximately 35 days) and covered five rounds. At the beginning of each round, the bottle was washed with basal medium and after filling with hydrogen production nutrient medium, it has been flashed by argon gas as described in material and methods.

At the end of rounds R1-R4 total hydrogen produced was ranging from 150-200 ml of hydrogen per bottle where glutamate concentration was 2 mM; Table A. 17 and Figure 3.13. Total hydrogen produced at the end of round R5 reached to 430 ml of hydrogen per bottle after glutamate concentration has been increased to 4 mM. Mode of pH change during all rounds of the experiment has shown stability trends between 6.8, the initial pH value, and 7.5 which was the highest pH value registered during round R5 (Figure 3.14 and Table A. 18).

3.1.2.4 Hydrogen production by *Rhodobacter capsulatus* YO3 immobilized in 6% agar

Around 30 ml of agar-bacteria complex were poured in 150 ml cell culture bottles. The operation conditions were same as explained in sections 3.1.1.1 and 3.1.1.2. In this part of study glutamate concentration was increased to 4 mM during fifth round. Incubation and illumination conditions were applied as described in materials and methods.

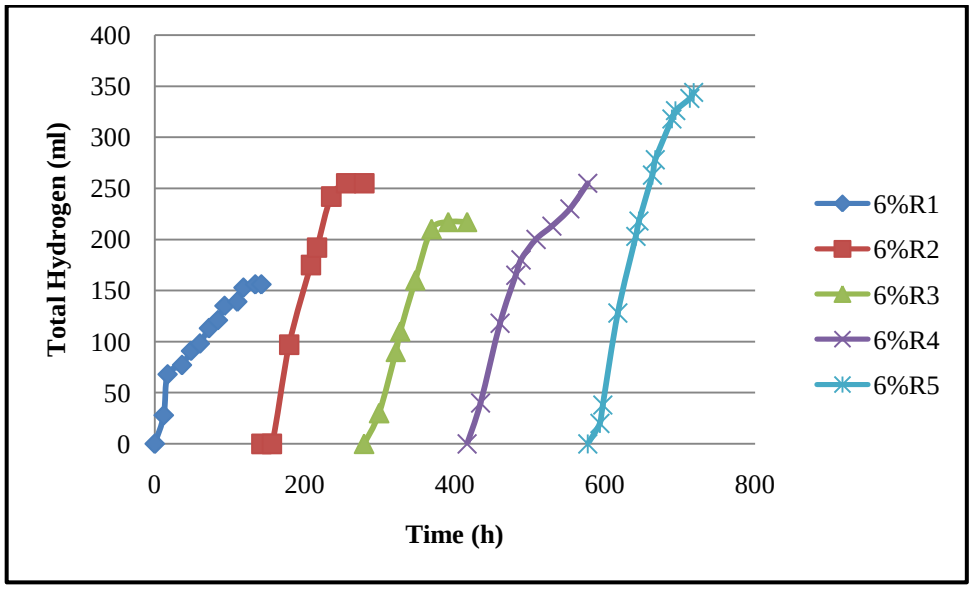


Figure 3.15 Total Hydrogen Production by *R. capsulatus* YO3 immobilized in 6% agar during all rounds

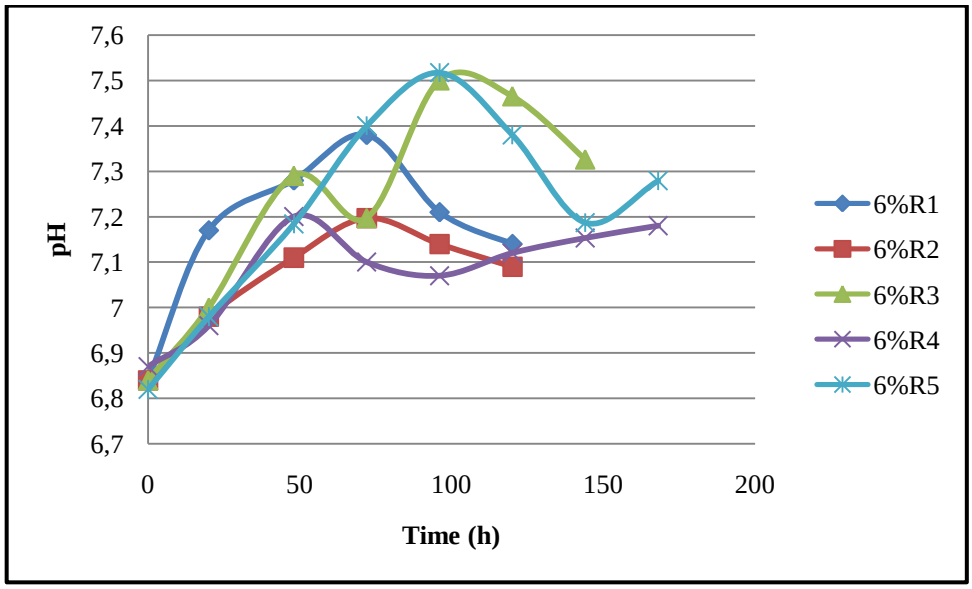


Figure 3.16 Mode of pH change during Hydrogen Production by *R. capsulatus* YO3 immobilized in 6% agar during all rounds

This experiment has continued for about 737 hours (approximately 31 days) and covered five rounds. Manipulation of immobilized bacteria inside the cell culture bottles was performed as described in previous sections as 3.1.2.3.

At the end of rounds R1-R4 total hydrogen produced was ranging from 156-255 ml of hydrogen per bottle where glutamate concentration was 2 mM; Table A. 19 and Figure 3.15. Total hydrogen produced at the end of round R5 reached to 351 ml of hydrogen per bottle after glutamate concentration has been increased to 4 mM. Mode of pH change during all rounds of the experiment has shown stability trends between 6.8, the initial pH value, and 7.5 which was the highest pH value observed during round R5 (Figure 3.16 and Table A. 20).

3.2 Hydrogen production by using different concentrations of acetate

This part of study investigated the effect of different concentrations of acetate (60, 80 and 100 mM) as organic acid while keeping concentration of glutamate at 4 mM on hydrogen production. Bacteria cell concentration was adjusted at 2.5 mg DCW/ml of gel.

Bacteria were immobilized inside 150 ml cell culture bottles. Agar-bacteria complex occupied around 30 ml while 120 ml of hydrogen production nutrient medium were used to fill the bottle and left headspace was 10 ml.

The effect of agar concentration on hydrogen production by immobilized bacteria indicated that 3% and 4% agar concentrations gave the best results in relation with total gas produced, rate of gas production and efficiency. During this part of work 4% agar concentration was applied.

Choice of 4% agar concentration was depending on the balance between the need for high total gas produced and the need for better gel mechanical stability for longer time. The experiments of acetate concentrations effect on hydrogen production were performed in a double for each concentration for both bacterial strains. The experiments have been allowed to work for three sequential batches (rounds) designated R1, R2 and R3.

3.2.1 Hydrogen production by using different concentrations of acetate by *Rhodobacter capsulatus* DSM1710

In this part of work *Rhodobacter capsulatus* DSM1710 has been immobilized in 4% agar inside 150 ml cell culture bottles. Immobilized bacteria were manipulated as described in section 3.2.

3.2.1.1 Hydrogen production by Rhodobacter capsulatus DSM1710 by using 60 mM acetate and 4mM glutamate

Immobilized bacteria were fed with 60 mM of acetate and 4mM glutamate, 60/4. Total hydrogen production results by double experiment of immobilized *R. capsulatus* DSM 1710 throughout the three rounds R1-R3are illustrated in Figure 3.17. Immobilized bacteria in experiment (x1) continued hydrogen production for 825 hours (approximately 35 days) and 916 hours (38 days) in experiment (x2).

Total hydrogen produced at the end of the three rounds of experiment one (x1), 60R1-x1, 60R2-x1 and 60R3-x1 were 405, 645 and 640 ml of hydrogen respectively, Figure 3.17. Total hydrogen produced at the end of the three rounds of experiment two (x2), 60R1-x2, 60R2-x2 and 60R3-x2 were 475, 550 and 520 ml of hydrogen respectively, (Figure 3.17, Table A. 21 and Table A. 23).

The observed behavior of pH change mode during the double experiment has shown general stability between 6.7 and 7.471except in 60R1-x2 were it reached up to 7.77 for once time (Figure 3.18, Table A. 22 & Table A. 24).

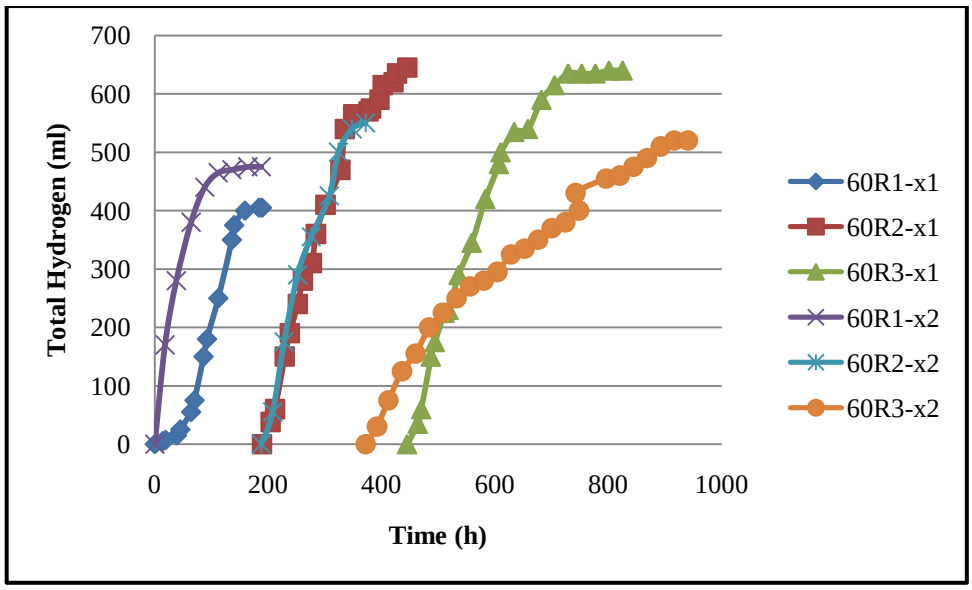


Figure 3.17 Total gas produced by double experiment during Hydrogen Production by *R. capsulatus* DSM1710 immobilized in 4% agar and fed with 60/4 acetate/glutamate

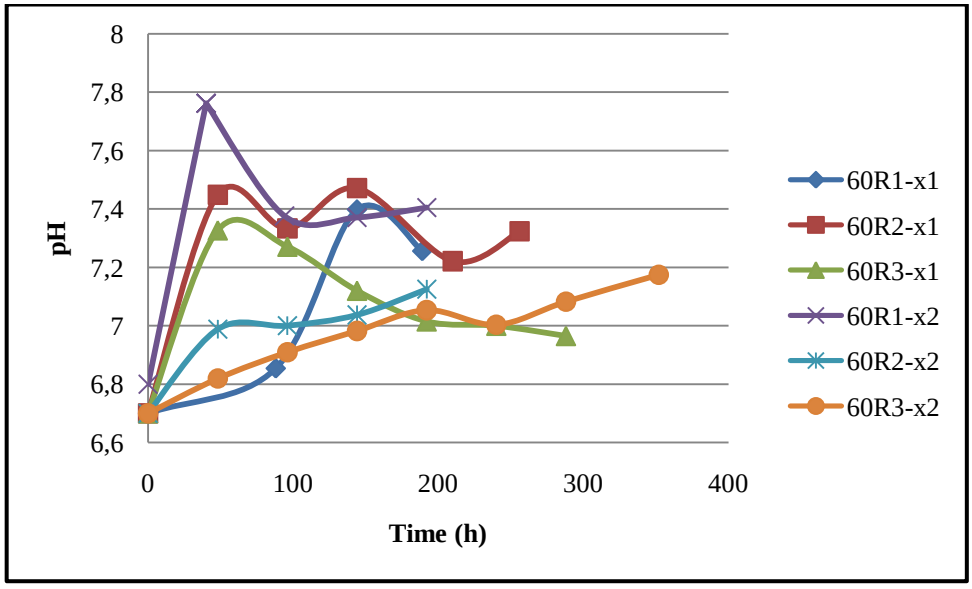


Figure 3.18 Mode of pH change by double experiment during Hydrogen Production by *R. capsulatus* DSM1710 immobilized in 4% agar and fed with 60/4 acetate/glutamate

3.2.1.2 Hydrogen production by *Rhodobacter capsulatus* DSM1710 by using 80 mM acetate and 4mM glutamate

Immobilized bacteria were fed with 80 mM of acetate and 4mM glutamate, 80/4. Total hydrogen production results by double experiment of immobilized *R. capsulatus* DSM 1710 throughout the three rounds R1-R3are illustrated in Figure 3.19.

Immobilized bacteria in experiment (x1) continued hydrogen production for 825 hours (approximately 35 days) and 916 hours (38 days) in experiment (x2).

The observed total hydrogen produced at the end of the three rounds of experiment one (x1), 80R1-x1, 80R2-x1 and 80R3-x1 were 475, 360 and 445 ml of hydrogen respectively (Figure 3.19, Table A. 21 and Table A. 23). The total hydrogen produced at the end of the three rounds of experiment two (x2), 80R1-x2, 80R2-x2 and 80R3-x2 were 475, 550 and 520 ml of hydrogen respectively (Figure 3.19, Table A. 21 and Table A. 23).

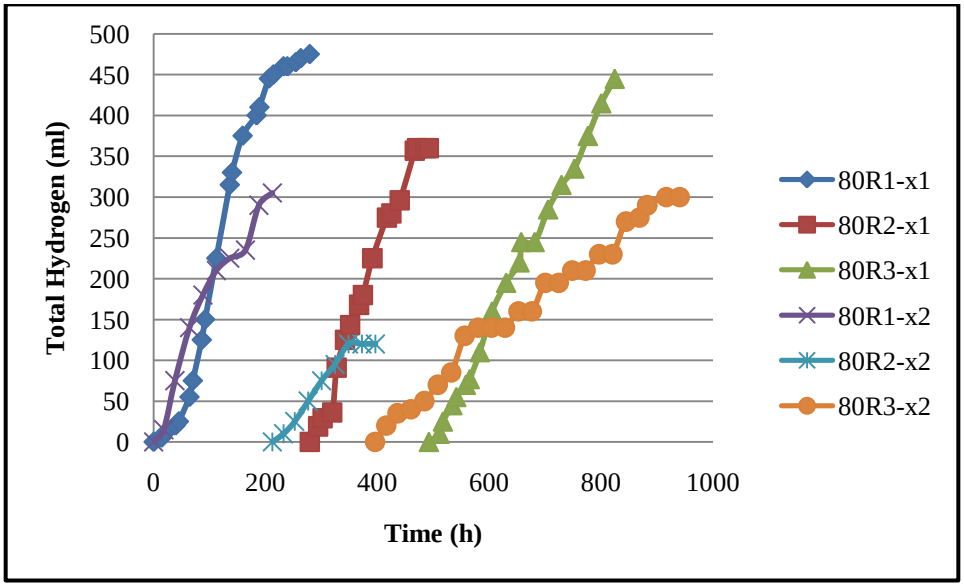


Figure 3.19 Total gas produced by double experiment during Hydrogen Production by *R. capsulatus* DSM1710 immobilized in 4% agar and fed with 80/4 acetate/glutamate

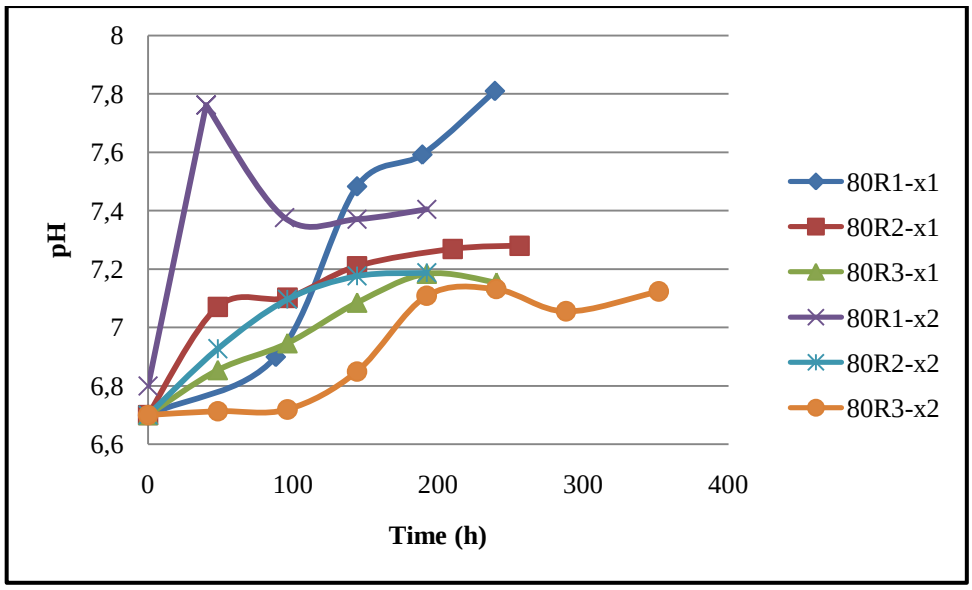


Figure 3.20 Mode of pH change by double experiment during Hydrogen Production by *R. capsulatus* DSM1710 immobilized in 4% agar and fed with 80/4 acetate/glutamate

The observed behavior of pH change mode during the double experiment has shown general stability between 6.7 and 7.48 except in 80R1-x2 were it reached up to 7.76 while in 80R1-x1 it reached 7.8 and 7.6, (Figure 3.20, Table A. 22 and Table A. 24). Change in pH mode shown more trends to develop higher values than was seen when using 40 and 60 mM acetate.

3.2.1.3 Hydrogen production by *Rhodobacter capsulatus* DSM1710 by using 100 mM acetate and 4mM glutamate

Immobilized bacteria were fed with 100 mM of acetate and 4mM glutamate, 100/4. Total hydrogen production results by double experiment of immobilized *R. capsulatus* DSM 1710 throughout the three rounds R1-R3are illustrated in Figure 3.21.

Total hydrogen produced at the end of the three rounds of experiment one (x1), 100R1-x1, 100R2-x1 and 100R3-x1 were 480, 320 and 420 ml of hydrogen respectively, Figure 3.21. The total hydrogen produced at the end of the three rounds of experiment two (x2), 100R1-x2, 100R2-x2 and 100R3-x2 were 325, 200 and 350 ml of hydrogen respectively, (Figure 3.21, Table A. 21 and Table A. 23).

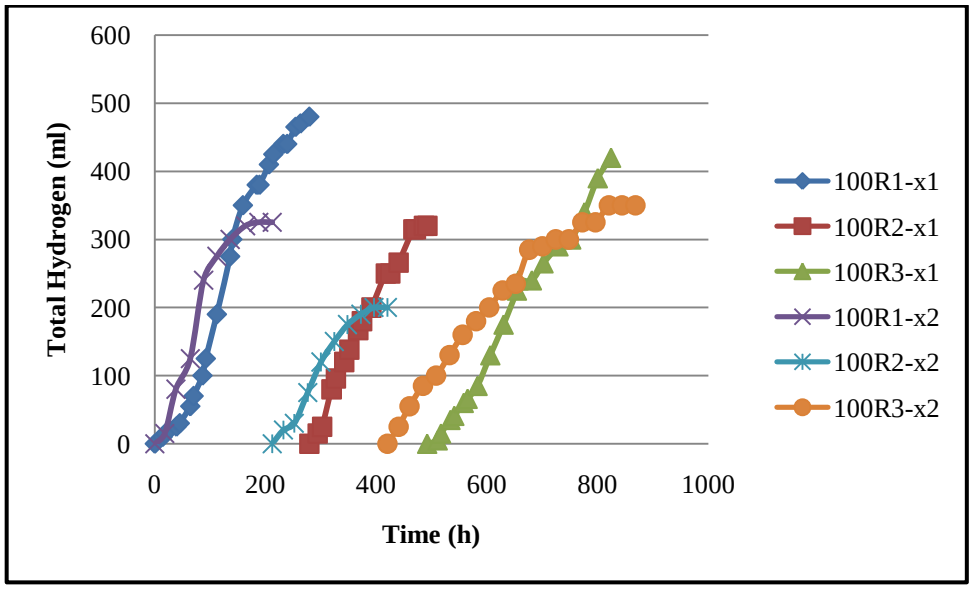


Figure 3.21 Total gas produced by double experiment during Hydrogen Production by *R. capsulatus* DSM1710 immobilized in 4% agar and fed with 100/4 acetate/glutamate

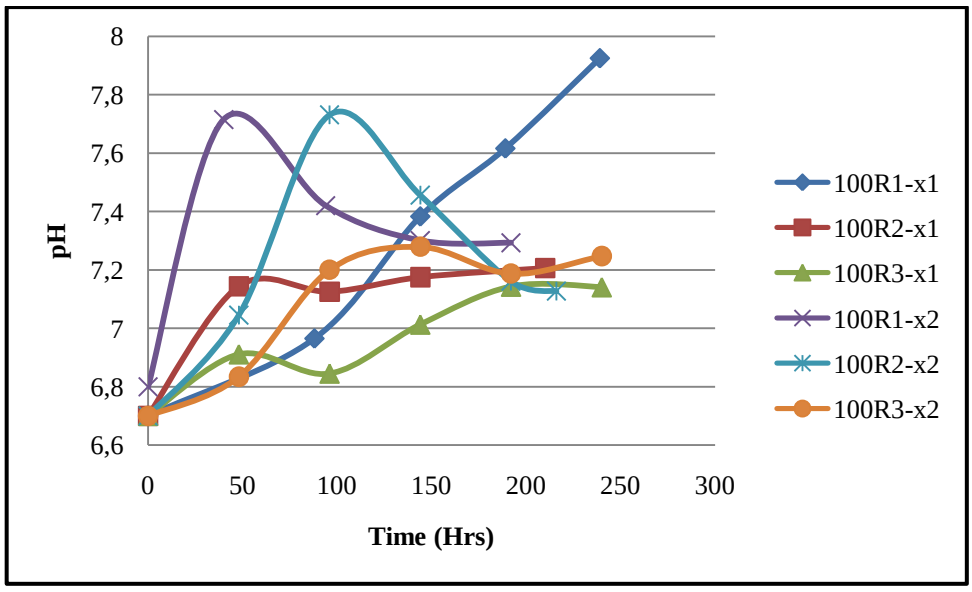


Figure 3.22 Mode of pH change by double experiment during Hydrogen Production by *R. capsulatus* DSM1710 immobilized in 4% agar and fed with 100/4 acetate/glutamate

The observed behavior of pH change mode during the double experiment has shown tendency to increase more than 7.5. Major pH values were between 6.7 and 7.483 but in 100R1-x2 reached up to 7.72 and in 100R2-x2 it reached 7.73, Figure 3.20. Observations also indicated that pH in 100R1-x1 increased more than 7.5 and continued up to 7.62 and 7.93 (Figure 3.20, Table A. 22 and Table A. 24). Change in pH mode shown more trends to develop higher values than was seen when using 40, 60 and 80 mM acetate.

3.2.2 Hydrogen production by using different concentrations of acetate by immobilized *Rhodobacter capsulatus* YO3

In this part of work *Rhodobacter capsulatus* YO3 has been immobilized in 4% agar inside 150 ml cell culture bottles. Immobilized bacteria were manipulated as described in section 3.2.

3.2.2.1 Hydrogen production by *Rhodobacter capsulatus* YO3 by using 60 mM acetate and 4mM glutamate

The immobilized bacteria were fed with hydrogen production nutrient medium containing 60 mM acetate while the glutamate concentration was kept on 4 mM value, 60/4. Operation of this experiment continued for about 832 hours (approximately 35 days) and covered three rounds for experiment 1 and 982 hours (approximately 41 days) for second experiment (x2) and covered three rounds.

The total hydrogen produced by end of each round during the two experiments (x1 & x2) is illustrated in (Figure 3.23, Table A. 25 & Table A. 27). Total hydrogen produced at the end of three rounds of experiment (x1), 60R1-x1, 60R2-x1 and 60R3-x1 was 640, 830 and 330 ml of hydrogen respectively while at the end of the three rounds of experiment (x2), 60R1-x2, 60R2-x2 and 60R3-x2, total hydrogen produced was 475, 550 and 520 ml of hydrogen.

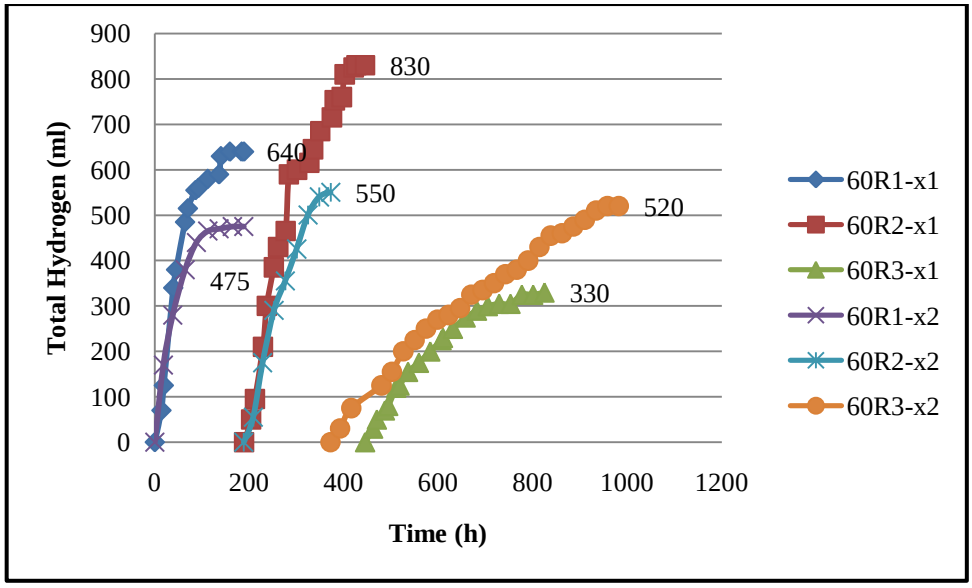


Figure 3.23 Total gas produced by double experiment during Hydrogen Production by *R. capsulatus* YO3 immobilized in 4% agar and fed with 60/4 acetate/glutamate

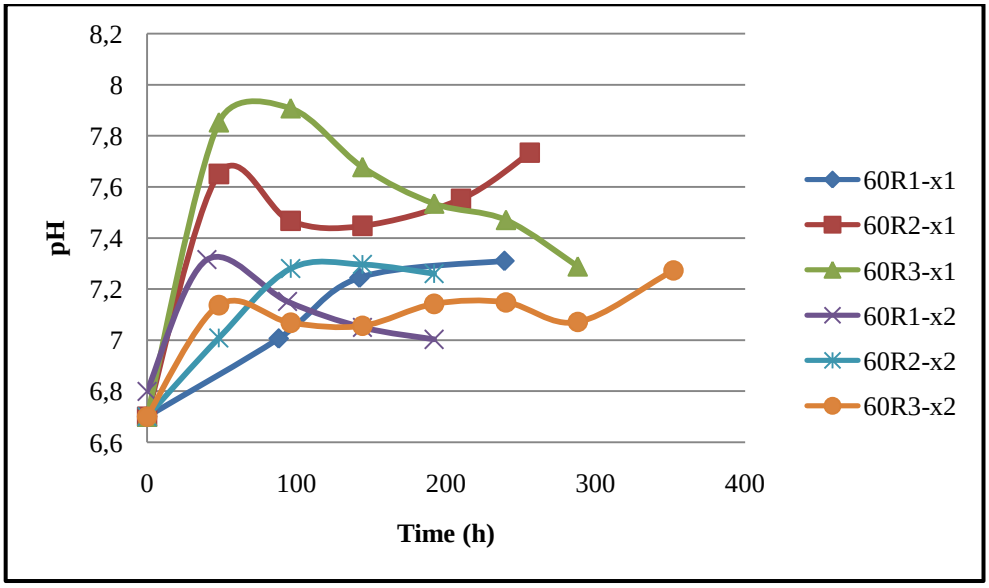


Figure 3.24 Mode of pH change by double experiment during Hydrogen Production by *R. capsulatus* YO3 immobilized in 4% agar and fed with 60/4 acetate/glutamate

The observed pH values shown tendency to exceed 7.5 in two cases, 60R1-x1, were it reached to 7.9 and declined again gradually to 7.552 and 60R2-x1 when pH was over 7.6 and declined to 7.45 then ended at 7.73. Other rounds of the two experiments have shown stable trends of pH between the initial value, 6.7, and 7.5, (Figure 3.24, Table A. 26 & Table A. 28).

3.2.2.2 Hydrogen production by *Rhodobacter capsulatus* YO3 by using 80 mM acetate and 4mM glutamate

The immobilized bacteria were fed with hydrogen production nutrient medium containing 80 mM acetate while the glutamate concentration was kept at 4 mM value, (80/4). Operation of the double experiment continued for about 825 hours (approximately 34 days) and covered three rounds for first experiment (x1) and 964 hours (approximately 40 days) for second experiment (x2) and covered three rounds.

The total hydrogen produced by end of each round during the two experiments (x1 & x2) is illustrated in Figure 3.23. Total hydrogen produced at the end of three rounds of experiment (x1), 80R1-x1, 80R2-x1 and 80R3-x1 was 685, 805 and 350 ml of hydrogen respectively while at the end of the three rounds of experiment (x2), 80R1-x2, 80R2-x2 and 80R3-x2, total hydrogen produced was 565, 330 and 320 ml of hydrogen, (Figure 3.25, Table A. 25 & Table A. 27) .

Observed pH values have shown tends to exceed 7.5 during rounds of first experiment where the highest registered pH was7.738 at the end of 80R2-x1 round. During second experiment, pH values remained less than 7.2 except once measured value at round 80R1-x2 which was 7.5 (Figure 3.26, Table A. 26 & Table A.28)

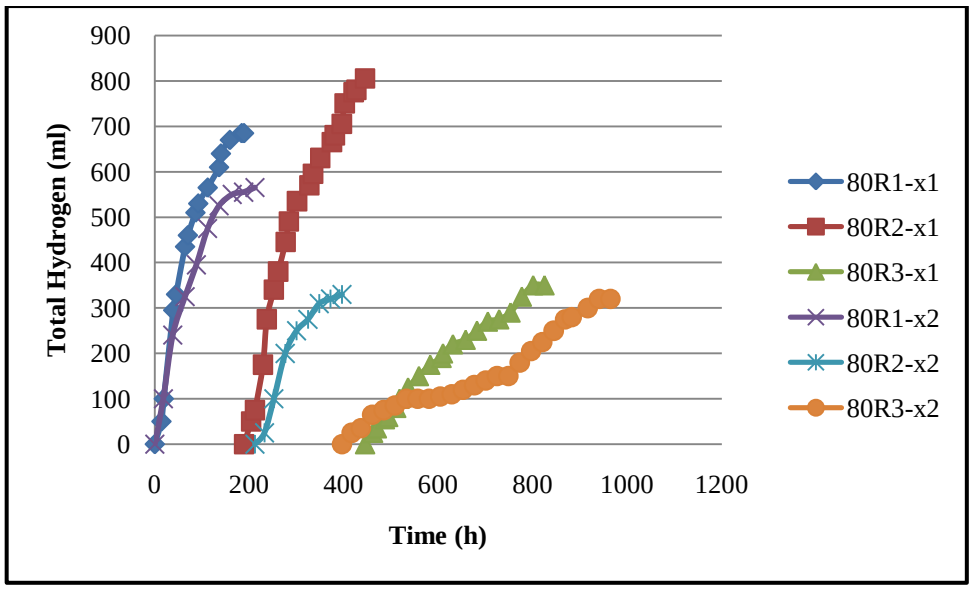


Figure 3.25 Total gas produced by double experiment during Hydrogen Production by *R. capsulatus* YO3 immobilized in 4% agar and fed with 80/4 acetate/glutamate

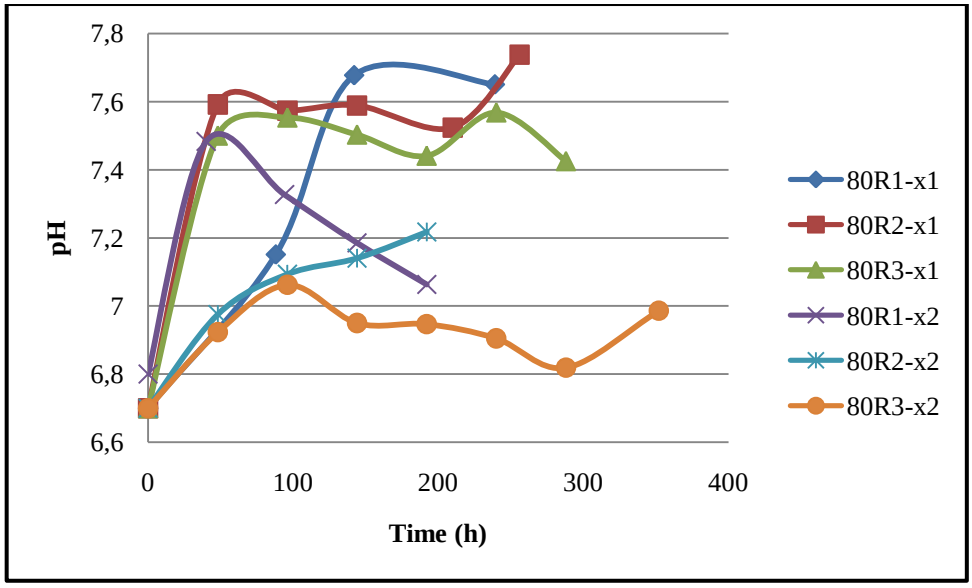


Figure 3.26 Mode of pH change by double experiment during Hydrogen Production by *R. capsulatus* YO3 immobilized in 4% agar and fed with 80/4 acetate/glutamate

3.2.2.3 Hydrogen production by *Rhodobacter capsulatus* YO3 by using 100 mM acetate and 4mM glutamate

The immobilized bacteria were provided with hydrogen production nutrient medium containing 100 mM acetate while the glutamate concentration was kept on 4 mM value, 100/4. Operation of this experiment continued for about 825 hours (approximately 34 days) and covered three rounds for first experiment (x1) and 964 hours (approximately 40 days) for second experiment (x2) and covered three rounds.

The total hydrogen produced by end of each round during the two experiments (x1 & x2) is illustrated in Figure 3.27, (Table A. 25 & Table A. 27). Total hydrogen produced at the end of three rounds of experiment (x1), 100R1-x1, 100R2-x1 and 100R3-x1 was 840, 555 and 300 ml of hydrogen respectively.

At the end of the three rounds of experiment (x2), 100R1-x2, 100R2-x2 and 100R3-x2, total hydrogen produced was 555, 450 and 210 ml of hydrogen. The pH values exhibited general stability during the two experiments (x1 & x2) except once time when it reached up to 7.68 at the end of 100R1-x1 round (Figure 3.28, Table A. 26 & Table A. 28).

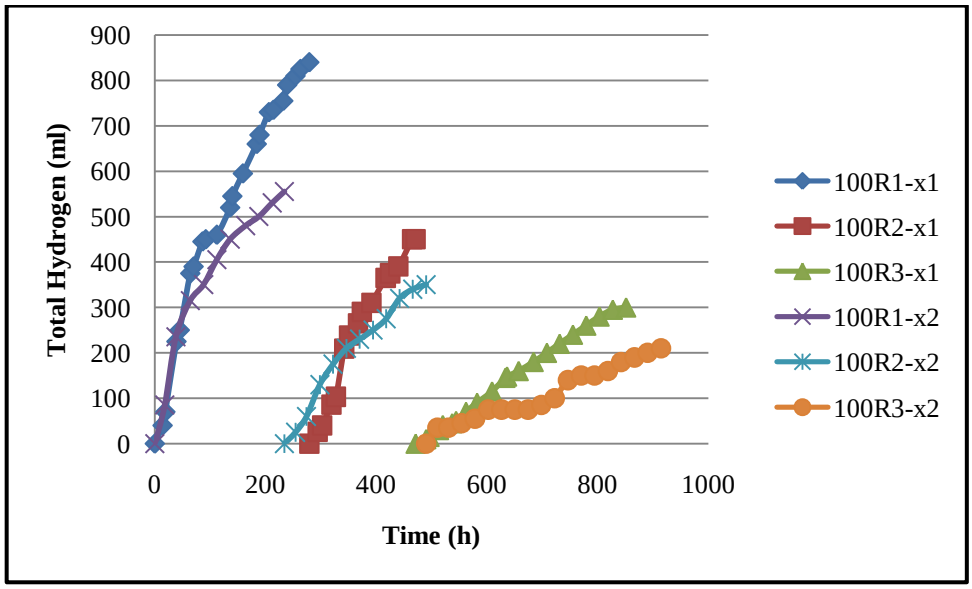


Figure 3.27 Total gas produced by double experiment during Hydrogen Production by *R. capsulatus* YO3 immobilized in 4% agar and fed with 100/4 acetate/glutamate

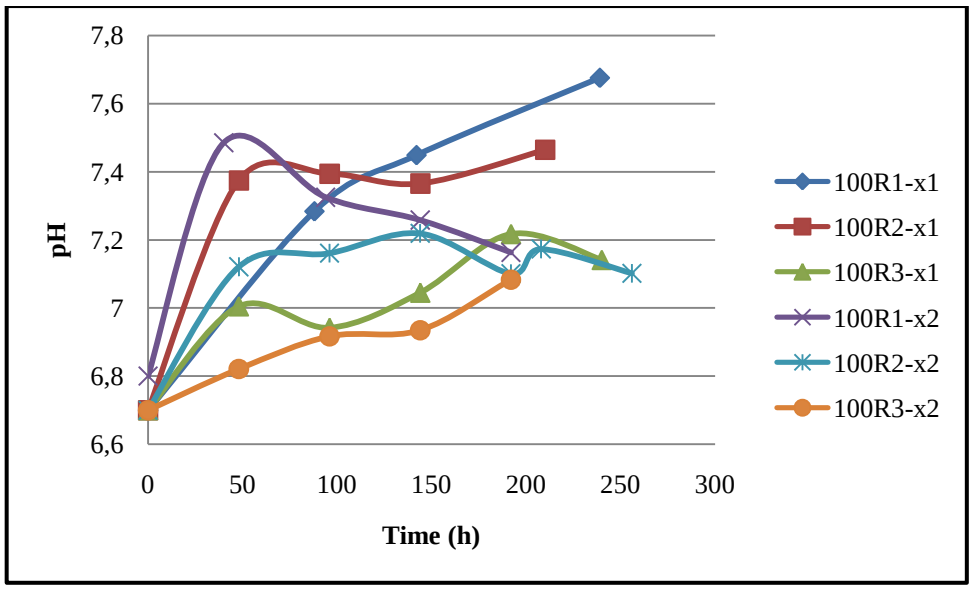


Figure 3.28 Mode of pH change by double experiment during Hydrogen Production by *R. capsulatus* YO3 immobilized in 4% agar and fed with 100/4 acetate/glutamate

3.3 Effect of doubling bacterial concentration immobilized in agar on hydrogen production by *Rhodobacter capsulatus*

In this part of work concentration of bacterial cells were doubled up to mg 5 DCW/ml of gel. The applied acetate concentrations were used as 60, 80, and 100 mM and glutamate concentration was 4 mM. During this part of work 4% agar concentration was depended. All experiments were performed in a double for each concentration of acetate for both bacterial strains. Bacteria were immobilized in 250 ml cell culture bottles. Immobilized bacteria inside the cell culture bottles were manipulated as described in section 3.2.

3.3.1 Effect of doubling the concentration of *Rhodobacter capsulatus* DSM1710 immobilized in agar on hydrogen production

In this part of work bacterial concentration was increased up to 5 mg DCW/ml agar gel. The bacteria were immobilized within 4% agar and provided with hydrogen production nutrient medium containing different concentrations of acetate including 60, 80, and 100 mM acetate while the glutamate concentration was kept on 4mM value.

3.3.1.1 Hydrogen production by *Rhodobacter capsulatus* DSM1710 by using 60 mM acetate and 4mM glutamate

The immobilized bacteria within were provided with hydrogen production nutrient medium containing 60 mM acetate and the 4 mM glutamate, 60/4. Operation of this experiment continued for about 540 hours (approximately 23 days) and covered three rounds for first experiment (x1) while it continued for 588 hours (approximately 25 days) in second experiment (x2) and covered three rounds.

The total hydrogen produced by end of each round during the two experiments (x1 & x2) is illustrated in Figure 3.29; see also Table A. 29 and Table A. 31. Total hydrogen produced at the end of three rounds of experiment (x1), 60R1-x1, 60R2-x1 and 60R3-x1 was 840, 555 and 300 ml of hydrogen respectively while it was 665, 450 and 425 at the end of the three rounds of second experiment (x2), 60R1-x2, 60R2-x2 and 60R3-x2, respectively.

The pH values have shown general trends toward stability between 6.7 and 7.399 during the whole period of process operation. At the end of each round it was observed that pH values went between 6.95 and 7.14, (Figure 3.30, Table A. 30 & Table A. 32).

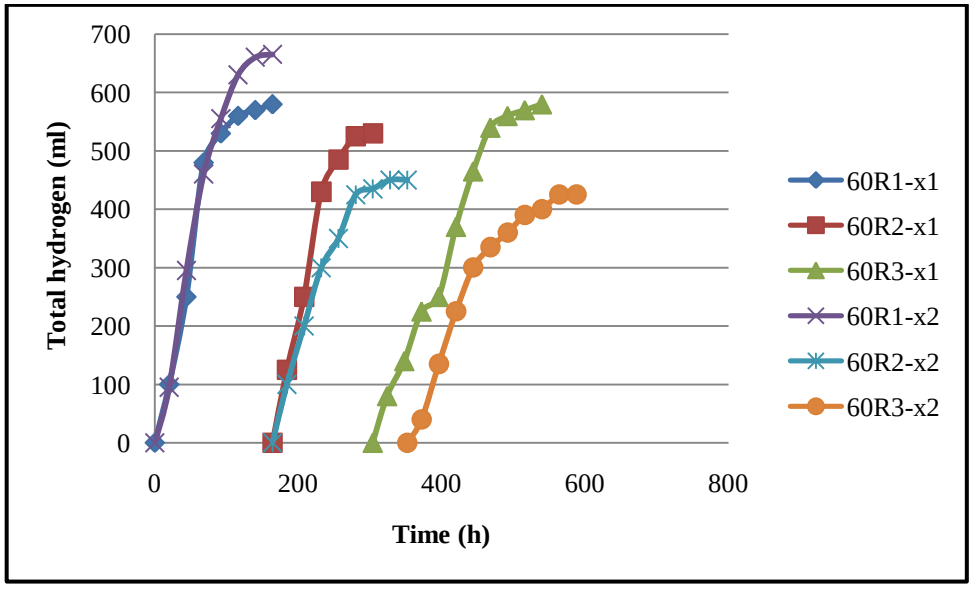


Figure 3.29 Total hydrogen produced by double experiment during Hydrogen Production by *R. capsulatus* DSM1710 immobilized in 4% agar and fed with 60/4 acetate/glutamate and 5 mg DCW/ml agar gel

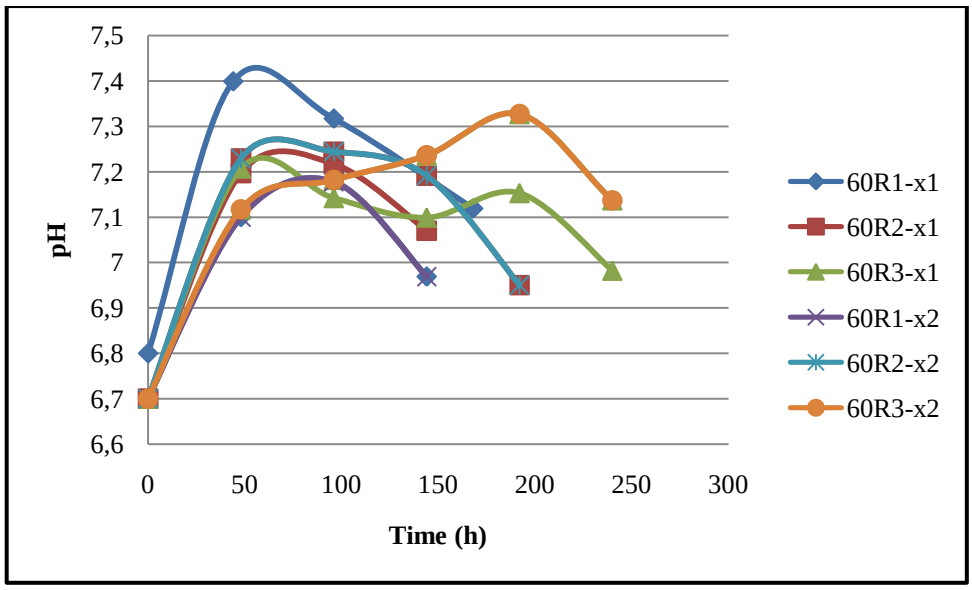


Figure 3.30 Mode of pH change by double experiment during Hydrogen Production by *R. capsulatus* DSM1710 immobilized in 4% agar and fed with 60/4 acetate/glutamate and 5 mg DCW/ml agar gel

3.3.1.2 Hydrogen production by *Rhodobacter capsulatus* DSM1710 by using 80 mM acetate and 4mM glutamate

The immobilized bacteria were provided with hydrogen production nutrient medium containing 80 mM acetate 4 mM glutamate, 80/4. Bacterial concentration was 5 mg DCW/ ml agar gel.

The experiment has been operated in a double form as mentioned in section 3.2. Operation of this experiment continued for about 878 hours (approximately 37 days) and covered three rounds for experiment 1 and 680 hours (approximately 28 days) for experiment 2and covered three rounds.

The total hydrogen produced by end of each round during the two experiments (x1 & x2) is illustrated in Figure 3.31; see also Table A. 29 and Table A. 31. Total hydrogen produced at the end of three rounds of experiment (x1), 80R1-x1, 80R2-x1 and 80R3-x1 was 500, 120 and 355 ml of hydrogen respectively while it was 815, 540 and 350 at the end of the three rounds of second experiment (x2), 80R1-x2, 80R2-x2 and 80R3-x2, respectively.

The pH values have shown general trends toward stability between 6.7 and 7.5 during the whole period of process operation, (Figure 3.32, Table A. 30 and Table A. 32).

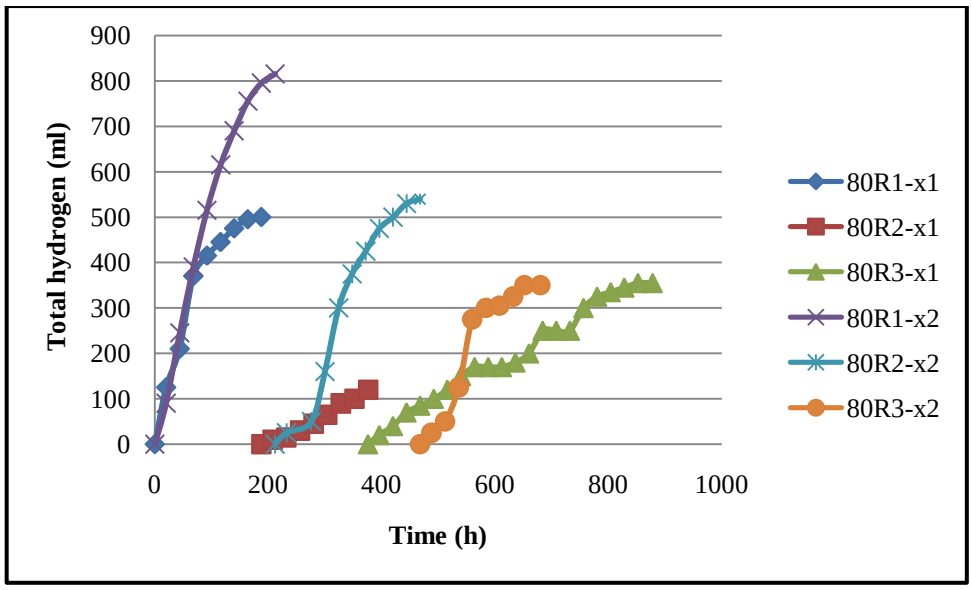


Figure 3.31 Total hydrogen produced by double experiment during Hydrogen Production by *R. capsulatus* DSM1710 immobilized in 4% agar and fed with 60/4 acetate/glutamate and 5 mg DCW/ml agar gel

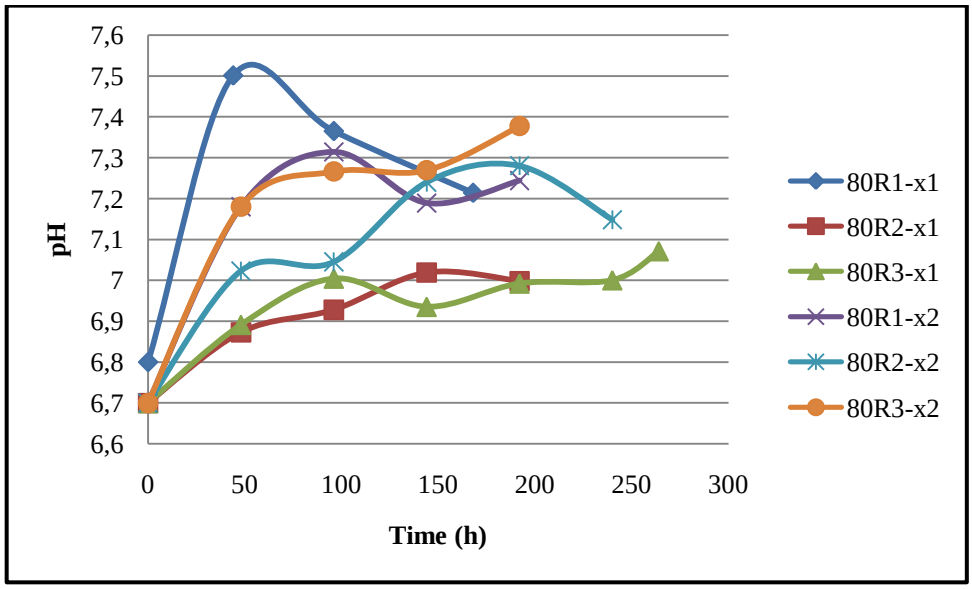


Figure 3.32 pH change by double experiment during Hydrogen Production by *R. capsulatus* DSM1710 immobilized in 4% agar and fed with 80/4 acetate/glutamate and 5 mg DCW/ml agar gel

3.3.1.3 Hydrogen production by *Rhodobacter capsulatus* DSM1710 by using 100 mM acetate and 4mM glutamate

The immobilized bacteria were provided with hydrogen production nutrient medium containing 100 mM acetate and 4 mM glutamate. Bacterial concentration was 5 mg DCW/ ml agar gel. The experiment has been operated in a double form as mentioned in section 3.2. Operation of this experiment continued for about 866 hours (approximately 36 days) and covered three rounds for experiment 1 and 540 hours (approximately 23 days) for experiment 2 and covered three rounds.

The total hydrogen produced by end of each round during the two experiments (x1 & x2) is illustrated in Figure 3.33; see also Table A. 29 and Table A. 31. Total hydrogen produced at the end of three rounds of experiment (x1), 100R1-x1, 100R2-x1 and 100R3-x1 was 420, 120 and 300 ml of hydrogen respectively while it was 550, 240 and 210 at the end of the three rounds of second experiment (x2), 100R1-x2, 100R2-x2 and 100R3-x2, respectively.

The pH values have shown general trends toward stability between 6.7 and 7.488 during the whole period of process operation, (Figure 3.34, Table A. 30 and Table A. 32).

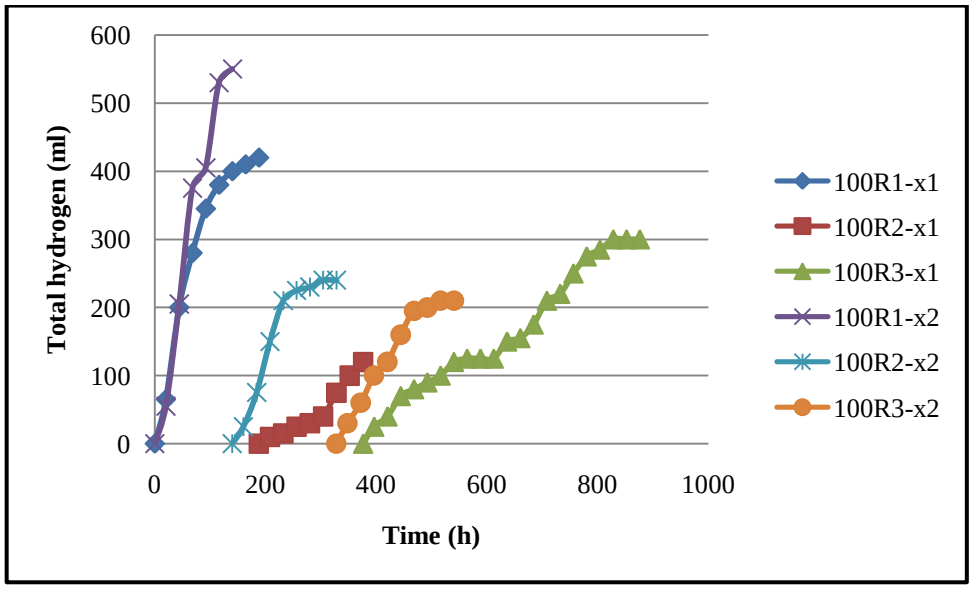


Figure 3.33 Total hydrogen produced by double experiment during Hydrogen Production by *R. capsulatus* DSM1710 immobilized in 4% agar and fed with 100/4 acetate/glutamate and 5 mg DCW/ml agar gel

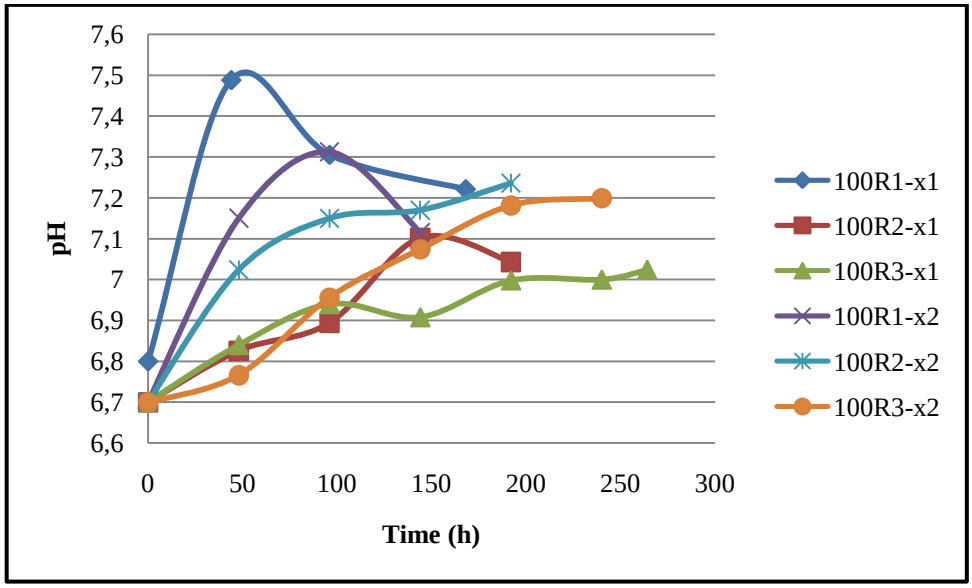


Figure 3.34 Mode of pH change by double experiment during Hydrogen Production by *R. capsulatus* DSM1710 immobilized in 4% agar and fed with 100/4 acetate/glutamate and 5 mg DCW/ml agar gel

3.3.2 Effect of doubling the concentration of *Rhodobacter capsulatus* YO3immobilized in agar on hydrogen production

In this part of work bacterial concentration was increased up to 5 mg DCW/ml agar gel. The bacteria were immobilized within 4% agar and provided with hydrogen production nutrient medium containing different concentrations of acetate including 60, 80, and 100 mM acetate while the glutamate concentration was kept on 4mM value. The experiment has been operated in double and the immobilized bacteria inside the cell culture bottles as mentioned in section 3.2.

3.3.2.1 Hydrogen production by *Rhodobacter capsulatus* YO3 by using 60 mM acetate and 4mM glutamate

The immobilized bacteria were provided with hydrogen production nutrient medium containing 60 mM acetate and 4 mM glutamate. Bacterial concentration was 5 mg DCW/ ml agar gel. The experiment has been operated in a double form as mentioned in section 3.2. Operation of this experiment continued for about 708 hours (approximately 30 days) and

covered three rounds for experiment 1 and 420 hours (approximately 18 days) for experiment 2and covered three rounds.

The total hydrogen produced by end of each round during the two experiments (x1 & x2) is illustrated in Figure 3.35, (Table A. 33 and Table A. 35). Total hydrogen produced at the end of three rounds of experiment (x1), 60R1-x1, 60R2-x1 and 60R3-x1 was 905, 905 and 615 ml of hydrogen respectively while it was 1045, 1100 and 800 at the end of the three rounds of second experiment (x2), 60R1-x2, 60R2-x2 and 60R3-x2, respectively.

The pH values have shown general trends toward stability between 6.7 and 7.5 during the whole period of process operation, (Figure 3.36, Table A. 34 and Table A. 36).

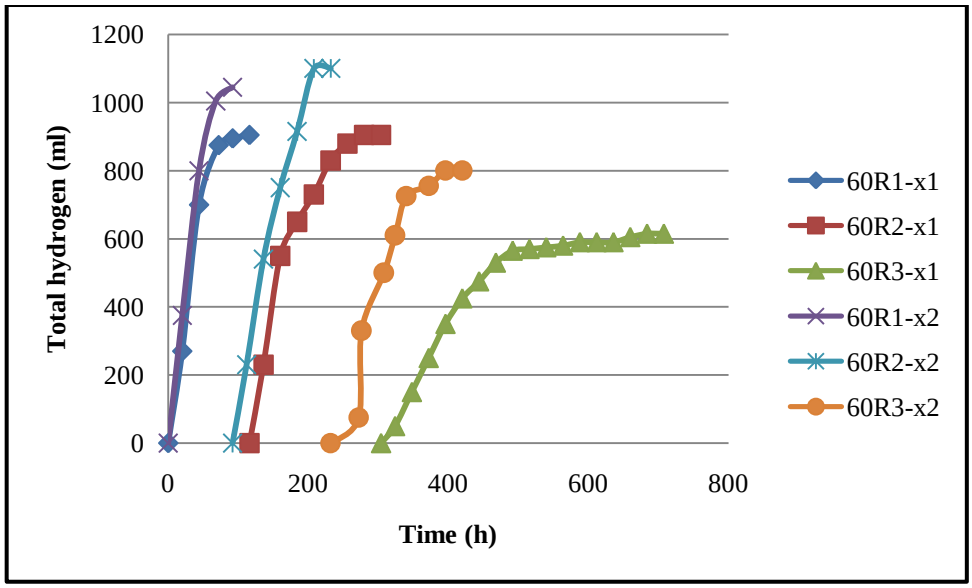


Figure 3.35 Total hydrogen produced by double experiment during Hydrogen Production by *R. capsulatus* YO3 immobilized in 4% agar and fed with 60/4 acetate/glutamate and 5 mg DCW/ml agar gel

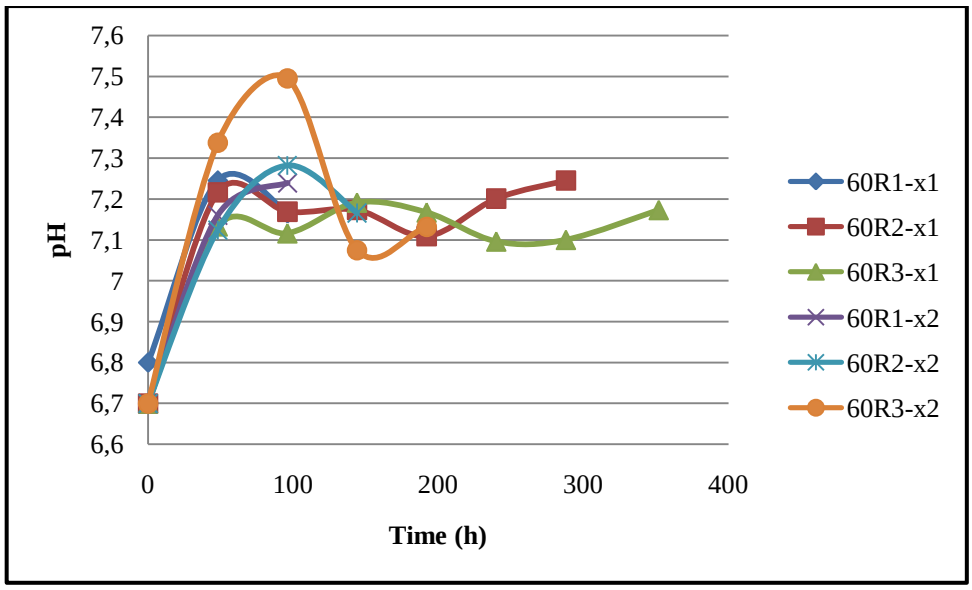


Figure 3.36 Mode of pH change by double experiment during Hydrogen Production by *R. capsulatus* YO3 immobilized in 4% agar and fed with 60/4 acetate/glutamate and 5 mg DCW/ml agar gel

3.3.2.2 Hydrogen production by *Rhodobacter capsulatus* YO3 by using 80 mM acetate and 4mM glutamate

The bacteria were immobilized within 4% agar and provided with hydrogen production nutrient medium containing 80 mM acetate while the glutamate concentration was kept on 4 mM value. Bacterial concentration was 5 mg DCW/ ml agar gel. The experiment has been operated in a double form as mentioned in section 3.2. Operation of this experiment continued for about 702 hours (approximately 29 days) and covered three rounds for first experiment (x1) and 420 hours (approximately 18 days) for experiment 2and covered three rounds.

The total hydrogen produced by end of each round during the two experiments (x1 & x2) is illustrated in Figure 3.37; see also Table A. 33 and Table A. 35. Total hydrogen produced at the end of three rounds of experiment (x1), 80R1-x1, 80R2-x1 and 80R3-x1 was 1120, 800 and 740 ml of hydrogen respectively while it was 1045, 1100 and 800 at the end of the three rounds of second experiment (x2), 80R1-x2, 80R2-x2 and 80R3-x2, respectively.

The pH values have shown general trends toward stability between 6.7 and 7.5 except two times during round 80R2-x2 when pH reached to 7.63 and round 80R3-x2 when pH reached to 7.78, (Figure 3.38, Table A. 34 and Table A. 36).

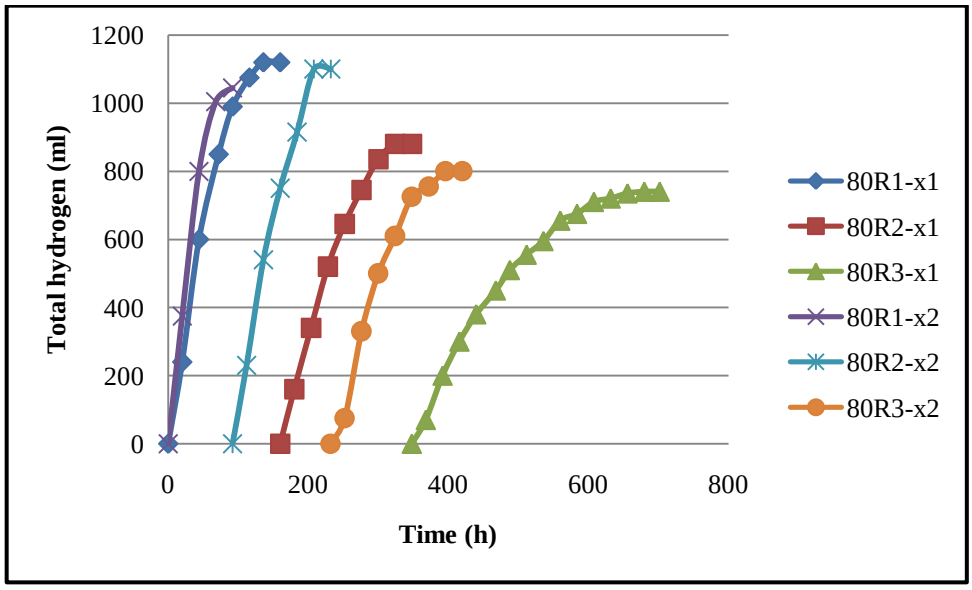


Figure 3.37 Total hydrogen produced by double experiment during Hydrogen Production by *R. capsulatus* YO3 immobilized in 4% agar and fed with 80/4 acetate/glutamate and 5 mg DCW/ml agar gel

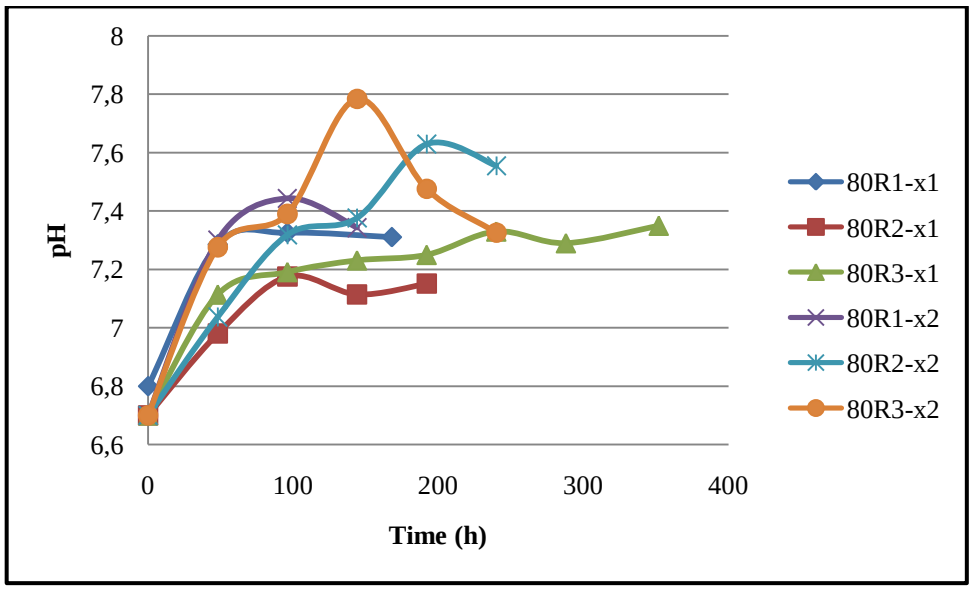


Figure 3.38 Mode of pH change by double experiment during Hydrogen Production by *R. capsulatus* YO3 immobilized in 4% agar and fed with 80/4 acetate/glutamate and 5 mg DCW/ml agar gel

3.3.2.3 Hydrogen production by *Rhodobacter capsulatus* YO3 by using 100 mM acetate and 4mM glutamate

The immobilized bacteria were fed with hydrogen production nutrient medium containing 100 mM acetate and 4 mM glutamate. Bacterial concentration was 5 mg DCW/ ml agar gel. The experiment has been operated in a double form as mentioned in section 3.2.

Operation of this experiment continued for about 964 hours (approximately 41 days) and covered three rounds for experiment 1 and 994 hours (approximately 42 days) for experiment 2 and covered three rounds.

The total hydrogen produced by end of each round during the two experiments (x1 & x2) is illustrated in Figure 3.39, (Table A. 33 and Table A. 35). Total hydrogen produced at the end of three rounds of experiment (x1), 100R1-x1, 100R2-x1 and 100R3-x1 was 1350, 1370 and 825 ml of hydrogen respectively while it was 1560, 1320 and 750 at the end of the three rounds of second experiment (x2), 100R1-x2, 100R2-x2 and 100R3-x2, respectively.

The pH values have shown common trends toward stability between 6.7 and 7.5, (Figure 3.40, Table A. 34 and Table A. 36).

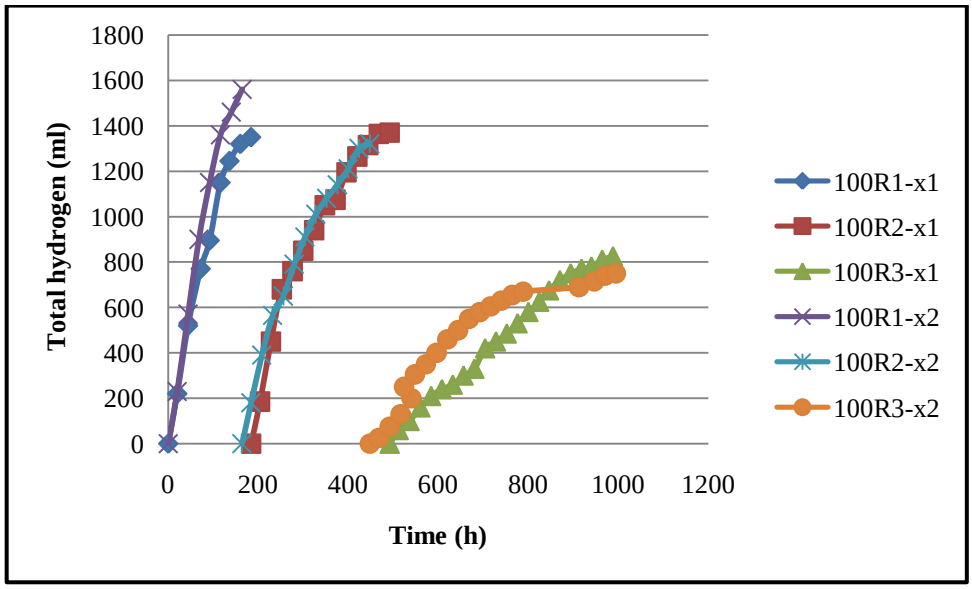


Figure 3.39 Total hydrogen produced by double experiment during Hydrogen Production by *R. capsulatus* YO3 immobilized in 4% agar and fed with 100/4 acetate/glutamate and 5 mg DCW/ml agar gel

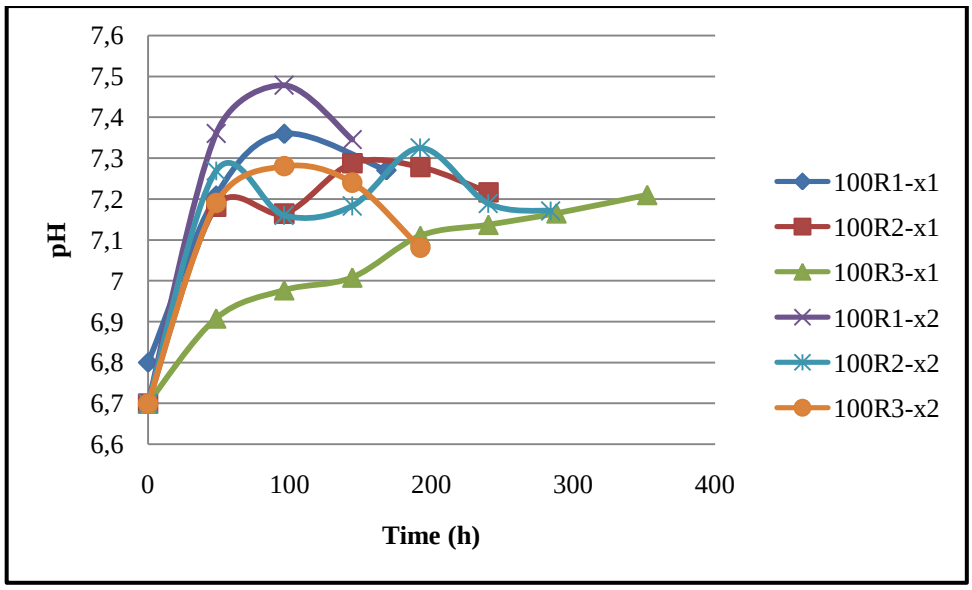


Figure 3.40 Mode of pH change by double experiment during Hydrogen Production by *R. capsulatus* YO3 immobilized in 4% agar and fed with 100/4 acetate/glutamate and 5 mg DCW/ml agar gel

3.4 Effect of glycerol on hydrogen production by photosynthetic bacteria

During this part of work effect of adding glycerol to the bacterial suspension before mixing with molten agar gel has been investigated. The aim of using glycerol was to provide protection for bacterial cell membrane during mixing with hot agar gel which has 45 °C before solidifying. Culture preparation methods were applied as described in section 2.7. Final glycerol concentrations were 2.5% and 5% v/v within the bacteria-agar gel complex. Bacterial concentration was used as 5 mg DCW for each strain.

The immobilized bacteria were fed with 60 mM acetate and 4 mM glutamate, 60/4. The immobilized bacteria inside the 250 ml cell cultures were divided into doubled groups for each glycerol concentration for both strains. Incubation and illumination were applied as described in materials and methods. The experiment is allowed to operate for three sequential batches (rounds; R1, R2 and R3).

3.4.1 Hydrogen production by *Rhodobacter capsulatus* DSM1710 manipulated with 2.5% and 5% glycerol

The immobilized *Rhodobacter capsulatus* DSM1710 which has been manipulated with glycerol was fed with 60/4 hydrogen production nutrient medium. This experiment continued for about 806 hours (approximately 34 days) and covered three sequential batches (rounds) for each concentration. The experiment was double and the sequential batches (rounds) for immobilized bacteria manipulated with 2.5% glycerol designated as 2.5-R1, 2.5-R2 and 2.5-R3 while those manipulated with 5% glycerol designated as 5-R1, 5-R2 and 5-R3.

The observed total hydrogen produced by immobilized bacteria manipulated with 2.5% and 5% glycerol is illustrated in Figure 3.41 and Table A. 37. At the end of the three rounds, 2.5-R1, 2.5-R2 and 2.5-R3, average total hydrogen produced for each round was 805, 500, and 505 ml of hydrogen, respectively, from immobilized bacteria manipulated with 2.5% glycerol. The observed total hydrogen produced by immobilized bacteria manipulated with 5% glycerol was 500, 400 and 685 ml of hydrogen from the three rounds 5-R1, 5-R2 and 5-R3, respectively, (Figure 3.41 and Table A. 37). During this experiment pH exhibited common stability between initial pH, 6.7, and the highest assigned value which was 7.3, (Figure 3.42 and Table A. 38).

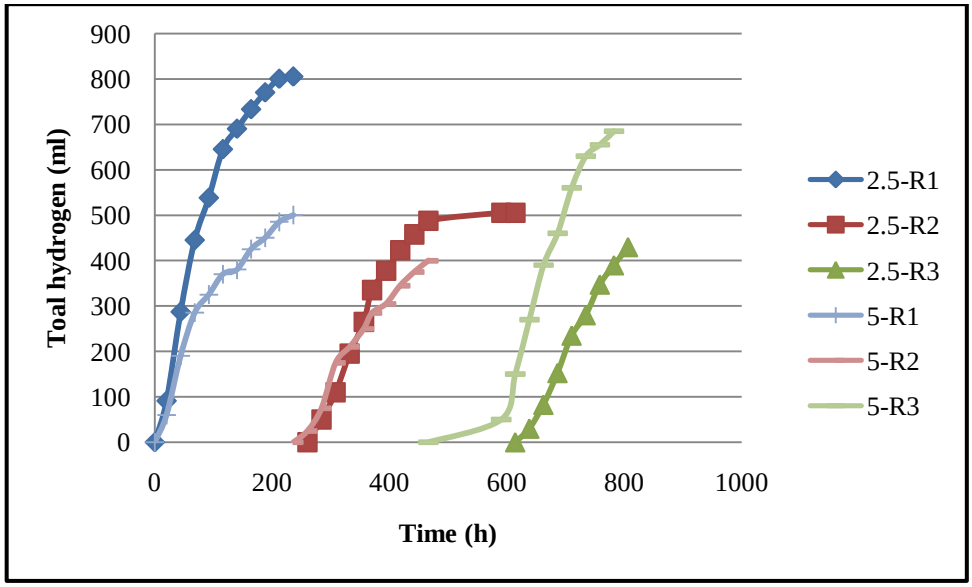


Figure 3.41 Total Hydrogen produced by double experiment using 5 mg DCW/ml agar gel of *R. capsulatus* DSM1710 manipulated with 2.5% and 5% glycerol and immobilized in 4% agar and fed with 60/4 acetate/glutamate

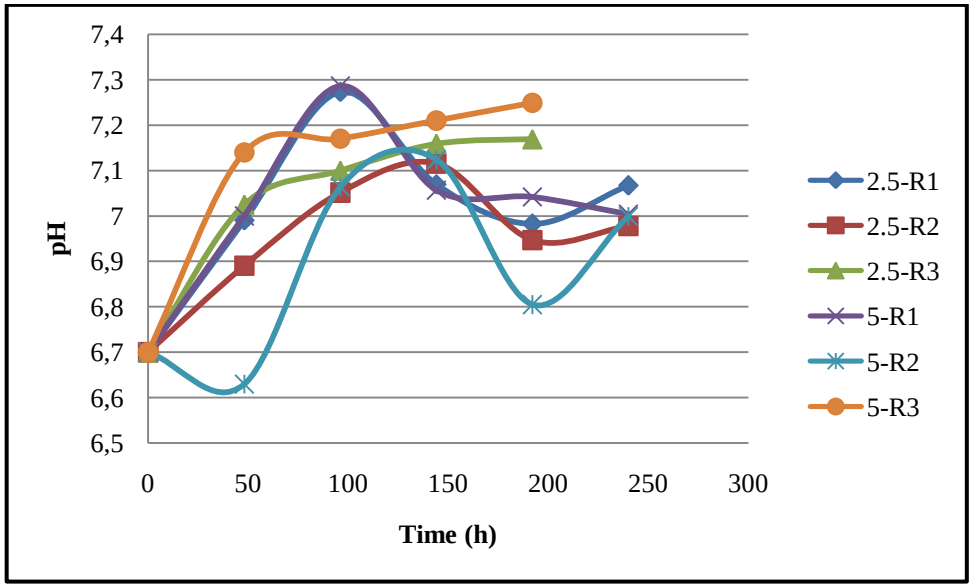


Figure 3.42 Mode of pH change during hydrogen production by double experiment using 5 mg DCW/ml agar-gel of *R. capsulatus* DSM1710 manipulated with 2.5% and 5% glycerol and immobilized in 4% agar and fed with 60/4 acetate/glutamate

3.4.2 Hydrogen production by *Rhodobacter capsulatus* YO3 manipulated with 2.5% and 5% glycerol

The immobilized *Rhodobacter capsulatus* YO3 which has been manipulated with glycerol was fed with 60/4 hydrogen production nutrient medium. This experiment continued for about 710 hours (approximately 30 days) and covered three rounds for each concentration.

As mentioned before the experiment was double and the sequential batches (rounds) for immobilized bacteria manipulated with 2.5% glycerol designated as 2.5-R1, 2.5-R2 and 2.5-R3 while those manipulated with 5% glycerol designated as 5-R1, 5-R2 and 5-R3.

The observed total hydrogen produced by immobilized bacteria manipulated with 2.5% and 5% glycerol is illustrated in Figure 3.43 and Table A. 39. At the end of the three rounds, 2.5-R1, 2.5-R2 and 2.5-R3, average total hydrogen produced for each round was 968, 843, and 680 ml of hydrogen, respectively, from immobilized bacteria manipulated with 2.5% glycerol. The observed total hydrogen produced by immobilized bacteria manipulated with 5% glycerol was 930, 835 and 780 ml of hydrogen from the three rounds 5-R1, 5-R2 and 5-R3, respectively, (Figure 3.43 and Table A. 39).

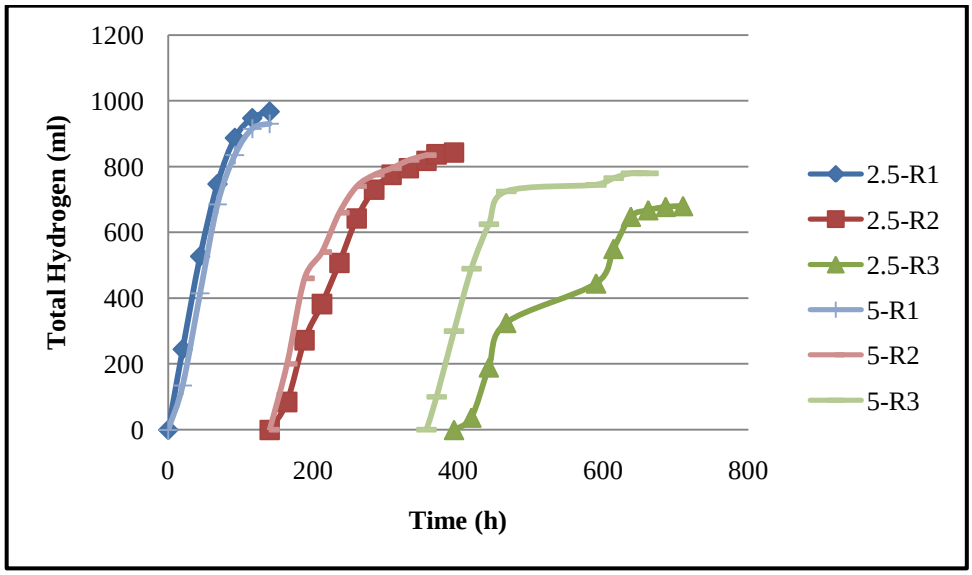


Figure 3.43 Total Hydrogen produced by double experiment using 5 mg DCW/ml agar gel of *R. capsulatus* YO3 manipulated with 2.5% and 5% glycerol and immobilized in 4% agar and fed with 60/4 acetate/glutamate

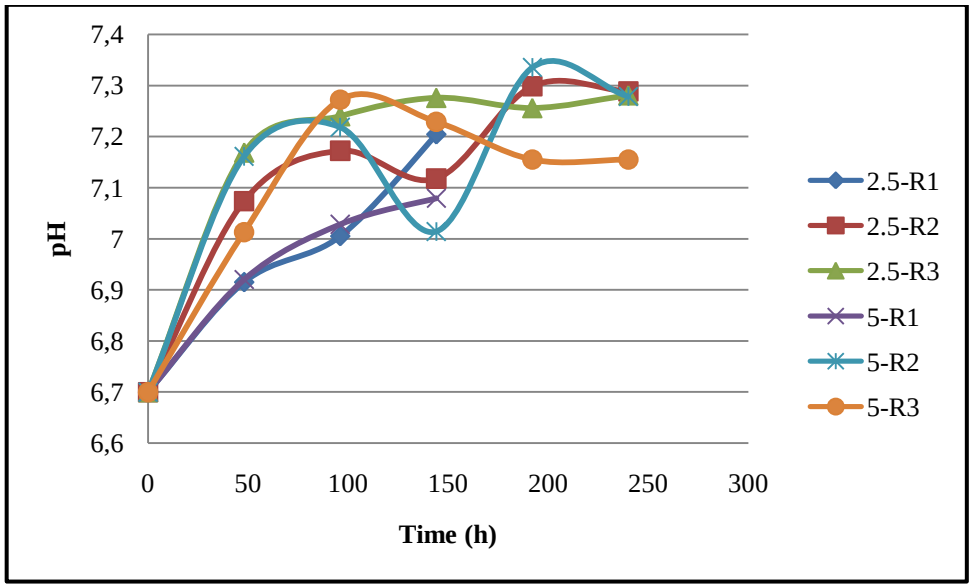


Figure 3.44 Mode of pH change during hydrogen production by double experiment using 5 mg DCW/ml agar-gel of *R. capsulatus* YO3 manipulated with 2.5% and 5% glycerol and immobilized in 4% agar and fed with 60/4 acetate/glutamate

The pH values during this experiment exhibited common stability between initial pH, 6.7, and the highest assigned value which was 7.34, (Figure 3.44 and Table A. 40).

3.5 Effect of different concentrations of sodium dithionite as reducing agent on hydrogen production by *Rhodobacter capsulatus*

Various concentrations of sodium dithionite (1.5, 3 and 4.5 mM) were added to bacteria-gel complex during mixing and before solidification of agar. Same concentrations of sodium dithionite were added to the nutrient medium (60/4). Immobilized bacteria were achieved in 250 ml cell culture bottles where the bacteria-agar complex occupied about 50 ml and 200 ml was occupied by the nutrient medium.

For each concentration of sodium dithionite, two cell culture bottles were specified and the experiment was allowed to work for three sequential batches (rounds) named designated as R1, R2 and R3.

The scanning experiment was conducted with *Rhodobacter capsulatus* YO3 by using 1.5, 3.0 and 4.5 mM of sodium dithionite added to the bacteria-gel complex and to the medium. Depending on the results from this scanning trail (data are not shown) the concentrations of sodium dithionite were assigned as 0.5, 1, and 1.5 mM of sodium dithionite and applied for both strains of photosynthetic bacteria. Sodium dithionite was added to the gel and the nutrient hydrogen production medium 60/4 as described above.

3.5.1 Effect of sodium dithionite on Hydrogen production by *Rhodobacter capsulatus* DSM1710

In this part of study three concentrations of sodium dithionite 0.5, 1.0, 1.5 mM were applied with immobilized *Rhodobacter capsulatus* DSM171. Bacteria were immobilized in 4% agar. Immobilized bacteria were manipulated as described in section 3.5.

Total hydrogen produced by immobilized bacteria manipulated with 1.5 mM sodium dithionite is shown in Figure 3.45. At the end of first and second rounds, 1.5-1R1, 1.5-2R21, 1.5-1R2 and 1.5-2R2; total hydrogen produced was 75, 70, 270 and 220 ml of hydrogen respectively, (Figure 3.45 and Table A. 45).

During third round, immobilized bacteria were fed with 60/4 nutrient medium without sodium dithionite. At the end of this round, total hydrogen produced by 1.5-1R3 and 1.5-2R3 was 380 and 545 ml of hydrogen, (Figure 3.45 and Table A. 45).

During this experiment, major pH values were trending to be between the initial pH, 6.7 and 7.44 except two times, the first was during round 1.5-2R3 when it went to 7.6 and then declined to 7.44, and the second during round 1.5-1R3 when pH reached to 7.55 and then went down steadily to 7.1, (Figure 3.46 and Table A. 46).

Total hydrogen produced by immobilized bacteria manipulated with 1.0 mM sodium dithionite is shown in Figure 3.47. At the end of first and second rounds, 1-1R1, 1-2R21, 1-1R2 and 1-2R2; total hydrogen produced was 45, 55, 150 and 200 ml of hydrogen respectively, (Figure 3.47 and Table A.43).

During third round, immobilized bacteria were fed with 60/4 nutrient medium without sodium dithionite. At the end of this round, total hydrogen produced by 1-1R3 and 1-2R3 was 300 and 280 ml of hydrogen, (Figure 3.47 and Table A. 43).

During this experiment, pH values were trending to be in stable values ranging between the initial pH, 6.7 and 7.4. No pH values observed higher than 7.4, (Figure 3.48 and Table A. 44).

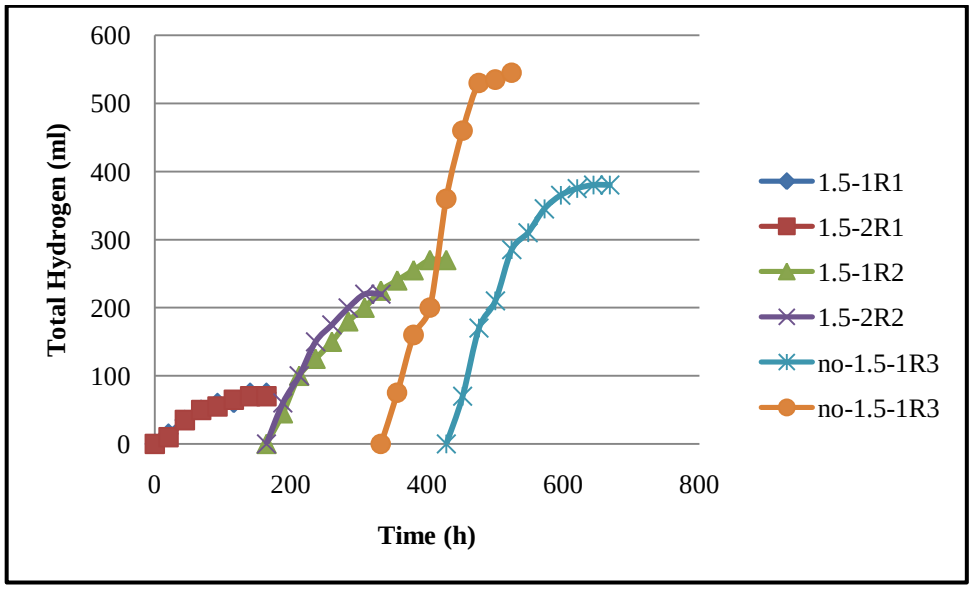


Figure 3.45 Total hydrogen produced during hydrogen production by double experiment using 5 mg DCW/ml agar-gel of *R. capsulatus* DSM1710 provided with 1.5 mM sodium dithionite

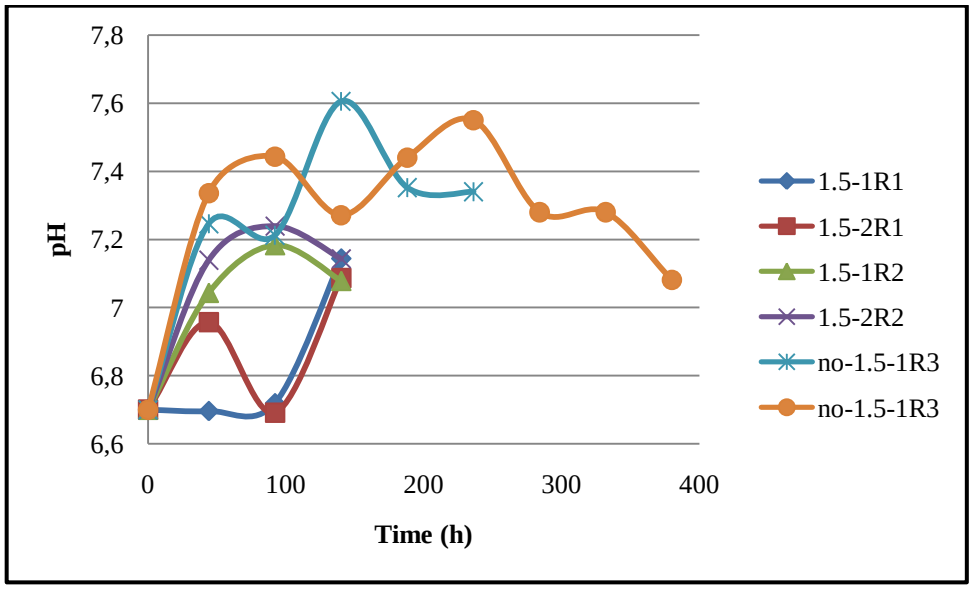


Figure 3.46 Mode of pH change during hydrogen production by double experiment using 5 mg DCW/ml agar-gel of *R. capsulatus* DSM1710 provided with 1.5 mM sodium dithionite

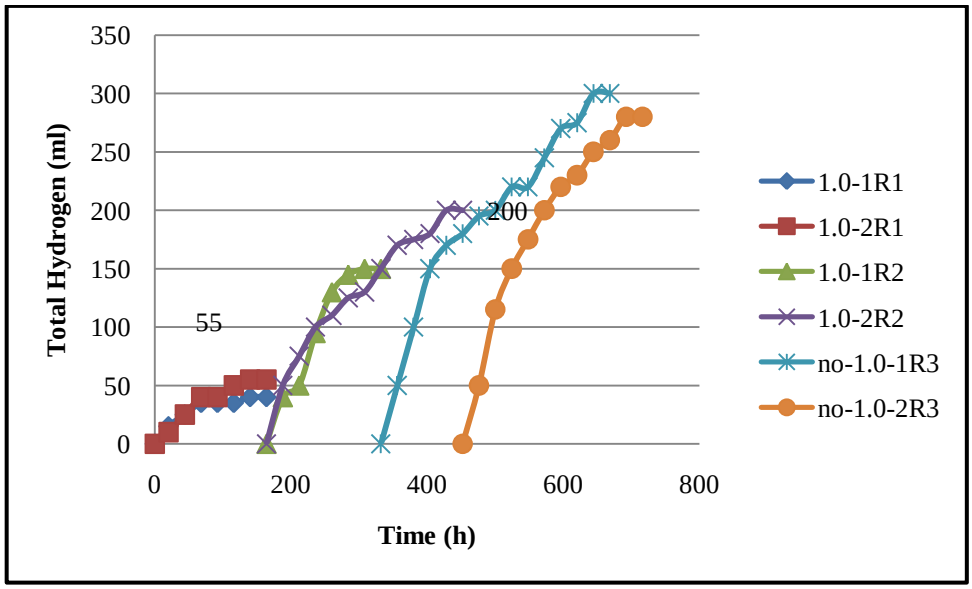


Figure 3.47 Total hydrogen produced during hydrogen production by double experiment using 5 mg DCW/ml agar-gel of *R. capsulatus* DSM1710 provided with 1.0 mM sodium dithionite

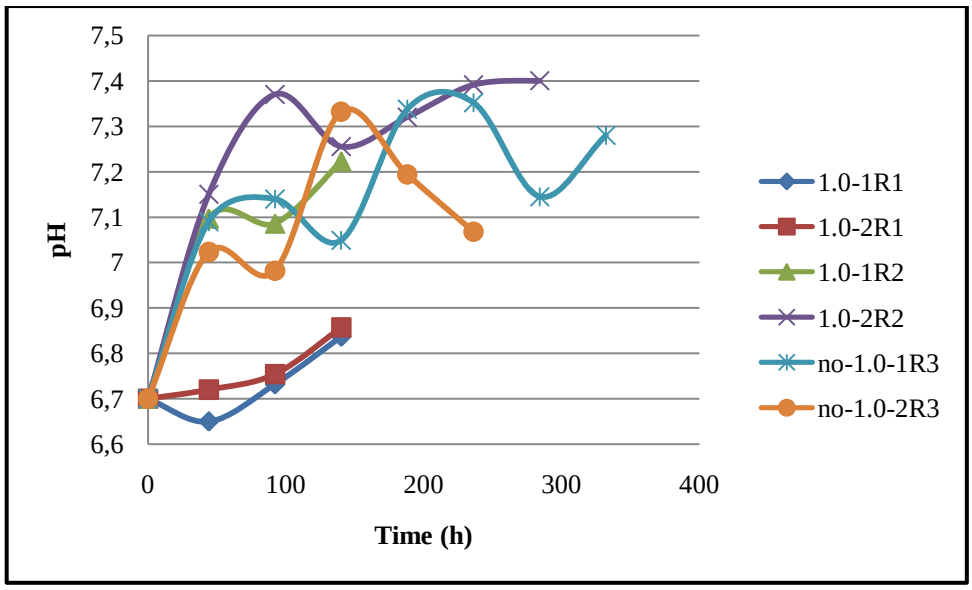


Figure 3.48 Mode of pH change during hydrogen production by double experiment using 5 mg DCW/ml agar-gel of *R. capsulatus* DSM1710 provided with 1.0 mM sodium dithionite

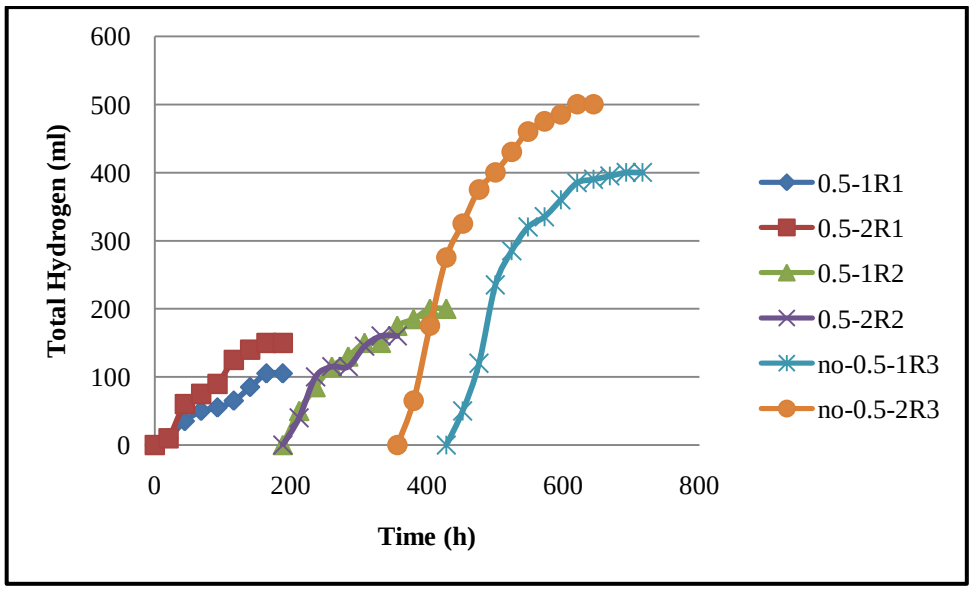


Figure 3.49 Total hydrogen produced during hydrogen production by double experiment using 5 mg DCW/ml agar-gel of *R. capsulatus* DSM1710 provided with 0.5 mM sodium dithionite

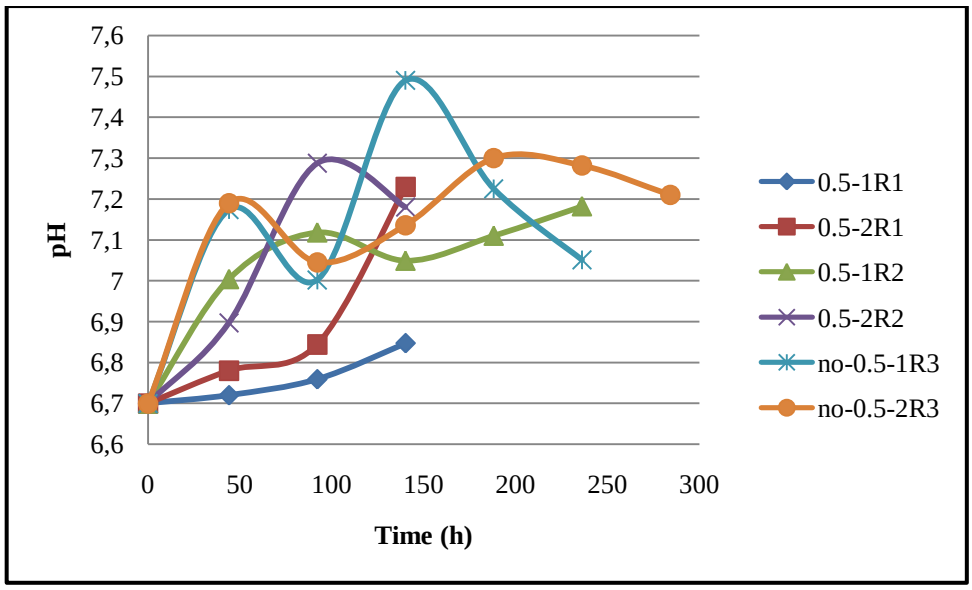


Figure 3.50 Mode of pH change during hydrogen production by double experiment using 5 mg DCW/ml agar-gel of *R. capsulatus* DSM1710 provided with 0.5 mM sodium dithionite

Total hydrogen produced by immobilized bacteria manipulated with 0.5 mM sodium dithionite is shown in Figure 3.49 and Table A. 41. At the end of first and second rounds, 0.5-1R1, 0.5-2R21, 0.5-1R2 and 0.5-2R2; total hydrogen produced was 105, 150, 200 and 160 ml of hydrogen respectively, (Figure 3.49 and Table A. 41).

During third round, immobilized bacteria were fed with 60/4 nutrient medium without sodium dithionite. At the end of this round, total hydrogen produced by 0.5-1R3 and 0.5-2R3 was 400 and 500 ml of hydrogen, (Figure 3.49 and Table A. 41).

During this experiment, major pH values were trending to stabilize between the initial pH, 6.7 and 7.5 and no higher pH values were observed, (Figure 3.50 and Table A. 42).

3.5.2 Effect of sodium dithionite on Hydrogen production by *Rhodobacter capsulatus* YO3

In this part of study three concentrations of sodium dithionite 0.5, 1.0, 1.5 mM were applied with immobilized *Rhodobacter capsulatus* YO3. For immobilizing bacteria 250 cell culture bottles were used and bacteria-agar complex occupied 40 ml of the bottles volume. The immobilized bacteria were provided with 60/4 hydrogen production medium plus the appropriate

concentration of sodium dithionite; 0.5, 1 and 1.5 mM. Six culture bottles were employed in this experiment, two bottles for each concentration.

Total hydrogen produced by immobilized bacteria manipulated with 1.5 mM sodium dithionite is shown in Figure 3.51. At the end of first and second rounds, 1.5-1R1, 1.5-2R1, 1.5-1R2 and 1.5-2R2; total hydrogen produced was 750, 770, 690 and 635 ml of hydrogen respectively, (Figure 3.51 and Table A. 51).

During third round, one of the two bottles (1.5-2) with immobilized bacteria were fed with 60/4 nutrient medium without sodium dithionite while the other bottle (1.5-1) still fed with 60/4 medium containing 1.5 mM sodium dithionite. At the end of this round, total hydrogen produced by 1.5-1R3 and 1.5-2R3 was 260 and 575 ml of hydrogen, (Figure 3.51 and Table A. 51).

During this experiment, pH values exceeded 7.5 several times. In round 1.5-1R3 pH exceeded 7.54 to 7.73 and 7.83 before coming down to 7.6 at the end of this round. During rounds 1.5-1R1 and 1.5-2R2 pH reached to 7.622 and 7.7 before it declines around 7.4 and slightly less. The other rounds enjoyed pH values not more than 7.5 with indication that the initial pH was around 6.7, (Figure 3.52 and Table A. 52).

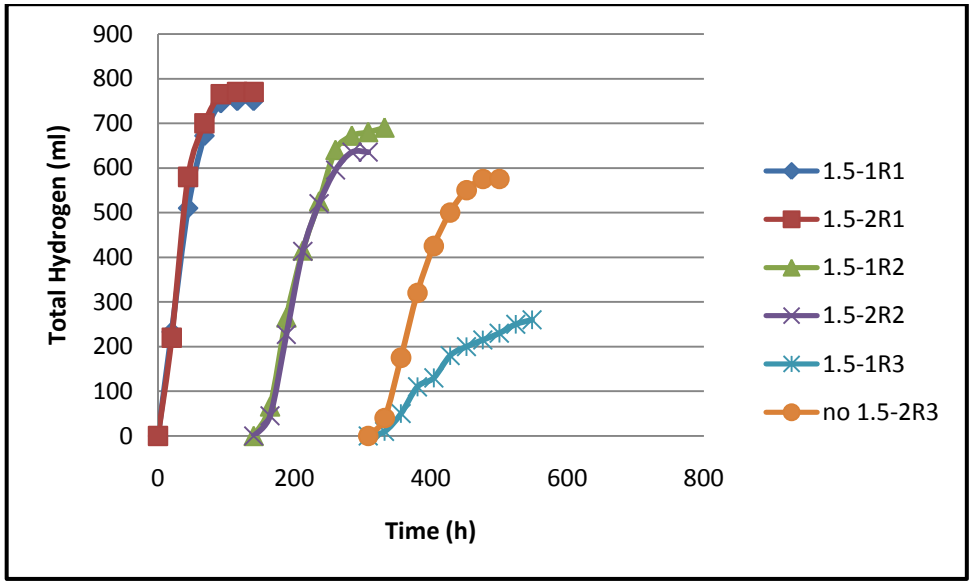


Figure 3.51 Total hydrogen produced during hydrogen production by double experiment using 5 mg DCW/ml agar-gel of *R. capsulatus* YO3 provided with 1.5 mM sodium dithionite

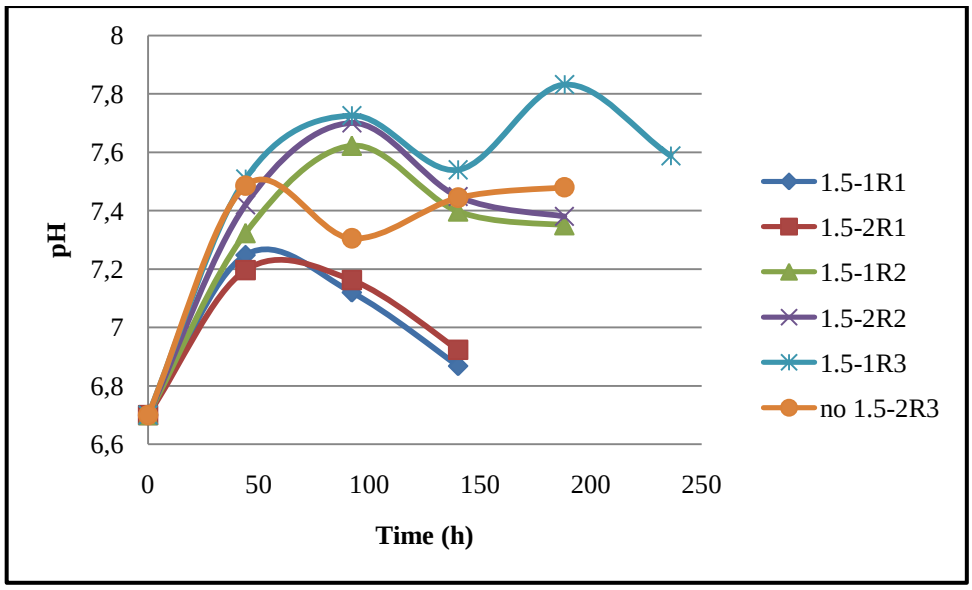


Figure 3.52 Mode of pH change during hydrogen production by double experiment using 5 mg DCW/ml agar-gel of *R. capsulatus* YO3 provided with 1.5 mM sodium dithionite

Total hydrogen produced by immobilized bacteria manipulated with 1.0 mM sodium dithionite is shown in Figure 3.53. At the end of first and second rounds, 1.0-1R1, 1.0-2R1, 1.0-1R2 and 1.0-2R2; total hydrogen produced was 840, 800, 815 and 690 ml of hydrogen respectively, (Figure 3.53 and Table A.49).

At the end of the second round of this experiment, immobilized bacteria start to precipitate black-colored precipitation with very bad smell characterizing the smell of rotten egg. This phenomenon has persisted during the third round and no hydrogen was produced during third round, so no available chance to isolate one bottle to be fed with 60/4 nutrient medium free of sodium dithionite.

During this experiment, pH values exceeded 7.5 just once time and reached to 7.6 before it come down to 7.3 during round 1.0-1R2. The other rounds enjoyed pH values not more than 7.476 with indication that the initial pH was around 6.7, (Figure 3.54 and Table A. 50).

Total hydrogen produced by immobilized bacteria manipulated with 0.5 mM sodium dithionite is shown in Figure 3.55 and Table A. 47. At the end of first and second rounds, 0.5-1R1, 0.5-2R1, 0.5-1R2 and 0.5-2R2; total hydrogen produced was 940, 895, 940 and 540 ml of hydrogen, respectively, (Figure 3.55 and Table A. 47).

During sequential batches (rounds) of this experiment it was observed that the agar gel texture became more softened and fragile. This was seen even when using 1.5 and 1.0 mM

sodium dithionite but with 0.5 mM sodium dithionite its effect caused fragmentation for agar in bottle 0.5-2 bottle. In addition to that, black precipitations and rotten egg smell was more intensified and hydrogen production by this bottle stopped completely in the third round. The black precipitation and rotten egg smell was observed also in the other bottle 0.5-1 and this was going more intensified during third round 0.5-1R3 which produced only 61% of hydrogen produced at the end of the first round 0.5-1R1, (Figure 3.55 and Table A. 47).

During this experiment, pH values did not exceed 7.44. All rounds enjoyed pH between the initial 6.7 pH 7.44. During major times of experiment operation pH values were less than 7.4, (Figure 3.56 and Table A. 48).

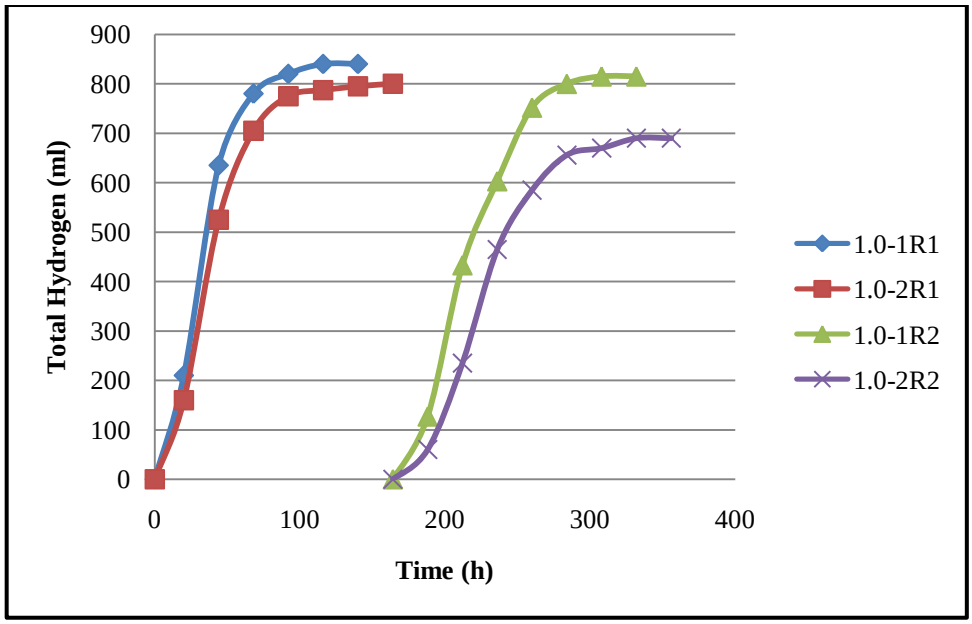


Figure 3.53 Total hydrogen produced during hydrogen production by double experiment using 5 mg DCW/ml agar-gel of *R. capsulatus* YO3 provided with 1.0 mM sodium dithionite

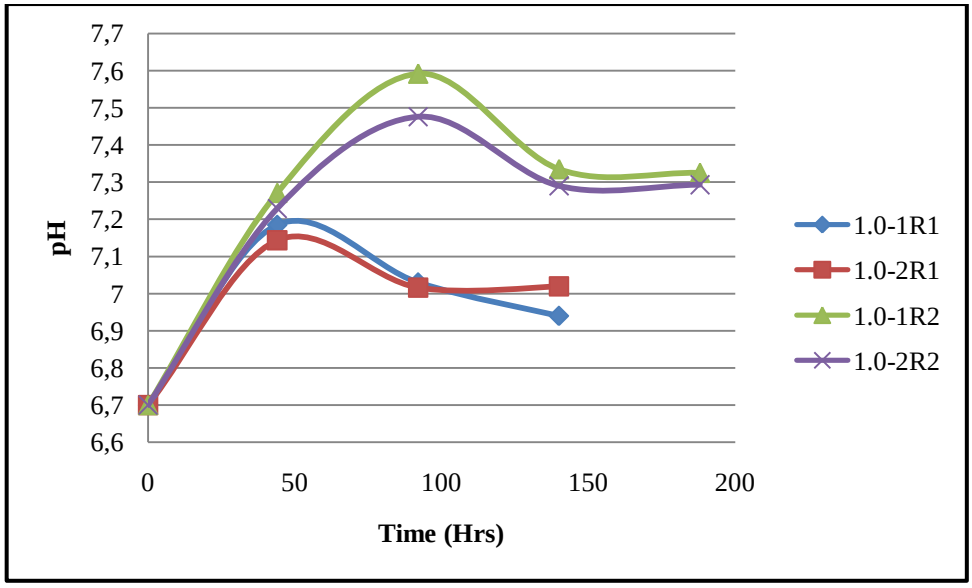


Figure 3.54 Mode of pH change during hydrogen production by double experiment using 5 mg DCW/ml agar-gel of *R. capsulatus* YO3 provided with 1.0 mM sodium dithionite

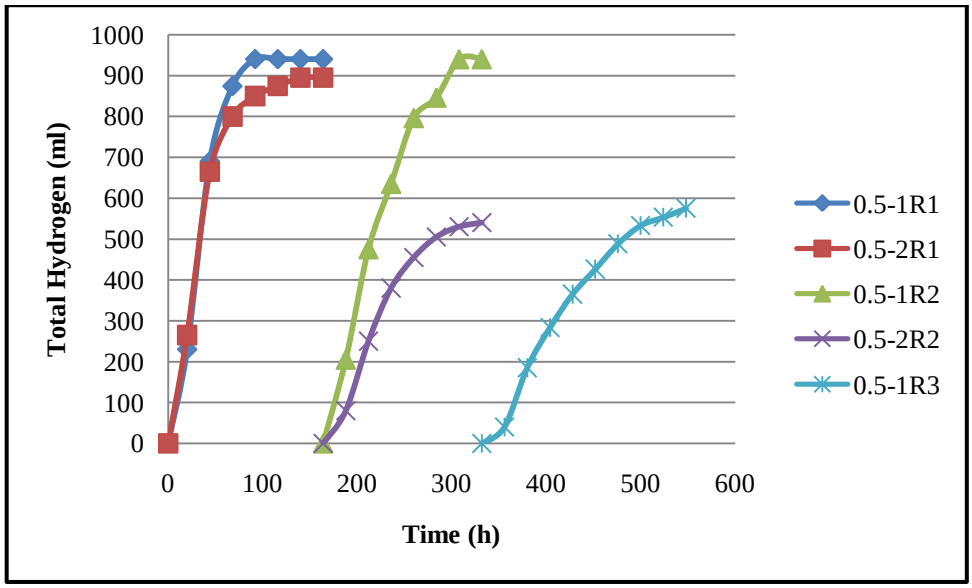


Figure 3.55 Total hydrogen produced during hydrogen production by double experiment using 5 mg DCW/ml agar-gel of *R. capsulatus* YO3 provided with 0.5 mM sodium dithionite

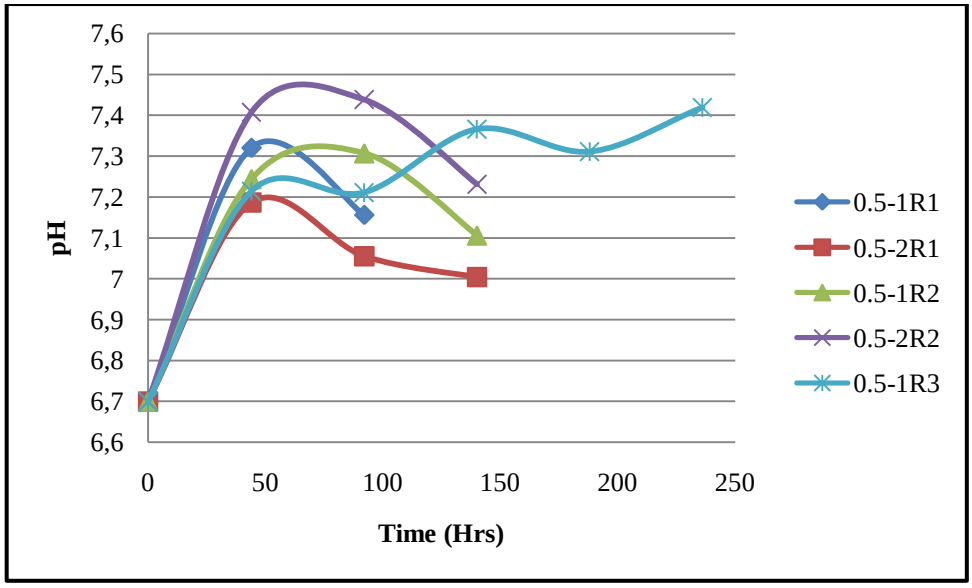


Figure 3.56 Mode of pH change during hydrogen production by double experiment using 5 mg DCW/ml agar-gel of *R. capsulatus* YO3 provided with 0.5 mM sodium dithionite

3.6 Effect of ammonium on hydrogen production by immobilized *Rhodobacter capsulatus*

To study effect of ammonium on hydrogen production by the two investigated strains of photosynthetic bacteria were grown and immobilized as described in materials and methods. Nutrient medium contained 60 mM acetate was used. Glutamate was replaced with the appropriate concentration of ammonium (2.5, 5, & 7.5 mM).

The modified medium was used for hydrogen production in this experiment. Each concentration of ammonium as ammonium chloride was examined two times (doubled) in parallel. Each of the bottles (250 ml cell culture bottles) containing the immobilized bacteria was operated in batch sequential manner for three rounds as described in materials and methods.

3.6.1 Effect of ammonium on hydrogen production by immobilized *Rhodobacter capsulatus* DSM 1710

Figure 3.57 and Table A. 53 show the cumulative hydrogen production by immobilized *Rhodobacter capsulatus* DSM 1710 strain which was fed with nutrient medium containing 2.5

mM of ammonium chloride as nitrogen source. The cumulative hydrogen production illustrated in Figure 3.57 and Table A.53 show the values through the whole sequential batch process covering three rounds for each bottle. The time period of the process continued for 468 hours (19.5 days). The doubled values for each round represent two bottles, 2.5-1 and 2.5-2. Designs R1, R2 and R3 indicate the rounds during sequential batch process.

At the end of the first round, cumulative hydrogen production from bottles 2.5-1 and 2.5-2 was 350 and 344 ml hydrogen per bottle respectively. Cumulative hydrogen produced at the end of second round recorded as 205 and 200 ml hydrogen per bottle for bottles 2.5-1 and 2.5-2 respectively. Round 3 shows the cumulative hydrogen production as 130 ml of hydrogen per bottle for each of the two bottles, 2.5-1 and 2.5-2.

Figure 3.58 and Table A. 54 illustrates the change mode of pH values during the whole time of the process. Values of pH during the three rounds of the process exhibited stability between 6.7 (initial pH value) and 7.34 which was the highest value reached during this process.

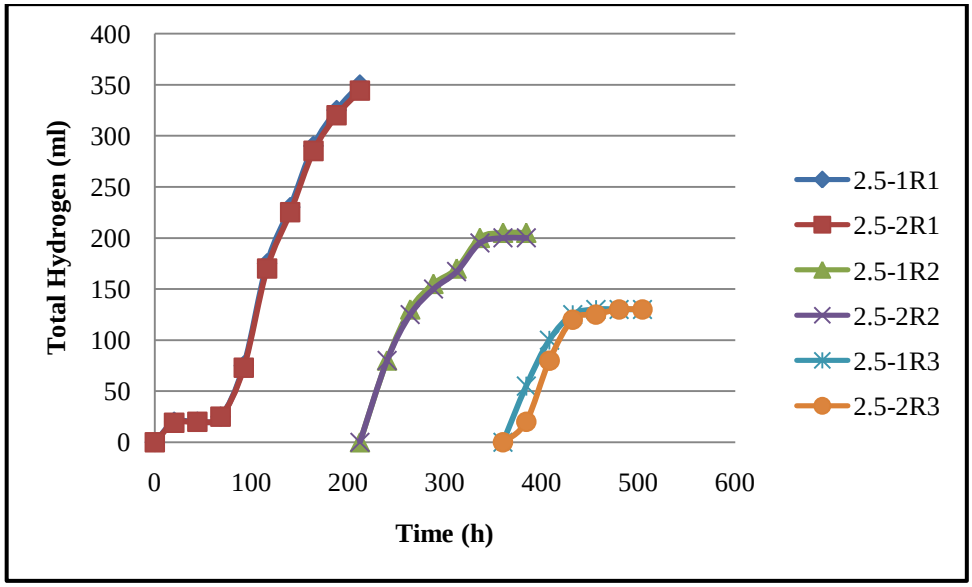


Figure 3.57 Total hydrogen produced by immobilized *Rhodobacter capsulatus* DSM 1710 strain provided with 2.5 mM of ammonium

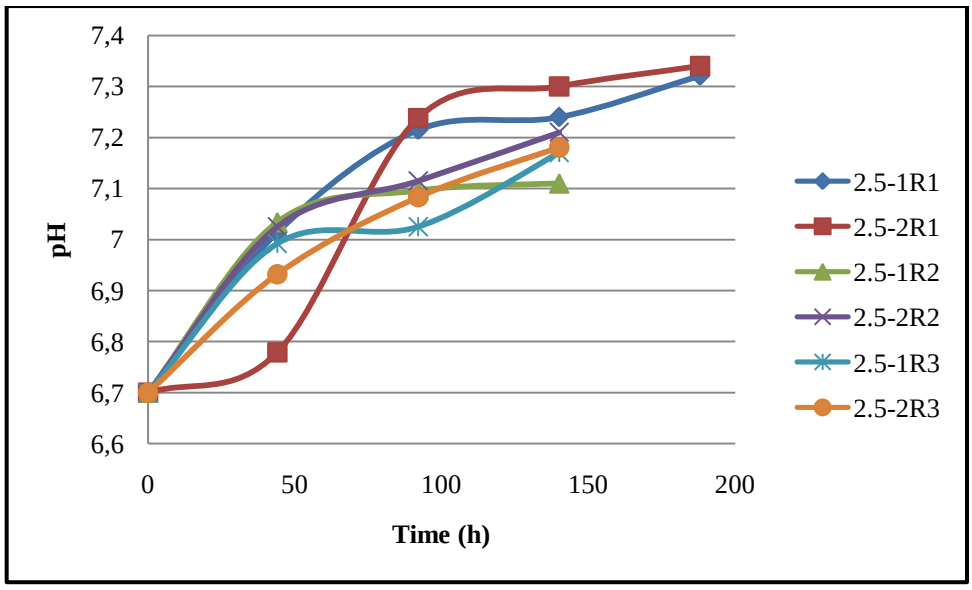


Figure 3.58 Mode of pH change during hydrogen production by immobilized *Rhodobacter capsulatus* DSM 1710 strain provided with 2.5 mM of ammonium

Figure 3.59 and Table A. 55 show the cumulative hydrogen production by immobilized *Rhodobacter capsulatus* DSM 1710 strain which was fed with nutrient medium containing 5 mM of ammonium chloride as nitrogen source.

The cumulative hydrogen production illustrated in Figure 3.59 and Table A. 55 show the values through the whole sequential batch process covering three rounds for each bottle. The time period of the process continued for 420 hours (17.5 days). The doubled values for each round represent two bottles, 5-1 and 5-2. Designs R1, R2 and R3 indicate the rounds during sequential batch process.

At the end of the first round, cumulative hydrogen production from bottles 5-1 and 5-2 was 225 and 220 ml hydrogen per bottle respectively. Cumulative hydrogen produced at the end of second round recorded as 225 and 220 ml hydrogen per bottle for bottles 5-1 and 5-2 respectively. Round 3 shows the cumulative hydrogen production as 75 and 80 ml of hydrogen per bottle for bottles 5-1 and 5-2 respectively.

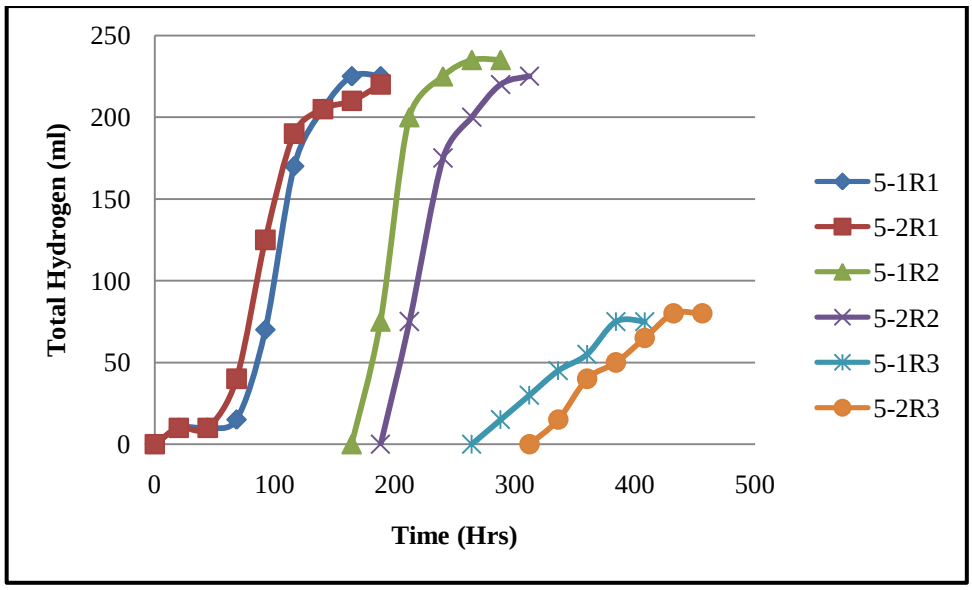


Figure 3.59 Total hydrogen produced by immobilized *Rhodobacter capsulatus* DSM 1710 strain provided with 5 mM of ammonium

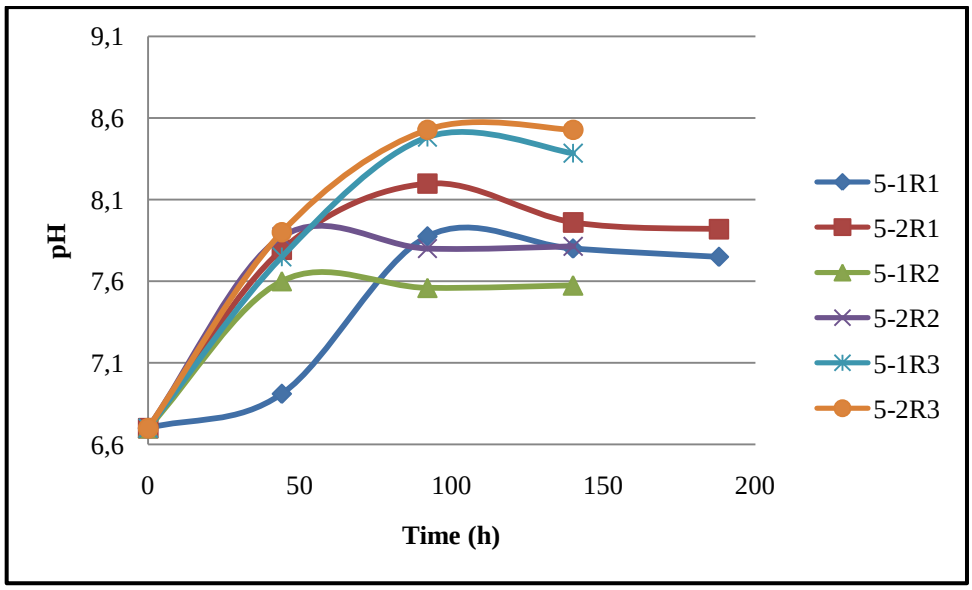


Figure 3.60 Mode of pH change during hydrogen production by immobilized *Rhodobacter capsulatus* DSM 1710 strain provided with 5 mM of ammonium

Figure 3.60 and Table A. 56 illustrates the change mode of pH values during the whole time of the process. Values of pH during the first round of the process exhibited increase of pH from 6.7 (initial pH value) up to 7.88 in bottle 5-1 and up to 8.2 in bottle 5-2. During the second round maximum pH value in bottle 5-1 ranged from 6.7 to 7.6 and 6.7 to 7.87 in bottle 5-2. Values of pH during round 3 acquired high values persisted more than 8.5. In bottle 5-1 it was from 6.7 to 8.5 and in bottle 5-2 from 6.7 to 8.53.

Figure 3.61 and Table A. 57 show the cumulative hydrogen production by immobilized *Rhodobacter capsulatus* DSM 1710 strain which was fed with nutrient medium containing 7.5 mM of ammonium chloride as nitrogen source.

The cumulative hydrogen production illustrated in Figure 3.61 and Table A. 57 show the values through the whole sequential batch process covering three rounds for each bottle. The time period of the process continued for 396 hours (16.5 days).

The doubled values for each round represent two bottles 7.5-1 and 7.5-2. Designs R1, R2 and R3 indicate the rounds during sequential batch process. At the end of the first round, cumulative hydrogen production from bottles 7.5-1 and 7.5-2 was 196 and 200 ml hydrogen per bottle respectively.

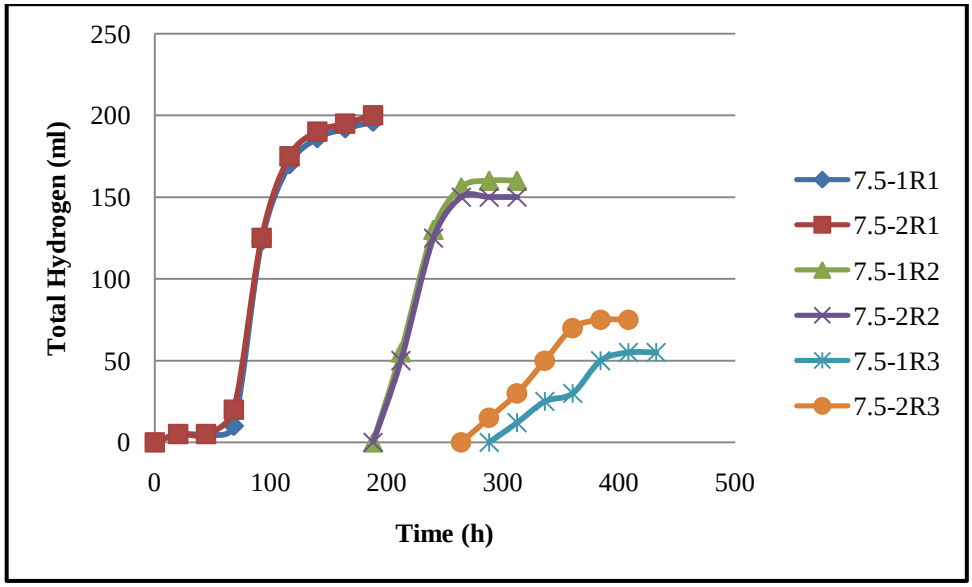


Figure 3.61 Total hydrogen produced by immobilized *Rhodobacter capsulatus* DSM 1710 strain provided with 7.5 mM of ammonium

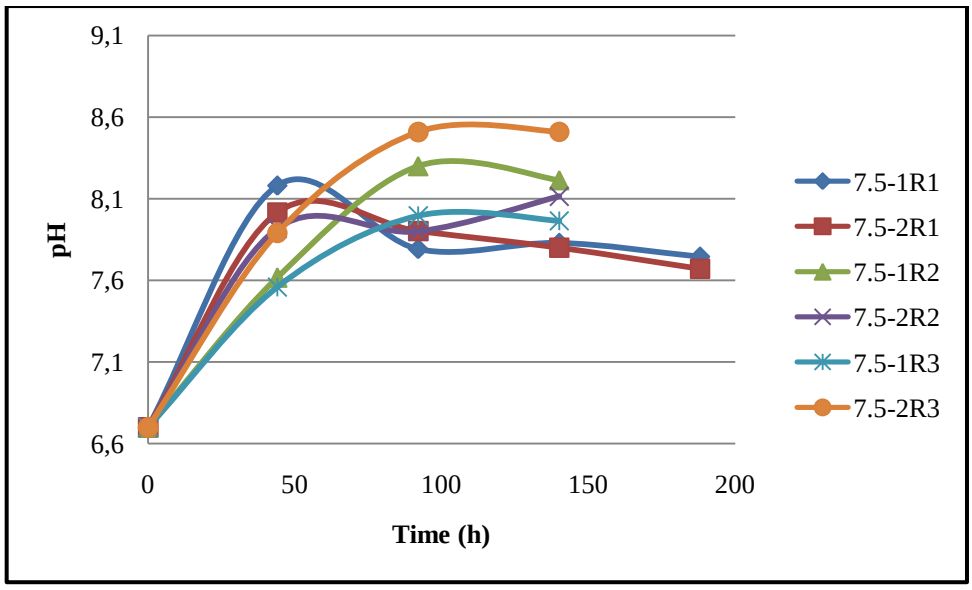


Figure 3.62 Mode of pH change during hydrogen production by immobilized *Rhodobacter capsulatus* DSM 1710 strain provided with 7.5 mM of ammonium

Cumulative hydrogen produced at the end of second round was recorded as 160 and 150 ml hydrogen per bottle from bottles 7.5-1 and 7.5-2 respectively. Round 3 shows the cumulative hydrogen production as 55 and 75 ml of hydrogen per bottle for bottles 7.5-1 and 7.5-2 respectively.

Figure 3.62 and Table A. 58 illustrates the change mode of pH values during the whole time of the process. Values of pH during the first round of the process exhibited increase of pH from 6.7 (initial pH value) up to 8.2 in bottle 7.5-1 and up to 8.0 in bottle 7.5-2. During the second round pH value in bottle 7.5-1 ranged from 6.7 to 8.3 and 6.7 to 8.1 in bottle 7.5-2. Values of pH during round 3 estimated as 6.7 to 8.00 in bottle 7.5-1 and 6.7 to 8.5 in the bottle 7.5-2.

3.6.2 Effect of ammonium on hydrogen production by immobilized *Rhodobacter capsulatus* YO3

Figure 3.63 and Table A. 59 show the cumulative hydrogen production by immobilized *Rhodobacter capsulatus* YO3 strain which was fed with nutrient medium containing 2.5 mM of ammonium chloride as nitrogen source. The cumulative hydrogen production illustrated in Figure 3.63 and Table A. 59 show the values through the whole sequential batch process

covering three rounds for each bottle. The time period of the process continued for 492 hours (20.5 days). The doubled values for each round represent two bottles, 2.5-1 and 2.5-2. Designs R1, R2 and R3 indicate the rounds during sequential batch process.

At the end of the first round, cumulative hydrogen production from bottles 2.5-1 and 2.5-2 was 840 and 860 ml hydrogen per bottle respectively. Cumulative hydrogen produced at the end of second round recorded as 500 and 512 ml hydrogen per bottle for bottles 2.5-1 and 2.5-2 respectively. Round 3 shows the cumulative hydrogen production as 110 and 115 ml of hydrogen per bottle for each of the two bottles, 2.5-1 and 2.5-2 respectively.

Figure 3.64 and Table A. 60 illustrate the change mode of pH values during hydrogen production by immobilized YO3 bacteria provided with 2.5 mM ammonium. Values of pH during the three rounds of the process exhibited stability between 6.7 (initial pH value) and 7.35 which was the highest value recorded during this process.

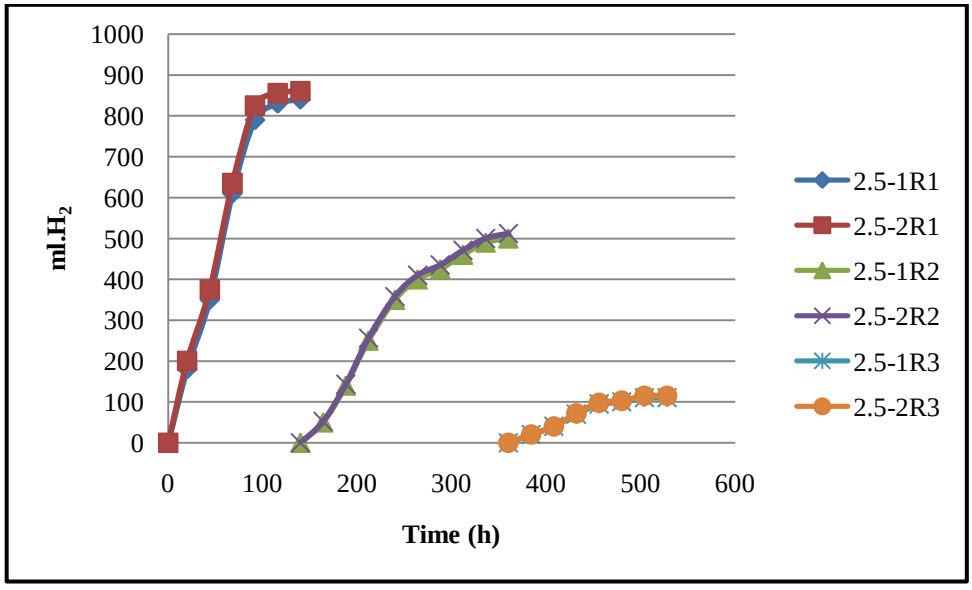


Figure 3.63 Total hydrogen produced by immobilized *Rhodobacter capsulatus* YO3 strain provided with 2.5 mM of ammonium

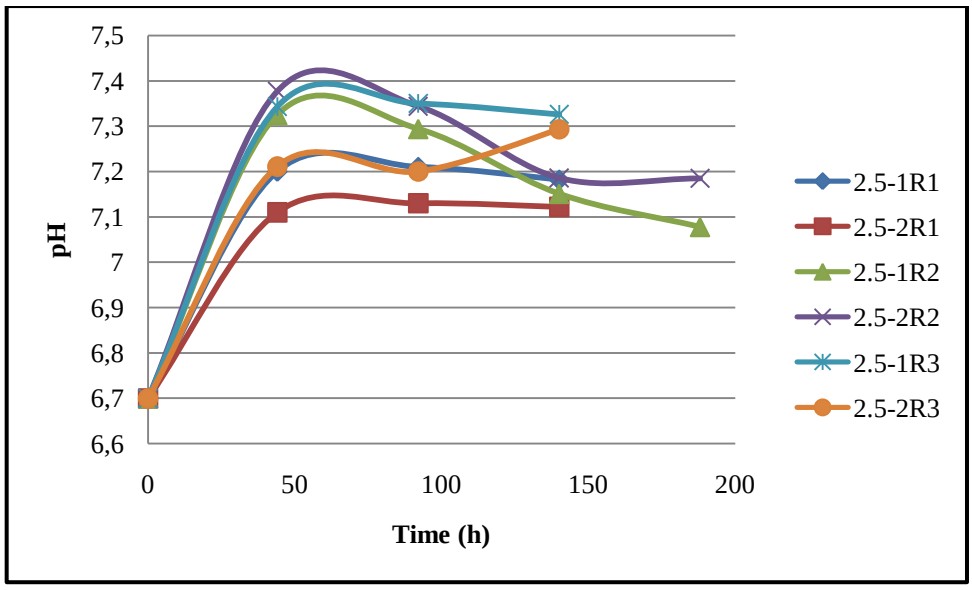


Figure 3.64 Mode of pH change during hydrogen production by immobilized *Rhodobacter capsulatus* YO3 strain provided with 2.5 mM of ammonium

Figure 3.65 and Table A. 61 show the cumulative hydrogen production by immobilized *Rhodobacter capsulatus* YO3 strain which was fed with nutrient medium containing 5 mM of ammonium chloride as nitrogen source.

The cumulative hydrogen production illustrated in Figure 3.65 and Table A. 61 shows the values through the whole sequential batch process covering three rounds for each bottle. The time period of the process continued for 444 hours (18.5 days). The doubled values for each round represent two bottles, 5-1 and 5-2. Designs R1, R2 and R3 indicate the rounds during sequential batch process.

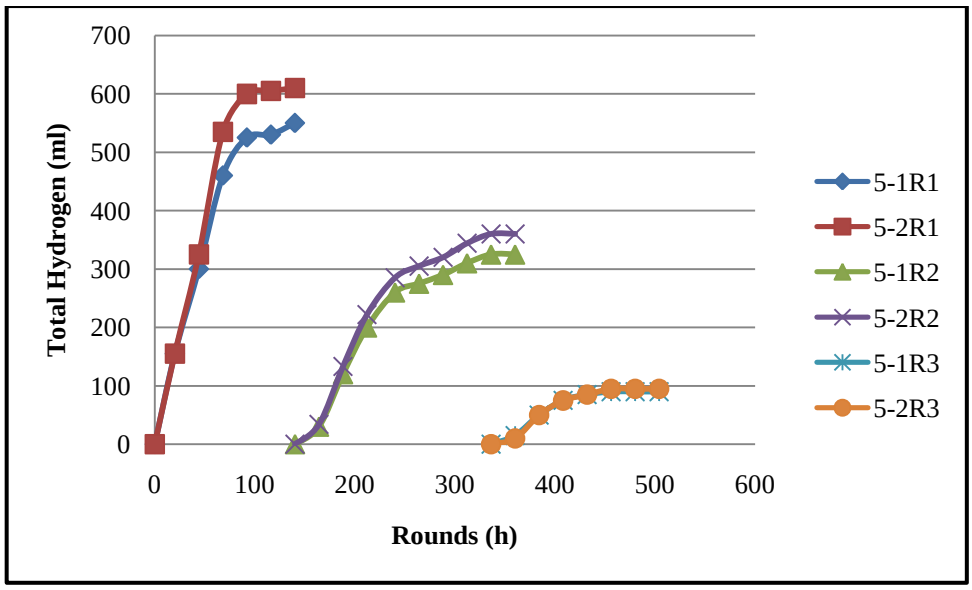


Figure 3.65 Total hydrogen produced by immobilized *Rhodobacter capsulatus* YO3 strain provided with 5 mM of ammonium

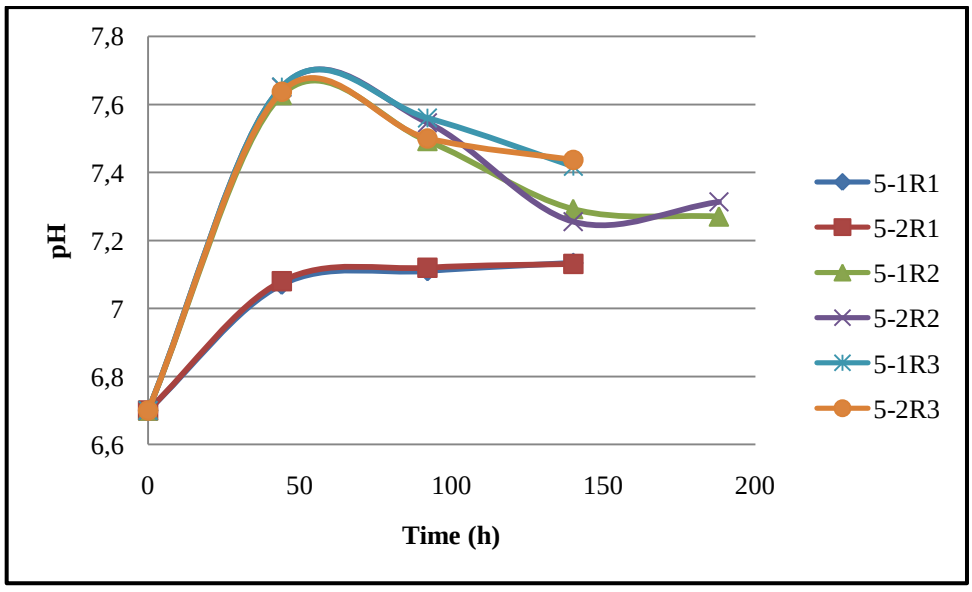


Figure 3.66 Mode of pH change during hydrogen production by immobilized *Rhodobacter capsulatus* YO3 strain provided with 5 mM of ammonium

At the end of the first round, cumulative hydrogen production from bottles 5-1 and 5-2 was 550 and 610 ml hydrogen per bottle respectively. Cumulative hydrogen produced at the end

of second round recorded as 325 and 360 ml hydrogen per bottle for bottles 5-1 and 5-2 respectively. Round 3 shows the cumulative hydrogen production as 90 and 95 ml of hydrogen per bottle for bottles 5-1 and 5-2 respectively.

Figure 3.66 and Table A. 62 illustrate mode of pH change during hydrogen production by immobilized YO3 bacteria provided with 5.0 mM ammonium.

Values of pH during the first round of the process exhibited stability of pH at moderate values from 6.7 (initial pH value) to 7.21 in bottle 5-1 and up to 7.13 in bottle 5-2. During the second round maximum pH value in bottle 5-1 ranged from 6.7 to 7.63 and 6.7 to 7.65 in bottle 5-2. Values of pH during round 3 were still acquiring moderate values. In bottle 5-1 it was from 6.7 to 7.65 and in bottle 5-2 from 6.7 to 7.64.

Figure 3.67 and Table A. 63 show the cumulative hydrogen production by immobilized *Rhodobacter capsulatus* YO3 strain which was fed with nutrient medium containing 7.5 mM of ammonium chloride as sole nitrogen source. The cumulative hydrogen production illustrated in Figure 3.67 and Table A. 63 shows the values through the whole sequential batch process covering three rounds for each bottle.

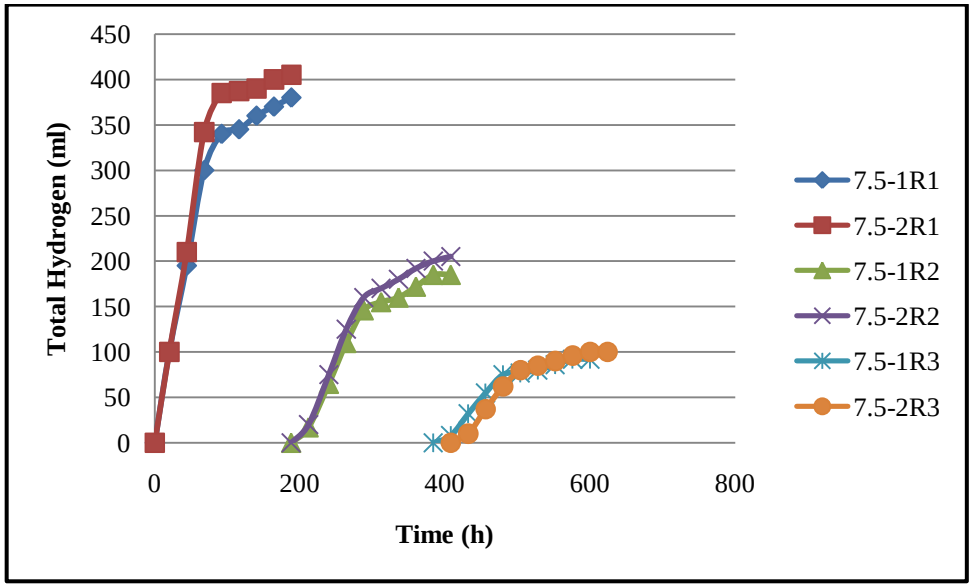


Figure 3.67 Total hydrogen produced by immobilized *Rhodobacter capsulatus* YO3 strain provided with 7.5 mM of ammonium

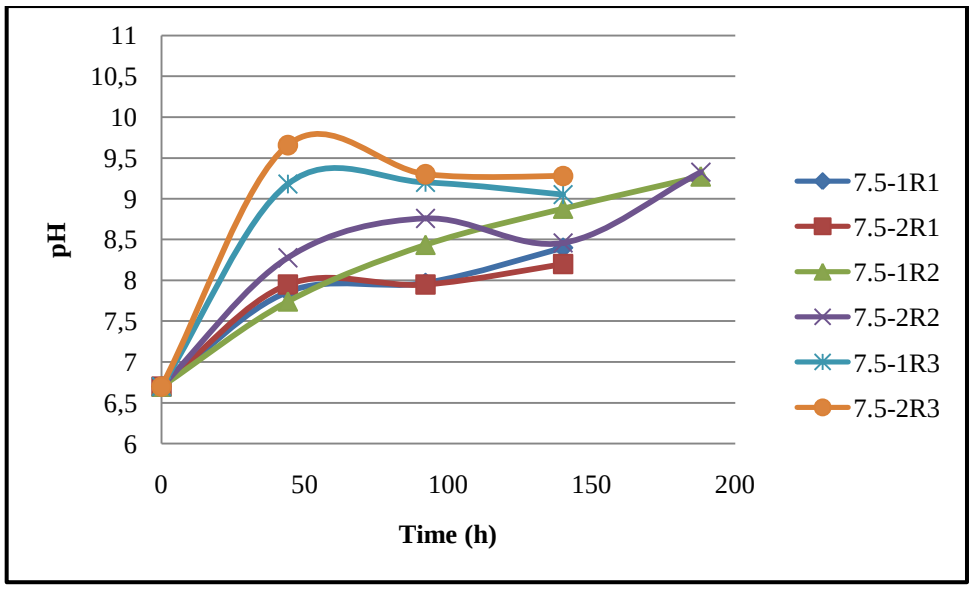


Figure 3.68 Mode of pH change during hydrogen production by immobilized *Rhodobacter capsulatus* YO3 strain provided with 7.5 mM of ammonium

The time period of the process continued for 580 hours (24.5 days). The doubled values for each round represent two bottles 7.5-1 and 7.5-2. Designs R1, R2 and R3 indicate the rounds during sequential batch process.

At the end of the first round, cumulative hydrogen production from bottles 7.5-1 and 7.5-2 was 380 and 405 ml hydrogen per bottle respectively. Cumulative hydrogen produced at the end of second round recorded as 185 and 205 ml hydrogen per bottle for bottles 7.5-1 and 7.5-2 respectively. Round 3 shows the cumulative hydrogen production as 92 and 100 ml of hydrogen per bottle for bottles 7.5-1 and 7.5-2 respectively.

Figure 3.68 and Table A. 64 illustrate the change mode of pH values during hydrogen production by immobilized YO3 bacteria provided by 7.5 mM ammonium.

Values of pH during the first round of the process exhibited increase of pH from 6.7 (initial pH value) up to 8.4 in bottle 7.5-1 and up to 8.2 in bottle 7.5-2. During the second round pH value in bottle 7.5-1 ranged from 6.7 to 9.273 and 6.7 to 9326 in bottle 7.5-2. Values of pH during round 3 estimated as 6.7 to 9.2 in bottle 7.5-1 and 6.7 to 9.657 in the bottle 7.5-2.

3.7 Effect of co-immobilizing photosynthetic bacteria *Rhodobacter capsulatus* and packed cells of *Halobacterium salinarium* S-9 on hydrogen production

The halophilic bacteria *Halobacterium salinarium* were used as packed cells to examine its effect on hydrogen production by photosynthetic bacteria *Rhodobacter capsulatus* (DSM 1710 & YO3 strains). To study effect co-immobilization of *H. salinarium* with the photosynthetic bacteria on hydrogen production by the two investigated strains YO3 and DSM 1710. Bacteria were grown and immobilized as described in materials and methods. Nutrient medium for hydrogen production was containing 60 mM acetate and 4 mM glutamate.

3.7.1 Hydrogen production by co-immobilizing photosynthetic bacteria *R. capsulatus* DSM 1710 strain and halophilic bacteria *Halobacterium salinarium* S-9

Figure 3.69 and Table A. 65 show the cumulative hydrogen production by *Rhodobacter capsulatus* DSM 1710 strain which was co-immobilized with packed cells of *H. salinarium* S-9. The co-immobilized bacteria were fed with nutrient medium containing 60 mM acetate and 4 mM of glutamate.

Each co-immobilized culture was doubled in parallel and a single culture of R. capsulatus without co-immobilization was employed for comparison. The cumulative hydrogen production illustrated in Figure 3.69 and Table A. 65 show the values through the whole sequential batch process covering three rounds for each bottle. The time period of the process continued for 684 hours (28.5 days). The doubled values for each round represent two bottles containing co-immobilized bacteria, D-HS1 and D-HS2. Designs R1, R2 and R3 indicate the rounds during sequential batch process. Symbol D stands for control immobilized DSM 1710 bacteria without packed cells of *H. salinarium*.

At the end of the first round, cumulative hydrogen production from bottles D-HS1 and D-HS2 was 770 and 740 ml hydrogen per bottle respectively while the non co-immobilized bacteria produced 550 ml of hydrogen per bottle. Cumulative hydrogen produced at the end of second round recorded as 650, 660 and 625 ml hydrogen per bottle for bottles D-HS1, D-HS2 and D bottles respectively.

At the end of third round (R3), the cumulative hydrogen production as 625, 635 and 615 ml of hydrogen per bottle for each of the three bottles, D-HS1, D-HS2 and D respectively.

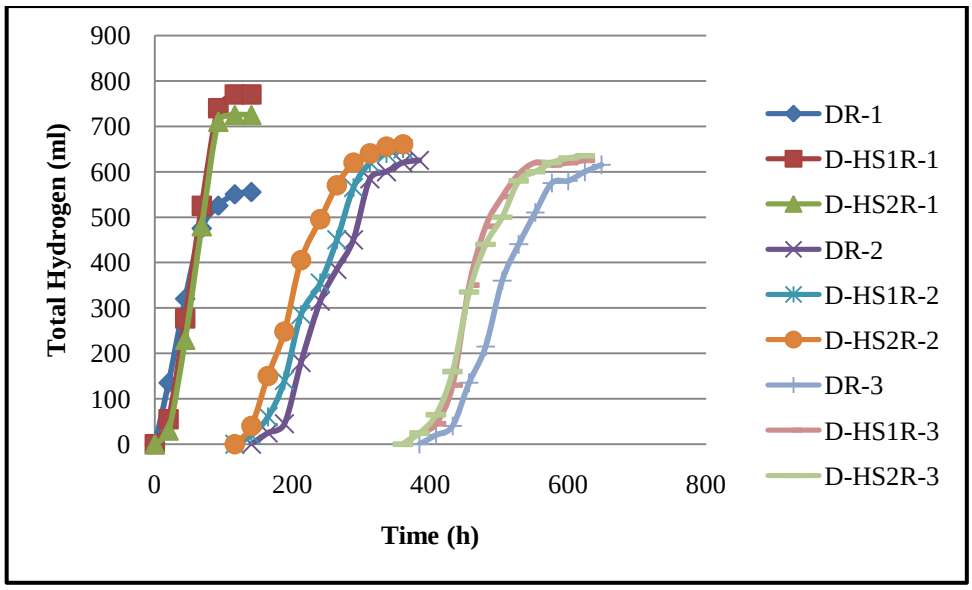


Figure 3.69 Total hydrogen produced by *Rhodobacter capsulatus* DSM 1710 strain co-immobilized with packed cells of *H. salinarium*

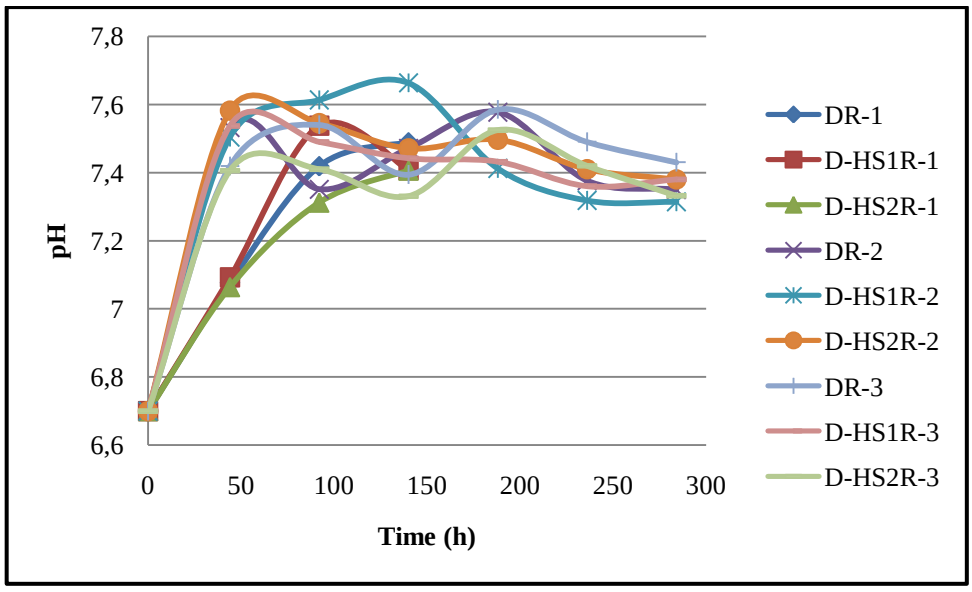


Figure 3.70 Mode of pH change during hydrogen production by *Rhodobacter capsulatus* DSM 1710 strain co-immobilized with packed cells of *H. salinarium*

Figure 3.70 and Table A. 66 illustrates the change mode of pH values during the whole time of the process. Values of pH during the first round of the process exhibited moderate increase of pH from 6.7 (initial pH value) up to less than 6.535 in all bottles. During the second round pH value in bottle D-HS1 increased up to 7.66 while other bottles exhibited lower values, mostly less than 7.5. Values of pH during round 3 did not exceed 7.585 in all bottles.

3.7.2 Hydrogen production by co-immobilizing photosynthetic bacteria *R. capsulatus* YO3 strain and halophilic bacteria *Halobacterium salinarium* S-9

Figure 3.71 and Table A. 67 show the cumulative hydrogen production by *Rhodobacter capsulatus* YO3 strain which was co-immobilized with packed cells of *H. salinarium* S-9. The co-immobilized bacteria were fed with nutrient medium containing 60 mM acetate and 4 mM of glutamate. Each co-immobilized culture was doubled in parallel and a single culture of *R. capsulatus* without co-immobilization was employed for comparison.

The cumulative hydrogen production illustrated in Figure 3.70 and Table A. 67 show the values through the whole sequential batch process covering three rounds for each bottle. The time period of the process continued for 516 hours (21.5 days). The doubled values for each round represent two bottles containing co-immobilized bacteria, Y-HS1 and Y-HS2.

The signs R1, R2 and R3 indicate the rounds during sequential batch process. Symbol Y stands for control immobilized YO3 strain bacteria without packed cells of *H. salinarium*.

At the end of the first round, cumulative hydrogen production from bottles Y-HS1 and Y-HS2 was 1260 and 1275 ml hydrogen per bottle respectively while the non-co-immobilized bacteria produced 905 ml of hydrogen per bottle. Cumulative hydrogen produced at the end of second round recorded as 1240, 1110 and 800 ml hydrogen per bottle for bottles Y-HS1, Y-HS2 and Y bottles respectively. Round 3 shows the cumulative hydrogen production as 725, 840 and 825 ml of hydrogen per bottle for each of the three bottles, Y-HS1, Y-HS2 and Y respectively.

Figure 3.72 and Table A. 68 illustrates the change mode of pH values during the whole time of the process. Values of pH during the first round of the process exhibited moderate increase of pH from 6.7 (initial pH value) up to less than 6.53 in all bottles. During the second round pH in all bottles enjoyed moderate optimum values and did not exceed 7.47 Values of pH during round 3 did not exceed 7.45 in all bottles and kept moderate optimum levels.

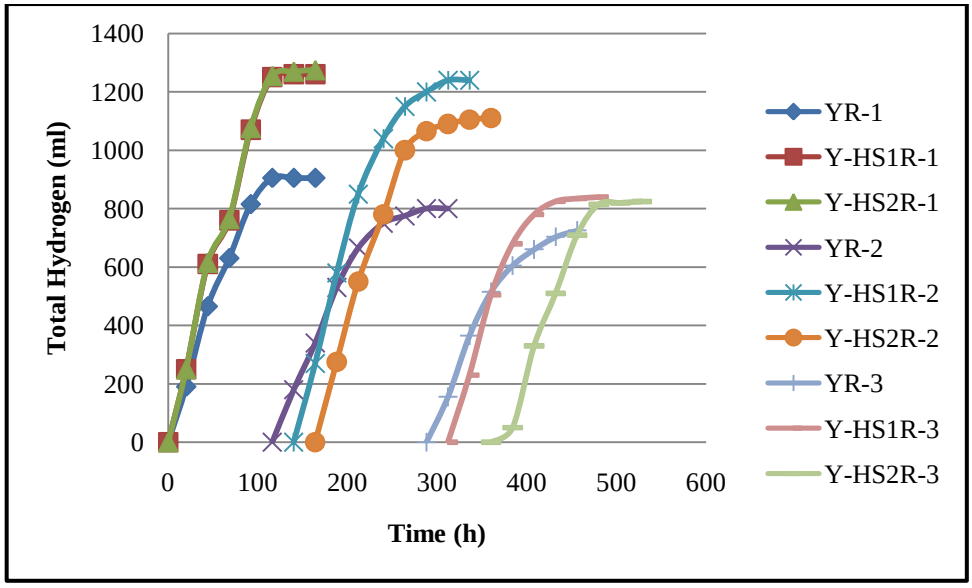


Figure 3.71 Total hydrogen produced by *Rhodobacter capsulatus* YO3 strain co-immobilized with packed cells of *H. salinarium*

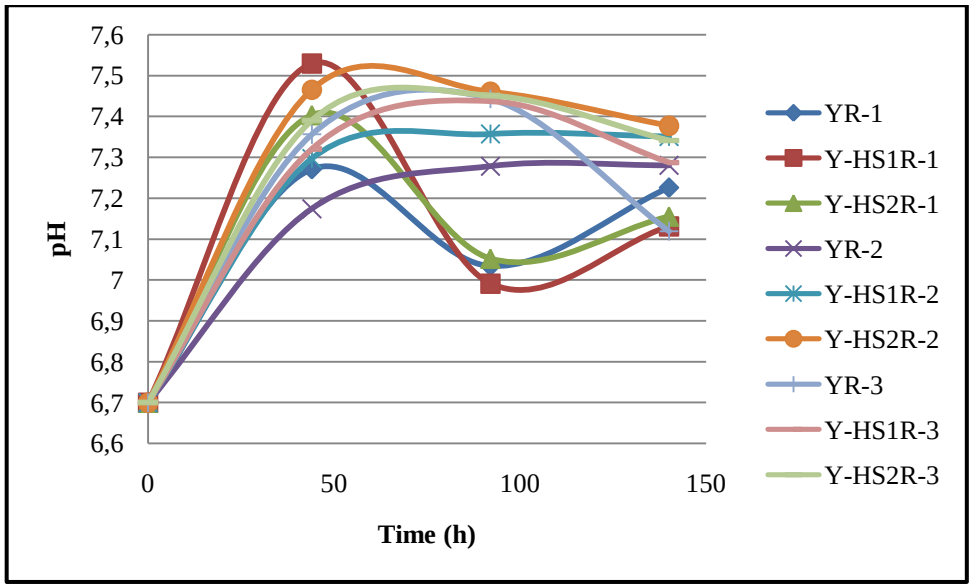


Figure 3.72 Mode of pH change during hydrogen production by *Rhodobacter capsulatus* YO3 strain co-immobilized with packed cells of *H. salinarium*

3.8 Hydrogen production by *Rhodobacter capsulatus* immobilized in panel photobioreactor

3.8.1 Hydrogen production by *R. capsulatus* YO3 bacteria immobilized by agar fixed inside wells on the inner surface of the panel reactor

As mentioned in section 2.9 two designs were tried for immobilizing the photosynthetic bacteria. Results of the first design which employed only YO3 strain bacteria involved using of two reactors, reactor A and reactor B. Illumination during this part was applied from one side of the reactor were the wells for immobilization are located. The two reactors were operated for about 576 hours (24 days). After operation of reactor A for 168 hours, it produced 1975 ml of hydrogen while reactor B has produced 3150 ml, (Figure 3.73 and Table A. 69).

At 168 hours, each reactor was fed with 200 ml of fresh hydrogen production medium 40/4 (acetate/glutamate). Feeding process was performed by removing equivalent amount of the liquid from the reactor and adding the same volume of fresh medium. Feeding process was repeated each 48 hours for both reactors.

In both reactors it was observed that agar gel was partially cracked as a result of gas pressure that cause partial detaching of the agar gel from the surface of reactor inside the wells where it was poured. This phenomena lead to design a new model (the second design) approach which allows keeping agar gel away from the reactor surface (7 mm distance).

Such approach involved a supporting material for agar to prevent its collapse. The new model involves a glass frame provided supporting Tulle cloth made from nylon. The frame with Tulle cloth was laid over a glass surface and agar was poured into the frame and left in the refrigerator for 10 minutes.

The frame with agar inside the reactor was supported by suitable grooved glass pieces. Such modification will allow free escape of gas from the gel without exerting any pressure on agar that may cause cracking. A model for this modification was done by the researcher. Figure 3.73 shows the cumulative hydrogen production mode during the 24 days of operation of each reactor.

After 576 hours (24 days) reactor A produced 6570 ml of hydrogen while reactor B produced about 7250 ml of hydrogen gas. The two reactors were stopped after agar cracking was more dominant and many parts of agar were lost to the liquid medium.

Mode of pH change during operation of the two reactors is illustrated in Figure 3.74 and Table A. 70. It was observed that pH levels during operation of reactor B has shown trends toward stability between 6.8 and 7.5 throughout the whole operation period.

The pH levels observed during operation of reactor B exhibited tendency to increase more than 7.5 during the first 144 hours when pH values reached to 8.5. After passing of 91 hours pH in reactor A started to decline to 7.5 and continued less to moderate values through the rest period of the experiment. Value of pH in both reactors continued below 7.5 and it was decreased slightly after feeding with fresh medium. Bacterial density in the liquid phase of the two reactors was below 0.75 all time.

It is worthy to mention that immobilized bacteria in this reactor were subjected to activation with growth medium 20/10 before setting up hydrogen production by 40/4 nutrient medium, (Figure 3.74 and Table A. 70).

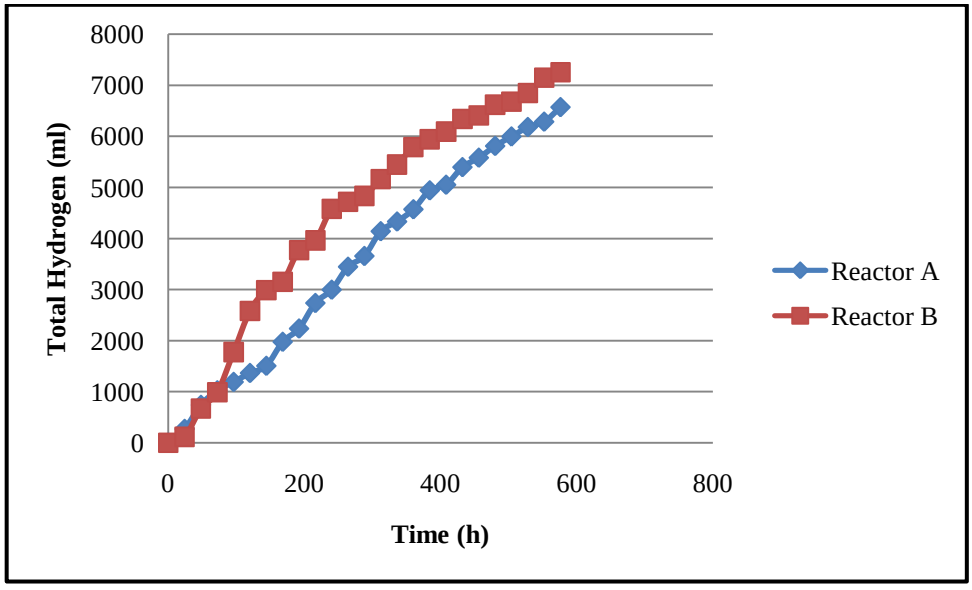


Figure 3.73 Total hydrogen produced by immobilized *Rhodobacter capsulatus* YO3 strain in panel reactor, first design

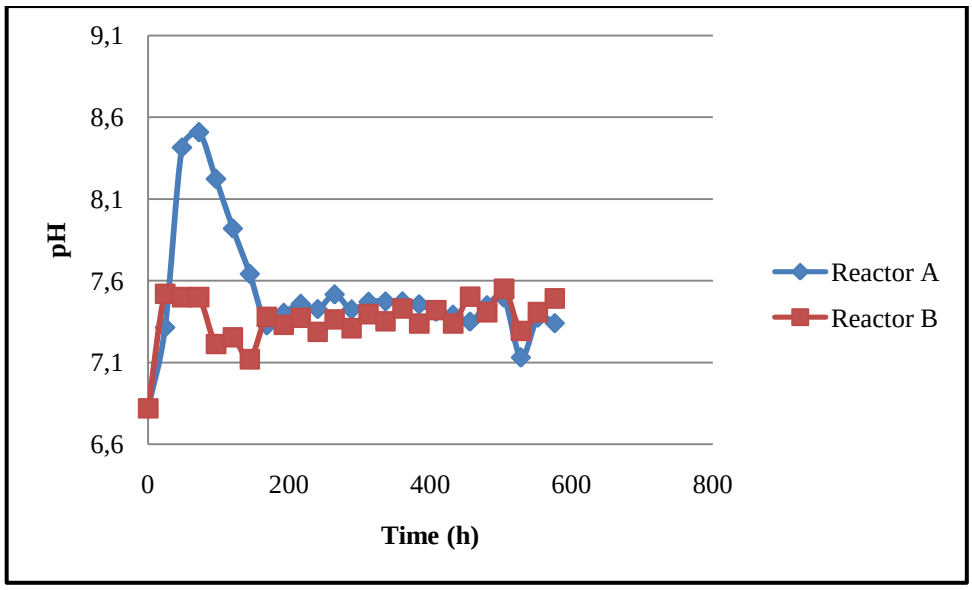


Figure 3.74 Mode of pH change during hydrogen production by immobilized *Rhodobacter capsulatus* YO3 strain in panel reactors A & B, first design

3.8.2 Hydrogen production by *R. capsulatus* immobilized in agar supported by framed-network cloth

The second design which was tried is explained in section 2.9. During the experiments done by using this design for immobilizing bacteria, the two strains YO3 and DSM 1710 were employed. It was clear from the long period of working time that the second design was more stable and no cracking of agar was observed. As mentioned in section 2.9, the operation of the reactors by using this design has depended sequential batch pattern.

3.8.2.1 Hydrogen production by immobilized *R. capsulatus* DSM 1710 strain

Figure 3.75 and Table 71 show the cumulative hydrogen production by immobilized *R. capsulatus* DSM 1710 strain in reactor 1. D1 symbol indicates reactor 1 with immobilized *R. capsulatus* DSM 1710 strain bacteria. This reactor was operated for four sequential batches designated R1-R4 for about 1608 hours (67 days) and fed with 60/4 hydrogen production medium described in materials and methods Through all rounds illumination was applied on both sides of the reactor except during second round (R-2). The first round R-1 produced around

3900 ml of hydrogen while total hydrogen produced was decreased to 2150. The third round R-4 shown better activity than R-2 and the reactor produced around 3640 ml of hydrogen. During the fourth round the system became more sluggish and total hydrogen produced decreased to 1470 ml.

The values of pH during operation of this reactor exhibited an increase over 7.6 and even more than 7.8 especially during R-2 and R-4 operation. During R-1 and R-3 pH was tending to be stable between 6.7 and 7.5, (Figure 3.76 and Table A. 72).

Figure 3.77 and Table A. 73 show the cumulative hydrogen production by immobilized *R. capsulatus* DSM 1710 strain in reactor 2. D2 symbol indicates reactor 2 with immobilized *R. capsulatus* DSM 1710 strain bacteria. This reactor was operated for five sequential batches designated R1-R5 for about 1968 hours (82 days) and fed with 60/4 hydrogen production medium described in materials and methods The reactor still active and will be introduced for further round.

Through all rounds illumination was applied on both sides of the reactor except during second round (R-2). The first round R-1 produced around 2600 ml of hydrogen while total hydrogen produced was decreased to 1650 ml at the end of R-2. The third round R-3 shown better activity than R-2 in term of rate of hydrogen production as it produced 1700 ml of hydrogen within 264 hours comparing to 504 hours to produced 1650 ml of hydrogen during R-2.

Regarding the low rate and total hydrogen produced by this reactor comparing to the second reactor with immobilized DSM 1710 strain, immobilized bacteria was fed with growth medium (20/10) containing 20 mM acetate and 10 mM glutamate. Bacteria were incubated for 48 hours before starting the fourth round.

The fourth round was resumed by re-feeding the reactor with nutrient medium for hydrogen production containing 60 mM acetate and 4 mM glutamate. The reactor was operated for two more rounds R-4 and R-5. At the end of R-4 total hydrogen produced was increased up to 3250 ml of hydrogen and 3000 ml after 432 hours of R-5 resuming.

The values of pH during operation this reactor exhibited an increase over 8.0 during R-2 and with less extent during R-4. During R-1, R-3 and R-5 pH has tendency to be stable between 6.7 and 7.6, (Figure 3.78 and Table A. 74).

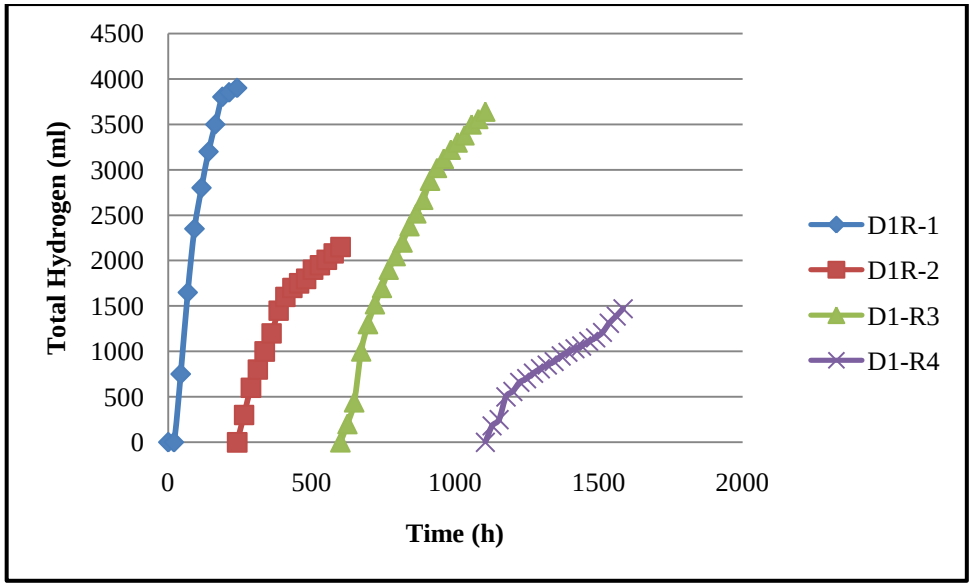


Figure 3.75 Total hydrogen produced by immobilized *Rhodobacter capsulatus* DSM 1710 strain in reactor D1, second design

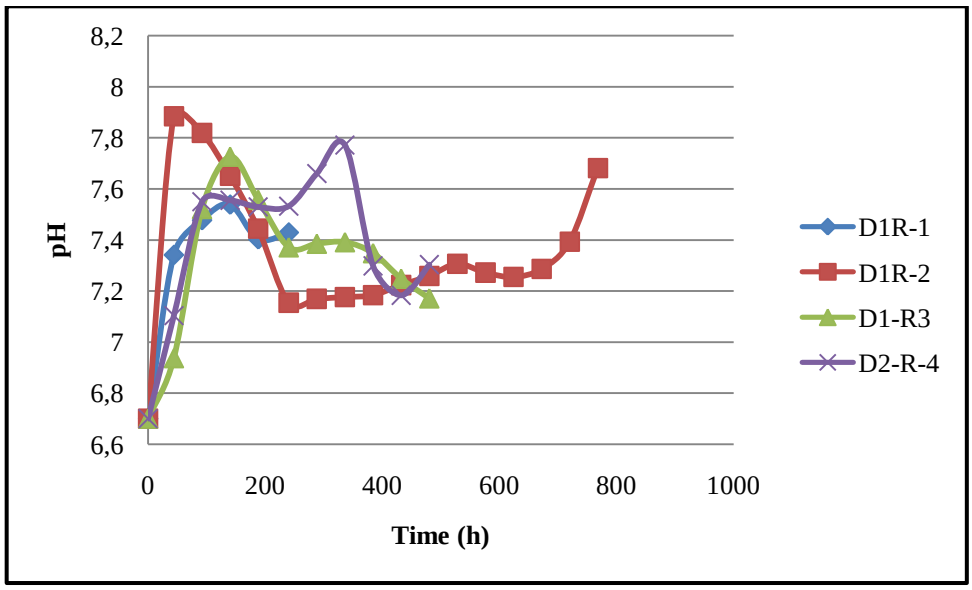


Figure 3.76 Mode of pH change during hydrogen production by immobilized *Rhodobacter capsulatus* DSM 1710 strain in reactor D1, second design

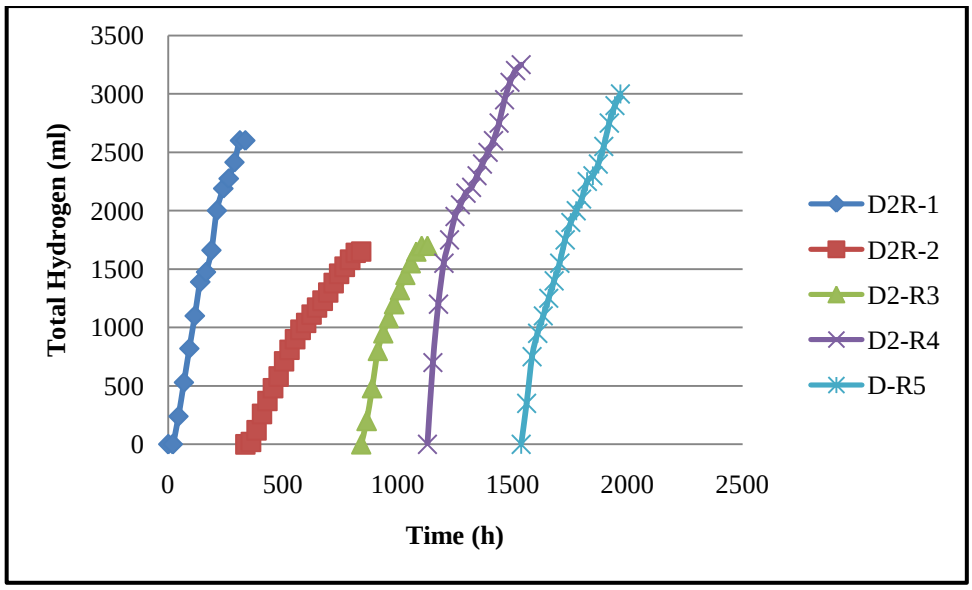


Figure 3.77 Total hydrogen produced by immobilized *Rhodobacter capsulatus* DSM 1710 strain in reactor D2, second design

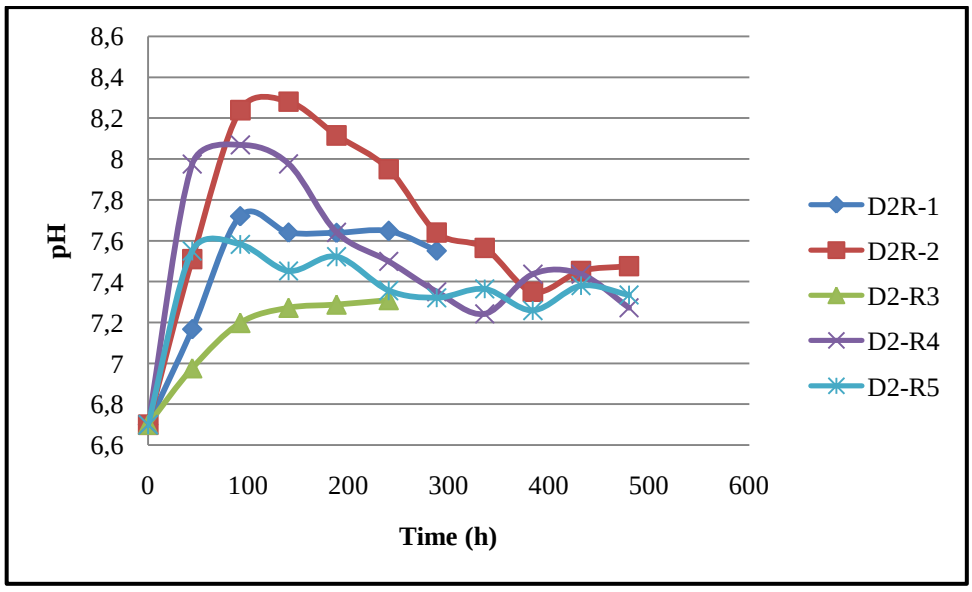


Figure 3.78 Mode of pH change during hydrogen production by immobilized *Rhodobacter capsulatus* DSM 1710 strain in reactor D2, second design

3.8.2.2 Hydrogen production by immobilized *R. capsulatus* YO3 strain

Figure 3.79 and Table A. 75 show the cumulative hydrogen production by immobilized *R. capsulatus* YO3 strain in reactor Y1. The symbol Y1 indicates reactor 1 with immobilized *R. capsulatus* YO3 strain bacteria. This reactor was operated for seven sequential batches designated R1-R7 for about 1644 hours (69 days) and fed with 60/4 hydrogen production medium described in materials and methods. Through all rounds illumination was applied on both sides of the reactor except during second round (R-2).

Regarding the low rate and total hydrogen produced by this reactor comparing to the second reactor with immobilized YO3 strain, immobilized bacteria was fed with growth medium (20/10) containing 20 mM acetate and 10 mM glutamate. Bacteria were incubated for 48 hours before re-feeding the reactor with nutrient medium for hydrogen production containing 60 mM acetate and 4 mM glutamate.

By the end of sixth round (R-6) black precipitation started to form in the reactor with rotten-egg smell. This black precipitation was persistent and more condensed during seventh round (R-7) which produced very low amount of hydrogen compared to other six rounds. Regarding period of operation from R-1 to R-6 the reactor produced 24950 ml of hydrogen with an average of total hydrogen produced per round per reactor as 4158 ml of hydrogen. During last round immobilized bacteria shown very low activity and produced only 1220 ml of hydrogen with heavy black precipitation accompanied with rotten-egg smell.

Figure 3.80 and Table A.76 illustrate mode of pH change during hydrogen production by immobilized bacteria YO3 strain in reactor Y1. It was observed that pH mode during operation of this reactor shown some high pH values exceeded 7.6 up to more than 8.0. This was clear during R-3, R-6 and R-7.

Figure 3.81 and Table A. 77 show the cumulative hydrogen production by immobilized *R. capsulatus* YO3 strain in reactor Y2. The symbol Y2 indicates reactor 2 with immobilized *R. capsulatus* YO3 strain bacteria. This reactor was operated for seven sequential batches designated R1-R7 for about 1720 hours (72 days) and fed with 60/4 hydrogen production medium described in materials and methods. Through all rounds illumination was applied on both sides of the reactor except during second round (R-2). This reactor was not manipulated by 20/10 growth medium for activation of bacteria like reactor Y1. Regarding period of operation from R-1 to R-7 the reactor produced 33090 ml of hydrogen with an average of total hydrogen

produced per round per reactor as 4727 ml of hydrogen. This reactor still working as mentioned above.

Figure 3.80 and Table A. 78 illustrate mode of pH change during hydrogen production by immobilized bacteria YO3 strain in reactor Y2. The observations illustrate that pH mode during operation of this reactor shown general pH stability below 7.6 with few exceptions. During R-5 pH went once time to 7.73. During round R-3 pH reached once time to 7.7 and also once time during round R-7 which was 7.65. In general it is acceptable to conclude that pH values during operation of this reactor were moderate and suitable for hydrogen production by the bacteria.

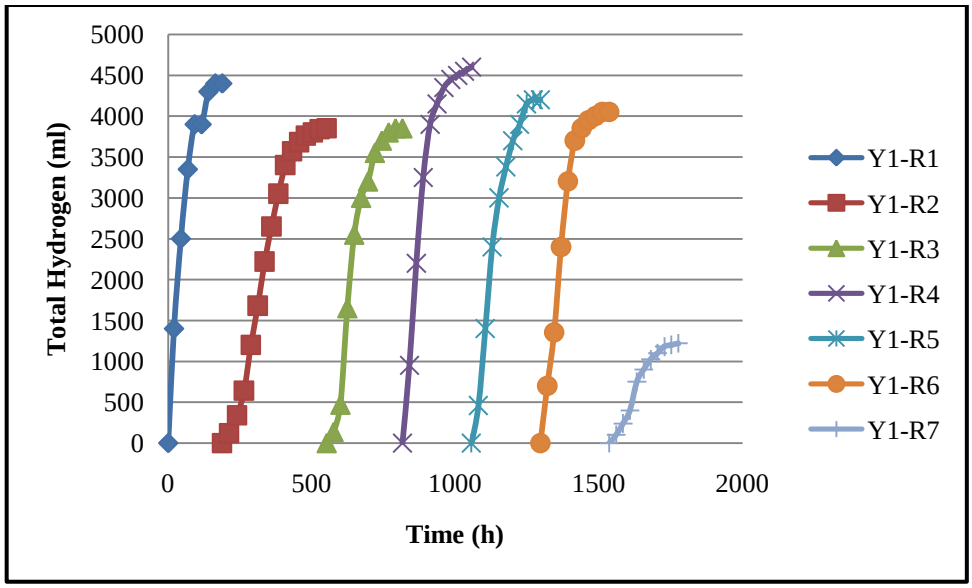


Figure 3.79 Total hydrogen produced by immobilized *Rhodobacter capsulatus* YO3 strain in reactor Y1, second design

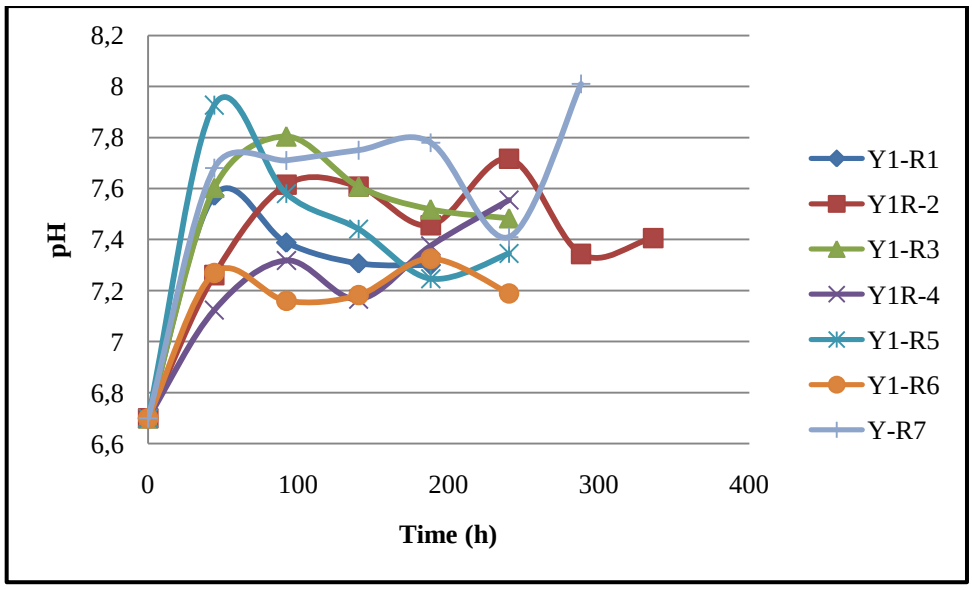


Figure 3.80 Mode of pH change during hydrogen production by immobilized *Rhodobacter capsulatus* YO3 strain in reactor Y1, second design

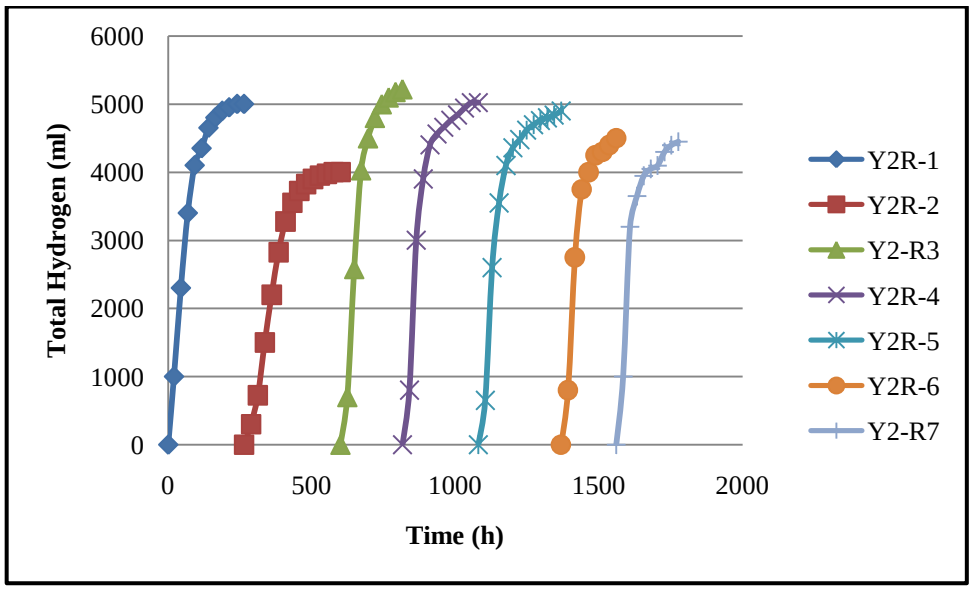


Figure 3.81 Total hydrogen produced by immobilized *Rhodobacter capsulatus* YO3 strain in reactor Y2, second design

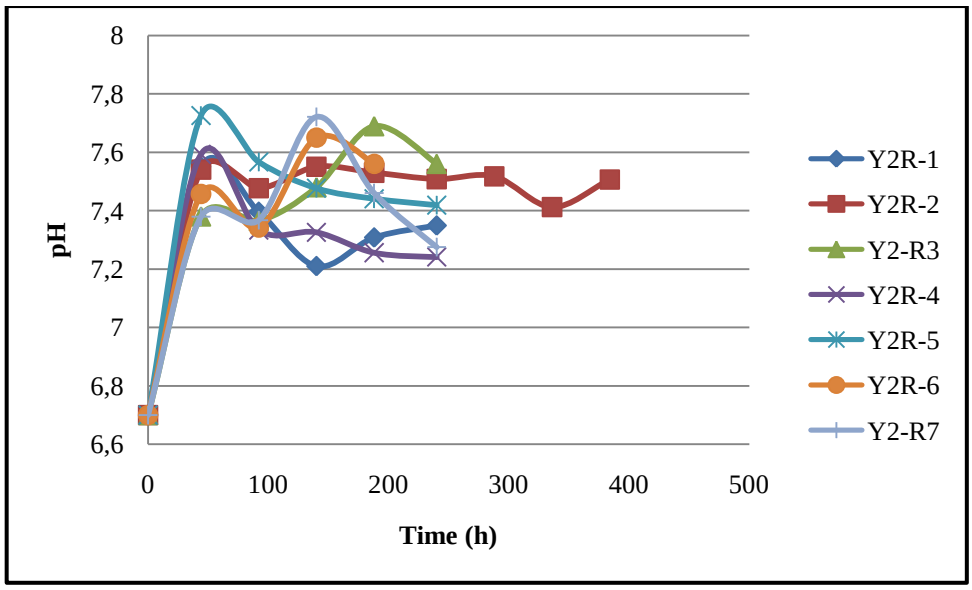


Figure 3.82 Mode of pH change during hydrogen production by immobilized *Rhodobacter capsulatus* YO3 strain in reactor Y2, second design

CHAPTER 4

DISCUSSION

4.1 Effect of different agar concentrations on hydrogen production by photosynthetic bacteria *Rhodobacter capsulatus*

During this part of work four different concentrations of agar were used to make agar gel for immobilizing bacteria. The use of those concentrations aimed to find the suitable candidate concentration of agar for applying during next studies of this work.

The results pointed 3% and 4% concentrations as suitable candidates to be used during next studies. The average of total hydrogen produced and the average rates of hydrogen production by cultures immobilized within the two concentrations shown insignificant differences. The need for adequate mechanical strength to keep stability and integrity of the gel preferred using 4% agar as the candidate concentration.

HPLC analysis has been conducted along the period of this work aiming to follow up the change of acetic acid concentration and production and accumulation of formate. Samples for analysis were collected at each 48 hours periodically, centrifuged and frozen until analysis.

4.1.1 Effect of different agar concentrations on hydrogen production by *Rhodobacter capsulatus* DSM1710

Figure 4.1.and Figure 4.2 show the total hydrogen produced and the rate of hydrogen production by *Rhodobacter capsulatus* DSM1710 immobilized by different concentrations of agar. The calculations in the Figure based on liter hydrogen per liter liquid.

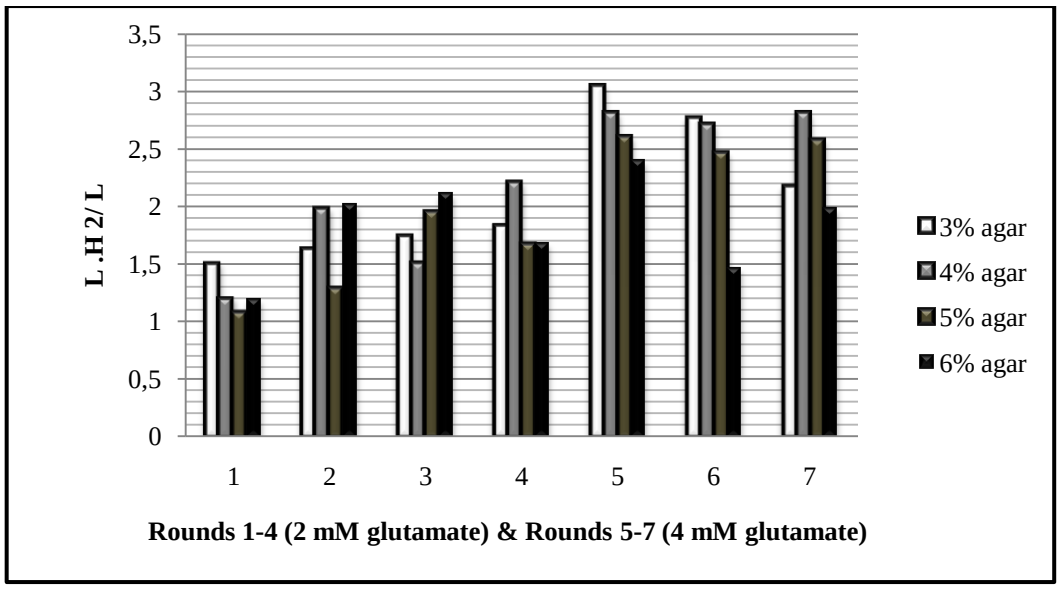


Figure 4.1 Total Hydrogen production by *Rhodobacter capsulatus* DSM1710 immobilized in different concentrations of agar and provided with different concentrations of glutamate (2 & 4 mM)

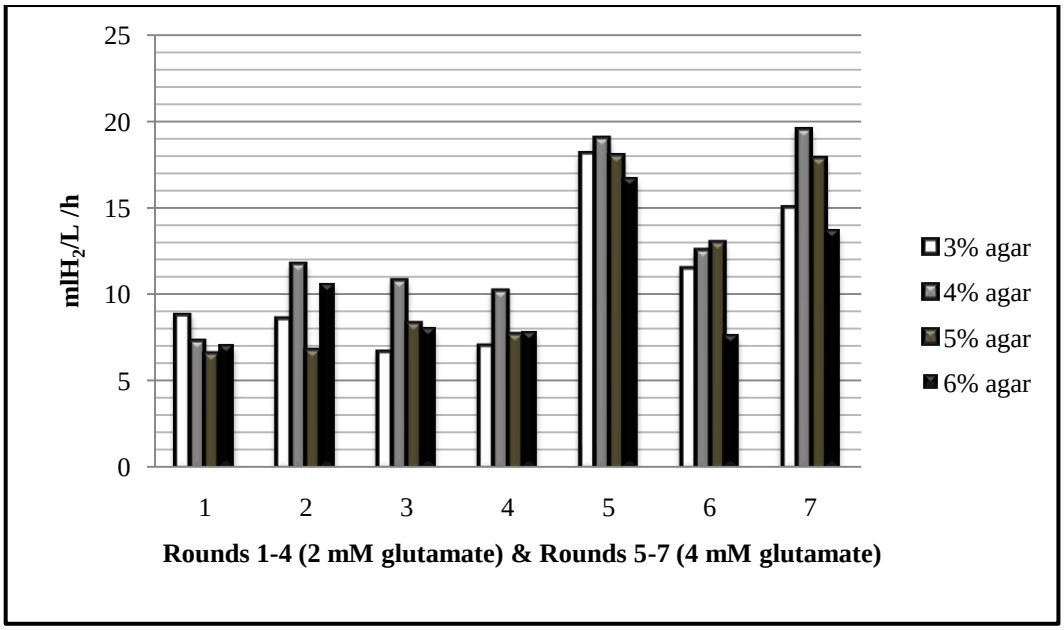


Figure 4.2 Average of Hydrogen production by *Rhodobacter capsulatus* DSM1710 immobilized in different concentrations of agar and provided with different concentrations of glutamate (2 & 4 mM)

The results in the Figure shows that 3% concentration has produced an average of total hydrogen about 1.7 L.H_2/L and the average rate of hydrogen production was 7.8 ml.H_2/L/h throughout the first four rounds were glutamate concentration was 2 mM and 2.7 L.H_2/L and the average rate of hydrogen production was 14.9 ml.H_2/L/h throughout the last three rounds were glutamate concentration was 4 mM.

Figure 4.1 also shows that for 4% agar used, average of total hydrogen produced was 1.7 L.H_2/L and the average rate was 10 ml.H_2/L/h for the first three rounds. The average of total hydrogen production was 2.8 L.H_2/L and the average rate was 17 ml.H_2/L/h throughout the last three rounds.

In case either hydrogen production rate or total hydrogen production was acceptable when using 5% and 6% agar concentrations, they still lower than 3% or 4% concentration, in addition to that using of 5% and 6% agar made some difficulties due to fast solidifying of the gel by such concentration more than seen by 3% and 4% agar. Fast solidifying of the gel need higher temperature manipulation more than 45°C to keep it liquid until finishing mixing of bacteria and pouring the gel into the culture bottles. High temperature can hurt bacteria which prefer lower temperatures.

Data in Figures 4.1 and 4.2 showed that when glutamate concentration increased from 2 mM up to 4 mM total hydrogen production and the rate of hydrogen production were both increased and improved by different ratios and also those results show that 3% and 4% agar kept their priority over other agar concentrations.

HPLC analysis data are shown in appendix B in Figure B. 5 to Figure B. 12 and Table B. 5 to Table B. 12. Figures 4.3-10 show that at the end of first round in all agar concentrations no detected acetate and by going to second, third and fourth round there was an increase in detected acetate. After increase of glutamate up to 4 mM it was observed an increase in hydrogen production which was reflected by very low or disappearance of acetate at the end of the fifth round down to the seventh round in all concentrations except 6% agar which registered slightly higher acetate remains comparing to other concentrations of agar.

According to HPLC data, formate was formed in all cases and all rounds and it was increasing by time for each round. No decrease in its amount could be seen except in case of 6% agar at fourth round and fifth round.

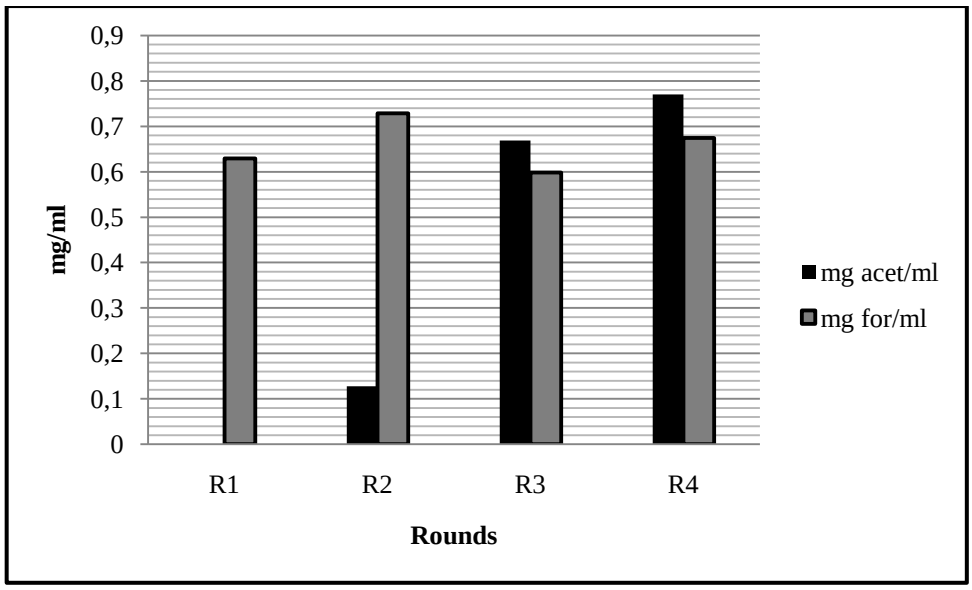


Figure 4.3 Final acetate and formate concentrations during hydrogen production by *R. capsulatus* DSM1710immobilized in 3% agar, R1 to R4, 2 mM glutamate

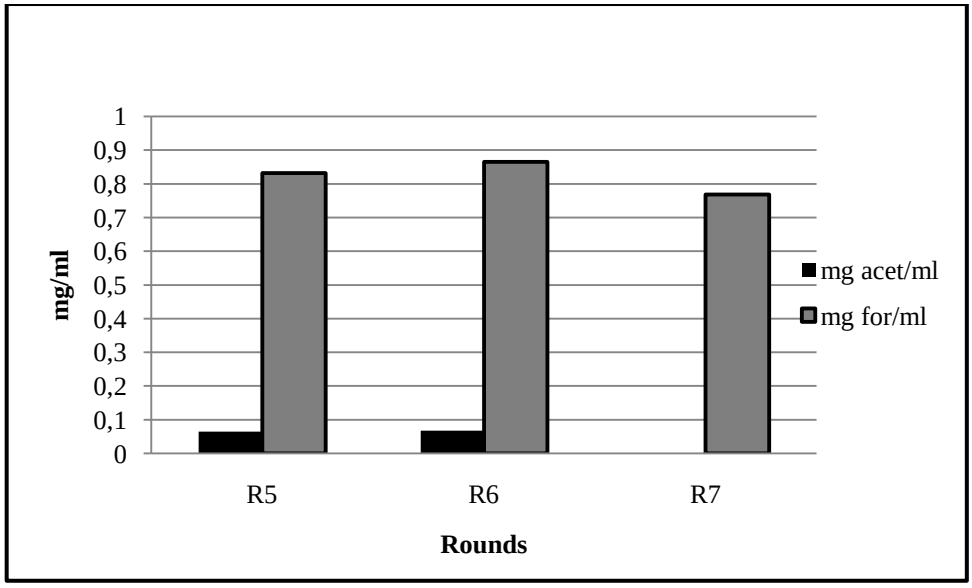


Figure 4.4 Final acetate and formate concentrations during hydrogen production by *R. capsulatus* DSM1710 immobilized in 3% agar, R5 to R7, 4 mM glutamate

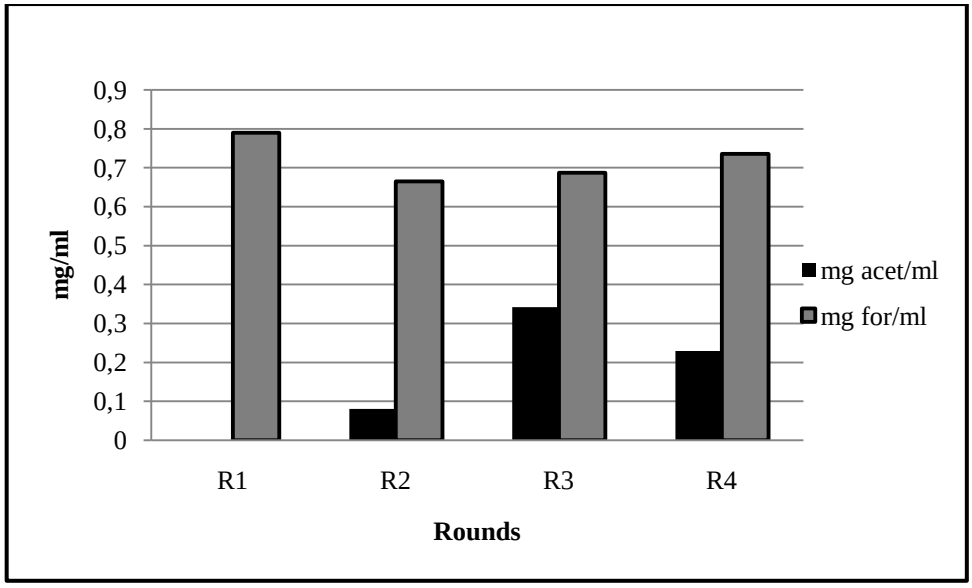


Figure 4.5 Final acetate and formate concentrations during hydrogen production by *R. capsulatus* DSM1710 immobilized in 4% agar, first four rounds, 2 mM glutamate

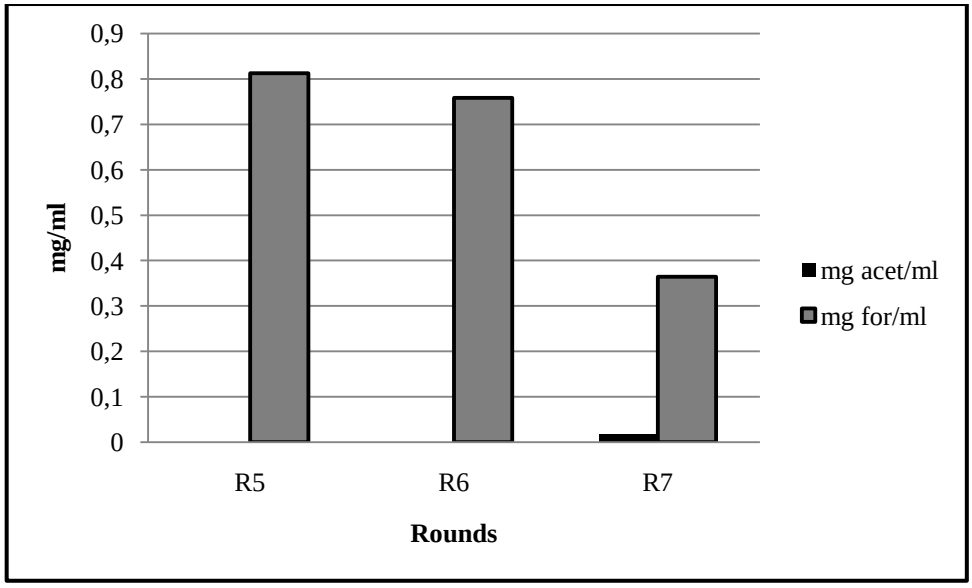


Figure 4.6 Final acetate and formate concentrations during hydrogen production by *R. capsulatus* DSM1710 immobilized in 4% agar, first four rounds, 4 mM glutamate

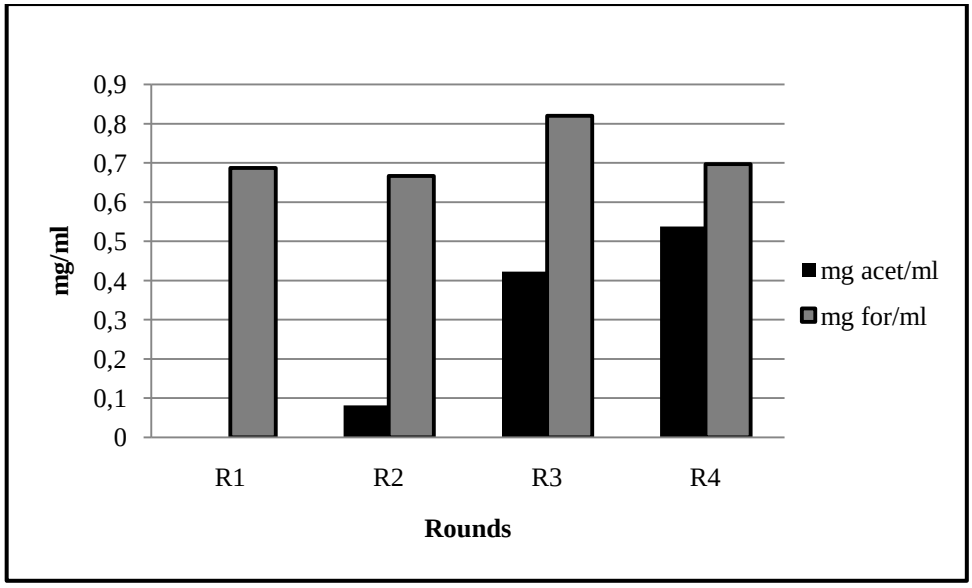


Figure 4.7 Final acetate and formate concentrations during hydrogen production by *R. capsulatus* DSM1710 immobilized in 5% agar, first four rounds, 2 mM glutamate

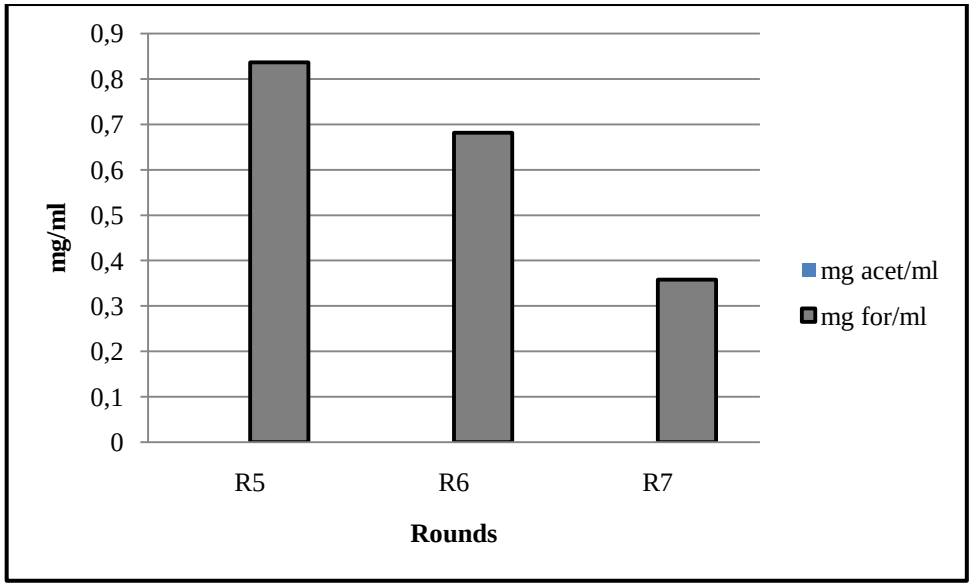


Figure 4.8 Final acetate and formate concentrations during hydrogen production by *R. capsulatus* DSM1710 immobilized in 5% agar, first four rounds, 4 mM glutamate

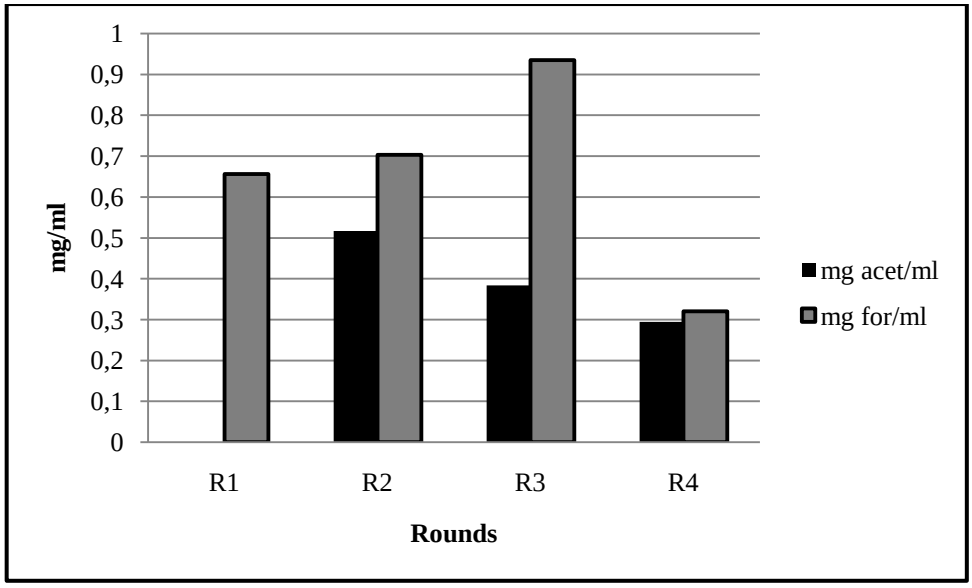


Figure 4.9 Final acetate and formate concentrations during hydrogen production by *R. capsulatus* DSM1710 immobilized in 6% agar, first four rounds, 2 mM glutamate

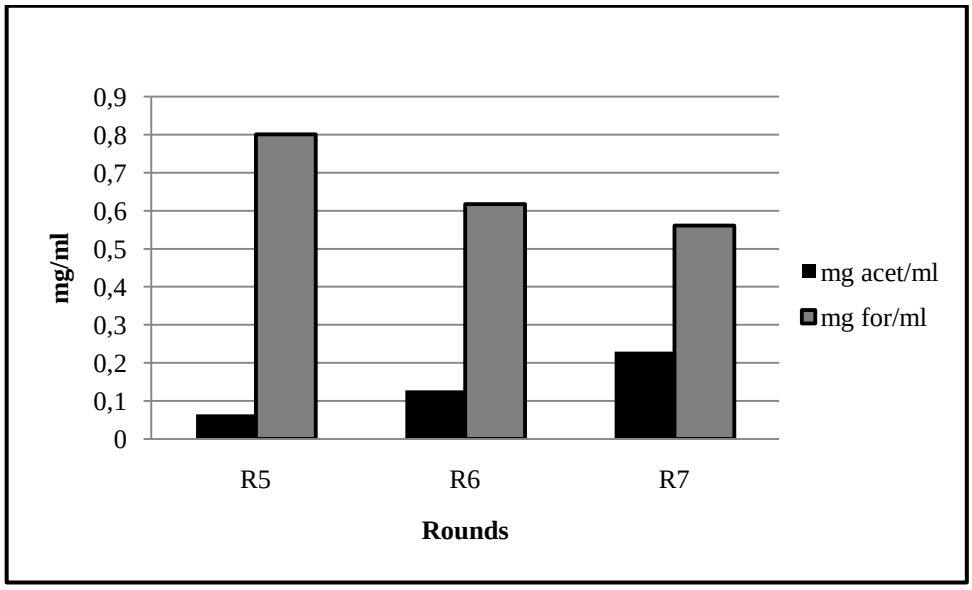


Figure 4.10 Final acetate and formate concentrations during hydrogen production by *R. capsulatus* DSM1710 immobilized in 6% agar, last three rounds, 4 mM glutamate

Consumption of formate was uncommon in other cases. HPLC analysis data conclude that rate of acetate consumption is connected to rate and amount of hydrogen production. This means faster hydrogen production means faster acetate consumption and more acetate consumed mostly more hydrogen produced.

4.1.2 Effect of different agar concentrations on hydrogen production by *Rhodobacter capsulatus* YO3

Figure 4.11 and Figure 4.12 show the total hydrogen produced and the rate of hydrogen production by *Rhodobacter capsulatus* YO3 immobilized by different concentrations of agar. The calculations in the Figure based on liter hydrogen per liter liquid.

The results in Figure 4.11 and Figure 4.12 show that 3% concentration has produced an average of total hydrogen about 1.8 $L.H_2/L$ and the average rate of hydrogen production was 14.7 $ml.H_2/L/h$ throughout the first four rounds when glutamate concentration was 2 mM and when glutamate concentration increased to 4 mM the average total hydrogen produced was 3.8 $L.H_2/L$ and the average rate of hydrogen production was 23.7 $ml.H_2/L/h$ throughout the last fifth round. The Figure also shows that when bacteria were immobilized in 4% agar, average total hydrogen produced was 2 $L.H_2/L$ and the average rate was 15.3 $ml.H_2/L/h$ during the first four rounds when glutamate concentration was 2 mM. After increasing glutamate concentration to 4 mM during R5, average total hydrogen produced was 3.6 and average rate of hydrogen production was 22.4 $ml.H_2/L/h$.

Exploring data in Figure 4.11 and Figure 4.12 may cause some confliction about the conclusion of choosing 4% as the accepted candidate to be used in the next studies as 5% and 6% agar concentrations given total hydrogen production and rate of hydrogen production values close to the output values given by 3% and 4% agar especially during third and fourth rounds.

Such confliction could be overcome by looking all over the process and calculate the average of total hydrogen produced and the average rate of hydrogen production. Such calculation indicates that results obtained by immobilizing bacteria in 3% or 4% agar concentration still preferred over 5% and 6% agar concentration. In addition to that, using of 5% and 6% agar made some difficulties due to fast solidifying of the gel when using 4% and 5% agar concentration. Increasing of glutamate concentration to 4 mM cleared the differences between results obtained from immobilizing bacteria in different agar concentrations.

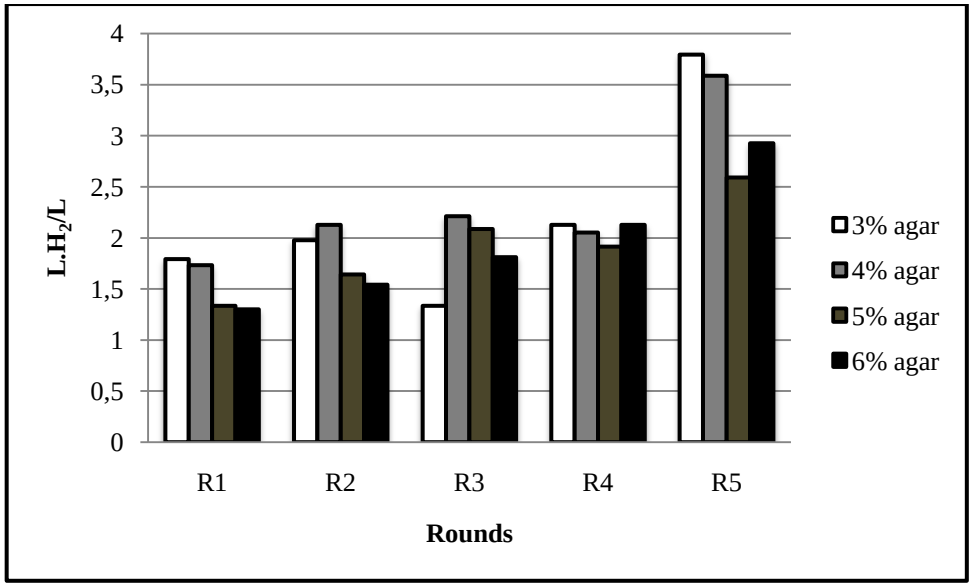


Figure 4.11 Total Hydrogen production by *Rhodobacter capsulatus* YO3 immobilized by different concentrations of agar (R1-R4 fed with 2 mM glutamate, and R5 fed with 4 mM glutamate)

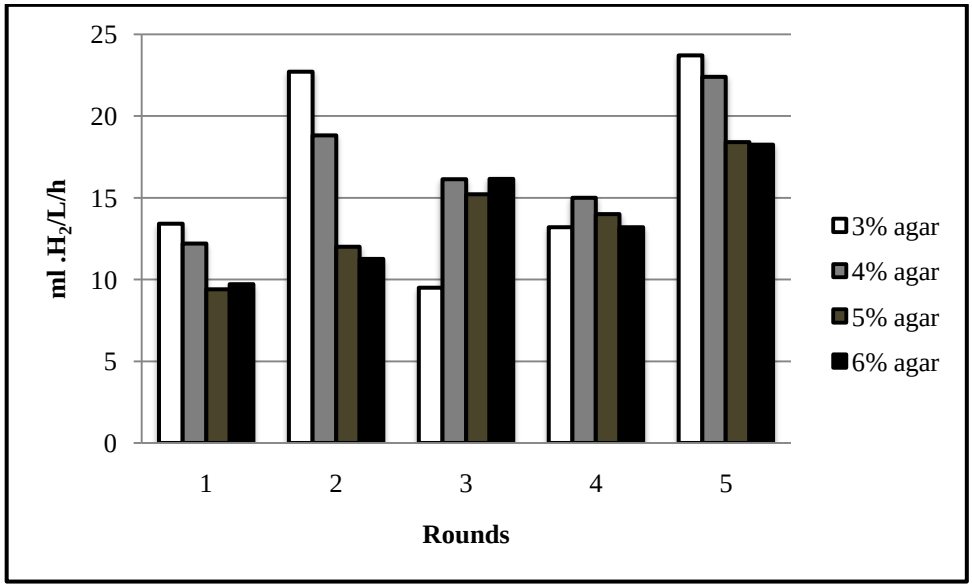


Figure 4.12 Rate of Hydrogen Production by *Rhodobacter capsulatus* YO3 immobilized by different concentrations of agar (R1-R4 fed with 2 mM glutamate, and R5 fed with 4 mM glutamate)

Fast solidifying of the gel with 5% and 6% agar need higher temperature manipulation more than 45°C to keep it liquid until finishing mixing of bacteria and pouring the gel into the culture bottles. High temperature can hurt bacteria which prefer lower temperatures.

Data observed in Figure 4.11 and Figure 4.12 show that when glutamate concentration increased from 2 mM to 4 mM total hydrogen production and the rate of hydrogen production were both increased and improved by different ratios and also those results show that 3% and 4% agar kept their priority over other agar concentrations.

HPLC analysis data (Appendix B, Figures B.1 to B. 5and Tables B .1 to B .5) and Figures 4.13-16 show that at the end of first round in all agar concentrations no detected acetate or very low amount (0.0235 mg/ml in case of 6%).

By advancing the process onto second, third and fourth rounds with all concentrations of agar no acetate was detected at the end of each round. The exception was observed at the end of second round of 4% agar with low remains (0.03068) and at the end of second round and third round of 3% agar (0.0715 and 0.2294 mg/ml respectively).

Relative high acetate remains at the end of third round of 3% agar reflects the lower hydrogen production comparing to other rounds of the same concentration of agar and other agar concentrations. By increasing of glutamate to 4 mM it was observed that in hydrogen production was increased and this was reflected by disappearance of acetate at the end of the fifth round in all agar concentrations.

According to HPLC data, formate was formed in all cases and all rounds and it was increasing by time for each round. At the end of the first rounds of all concentrations it was observed a steady increase of formate concentration by increasing of agar concentration in a proportional mode.

Consumption of formate was observed near end of fifth round of 3% agar and near end of second round of 6% agar. HPLC analysis data conclude that rate of acetate consumption is connected to rate and amount of hydrogen production. This means faster hydrogen production means faster acetate consumption and more acetate consumed mostly more hydrogen produced.

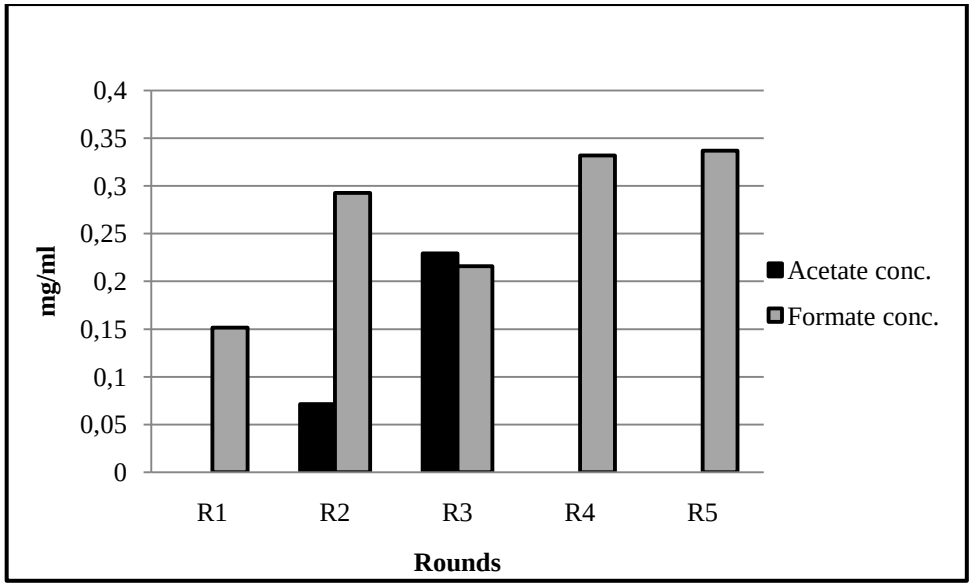


Figure 4.13 Final acetate and formate concentrations during hydrogen production by *R. capsulatus* YO3 immobilized in 3% agar, first four rounds, 2 mM glutamate & fifth round 4mM glutamate

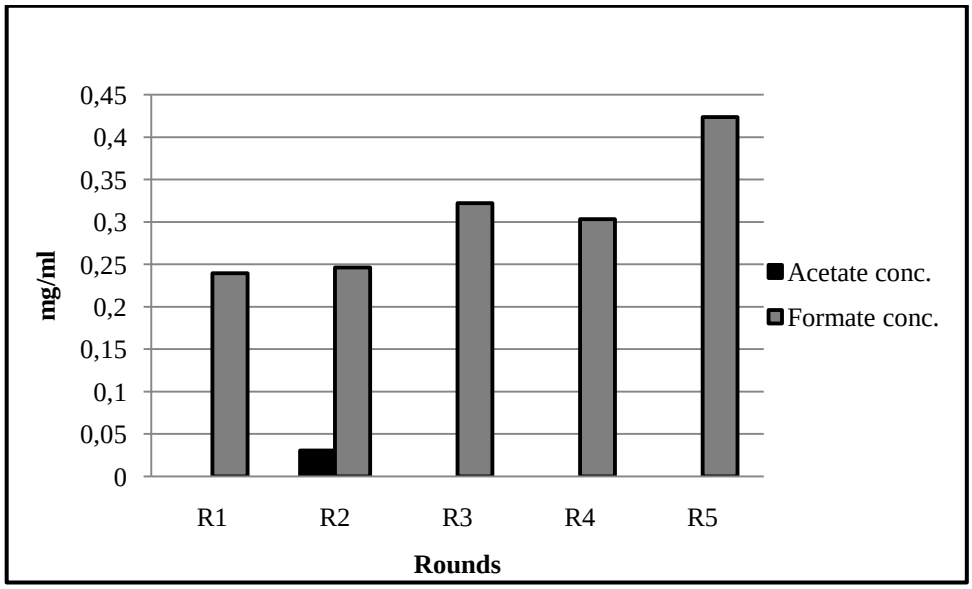


Figure 4.14 Final acetate and formate concentrations during hydrogen production by *R. capsulatus* YO3 immobilized in 4% agar, first four rounds, 2 mM glutamate & fifth round 4mM glutamate

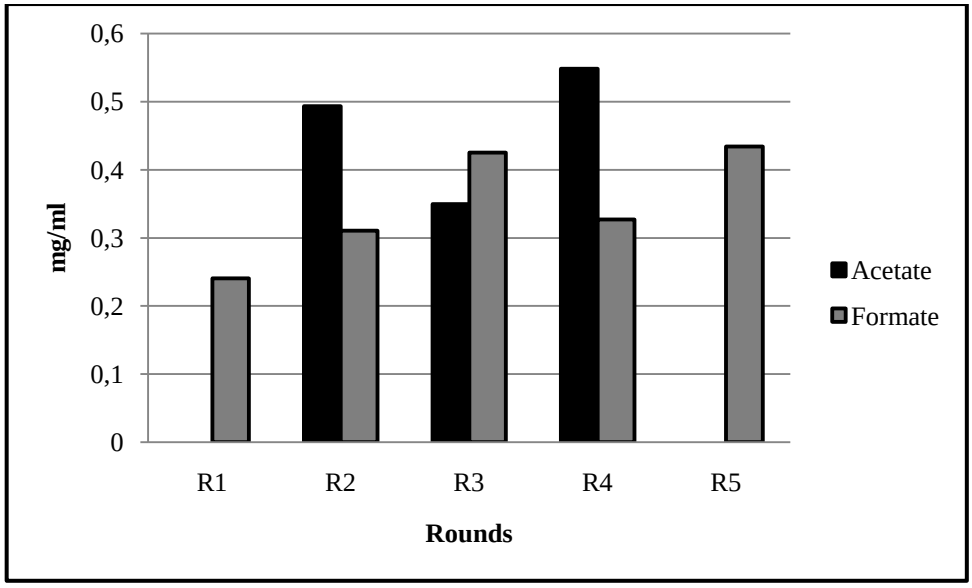


Figure 4.15 Final acetate and formate concentrations during hydrogen production by *R. capsulatus* YO3 immobilized within 5% agar, first four rounds 2 mM glutamate & fifth round 4mM glutamate

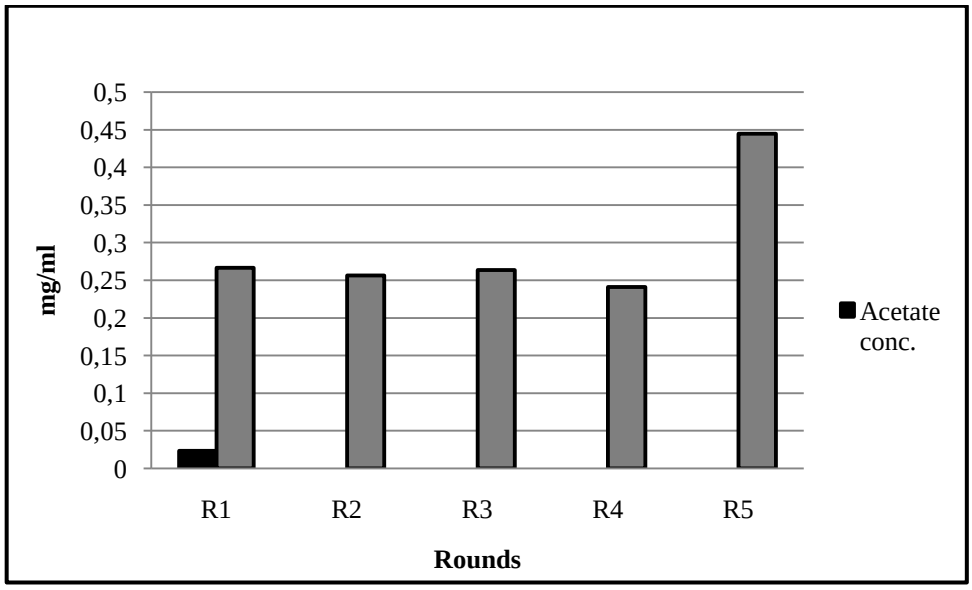


Figure 4.16 Final acetate and formate concentrations during hydrogen production by *R. capsulatus* YO3 immobilized in 6% agar, first four rounds, 2 mM glutamate & fifth round 4mM glutamate

Comparing HPLC data for acetate and formate among the two bacterial strains used in this work, it was observed that acetate was consumed by YO3 strain faster than DSM1710 strain and in most cases at end of each round for any concentration no acetate was observed or it was less than observed in opposite cases of DSM 1710 strain.

Accumulation of formate by YO3 strain cultures was all times less by considerable ratios than found in DSM1710 strain cultures. Such observation could be attributed to the fact that YO3 strain is a hup^- mutant strain which can accumulate higher amounts of hydrogen and shows high rate of hydrogen production comparing to DSM1710 strain. This was reflected when major of rounds in most used agar concentrations exhibited very little or no remains of acetate at the end of each round.

4.2 Hydrogen production by immobilized *Rhodobacter capsulatus* by using different concentrations of acetate

During this part of work hydrogen production by immobilized bacteria was investigated by using a range of different concentrations of acetate included 60, 80, and 100 mM. Bacteria immobilized within 4% agar in the gel. The bacterial concentration was fixed on 2.5 mg DCW/ml agar-gel. The process was performed in double experiments for each acetate concentration and for both bacterial strain.

4.2.1 Hydrogen production by immobilized *Rhodobacter capsulatus* DSM1710 by using different concentrations of acetate

Hydrogen production in this part of work was measured by double experiment for each concentration used. Each experiment was conducted for three rounds as mentioned in material and methods. The following Figures illustrate total hydrogen production and rate of hydrogen production.

Figure 4.17 and Figure 4.18 show total hydrogen production and rate of hydrogen production throughout three rounds for all applied concentrations. Each value in the Figures represent the output average of two values resulted from two parallel experiment for each concentration.

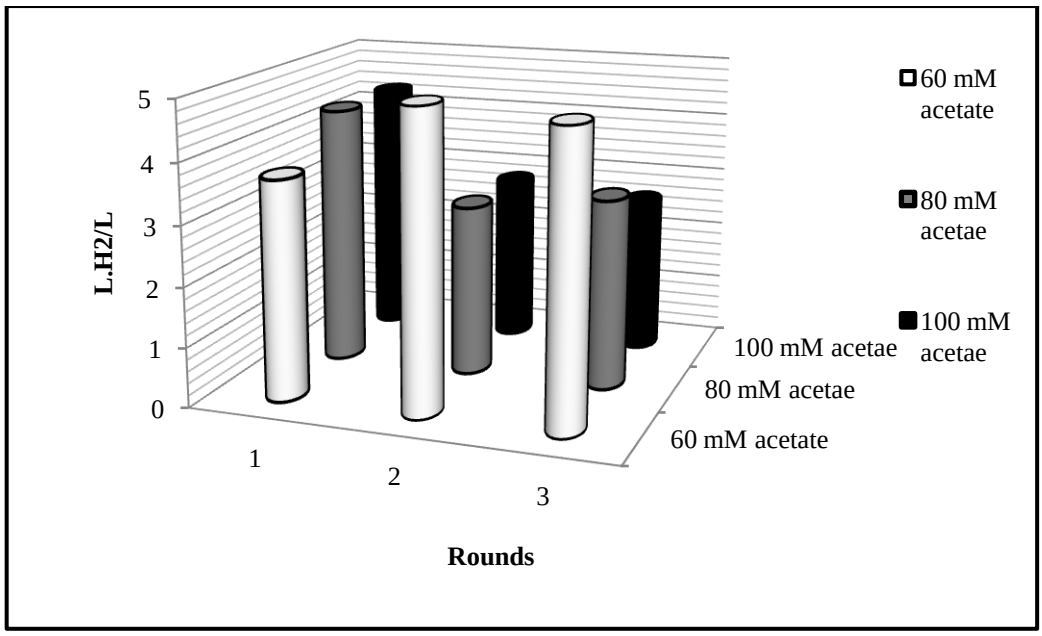


Figure 4.17 Average of total hydrogen produced by *Rhodobacter capsulatus* DSM1710 immobilized in 4% agar and fed with 60, 80, 100 mM of acetate

By exploring the values in Figure 4.17 and Figure 4.18, it is possible to observe that the average total hydrogen produced by the three rounds of all concentrations. For 60 mM acetate was around 4.2 liter of hydrogen per liter of liquid while the average rate of hydrogen production was 17.3 ml of hydrogen per liter of liquid per hour.

For 80 mM acetate, total hydrogen produced was 3.2 liter of hydrogen per liter of liquid while the average rate of hydrogen production was 12.9 ml of hydrogen per liter of liquid per hour. For 100 mM acetate total hydrogen produced was 3.00 liter of hydrogen per liter of liquid while the average rate of hydrogen production was 11.3 ml of hydrogen per liter of liquid per hour.

Data in Figure 4.17 and 4.18 indicated that 60 mM acetate was preferred over other two concentrations in terms of total hydrogen production and rate of hydrogen production.

Comparing this result with 40 mM acetate concentration result which was 2.8 liter of total hydrogen produced per liter of liquid and 17 ml of hydrogen per liter of liquid per hour, it is possible to conclude that 60 mM acetate has given 1.5 time hydrogen more than 40 mM with slightly higher rate.

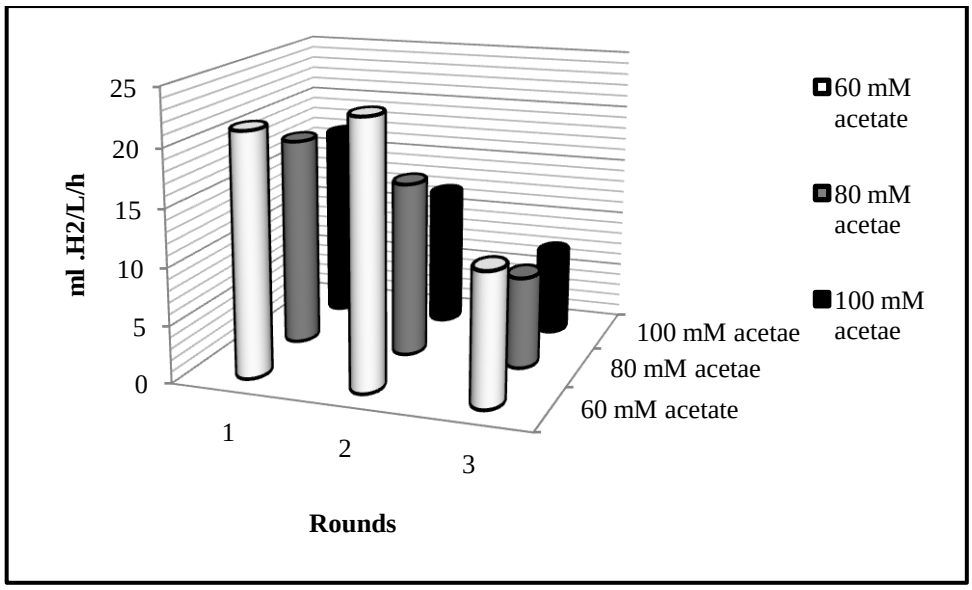


Figure 4.18 Average rate of hydrogen production by *Rhodobacter capsulatus* DSM1710 immobilized in 4% agar and fed with 60, 80, 100 mM of acetate

Those results suggested 60 mM acetate as a good candidate concentration to be applied with immobilized *Rhodobacter capsulatus* DSM1710 under the present work conditions

4.2.2 Hydrogen production by immobilized *Rhodobacter capsulatus* YO3 by using different concentrations of acetate

Hydrogen production study by using different concentrations of acetate was extended to cover working with the mutated hup- bacteria *Rhodobacter capsulatus* YO3. Each experiment was repeated two times and was allowed to work for three rounds as mentioned in material and methods.

Figure 4.19 and Figure 4.20 show total hydrogen production and rate of hydrogen production throughout three rounds for all applied concentrations. Each value in the Figures represents the output average of two values extracted from two parallel experiments for each concentration.

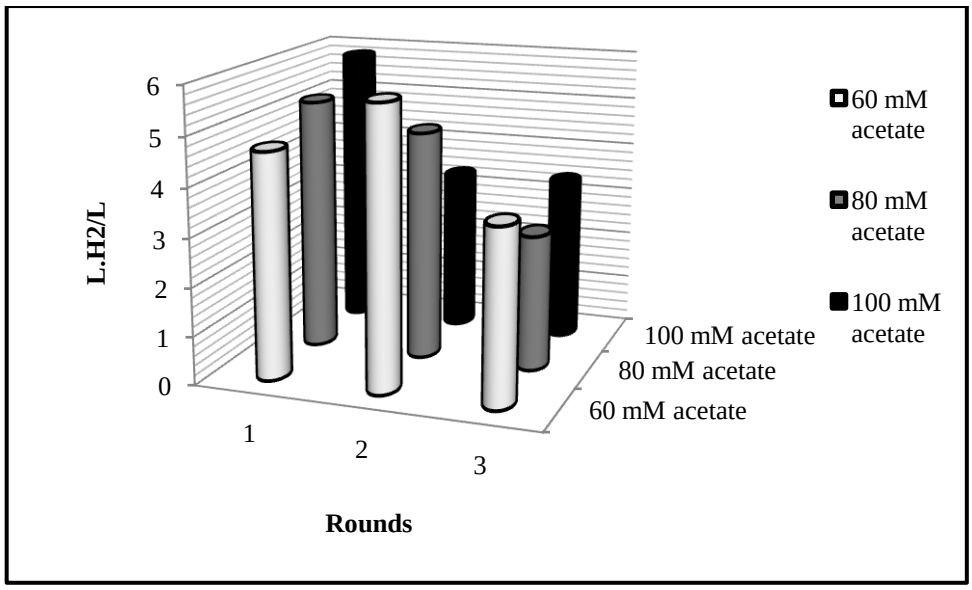


Figure 4.19 Average total hydrogen produced by *Rhodobacter capsulatus* YO3 immobilized in 4% agar and fed with 60, 80, 100 mM of acetate

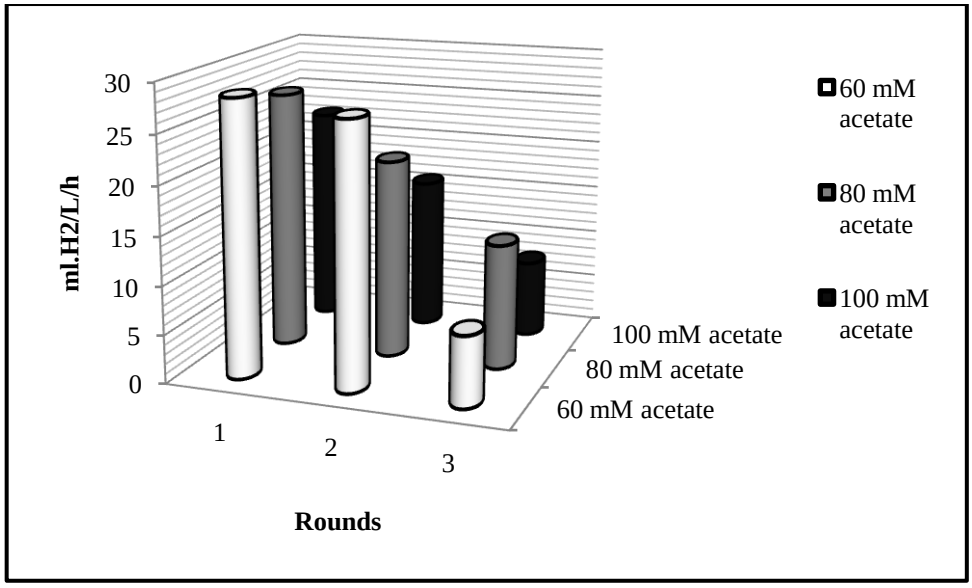


Figure 4.20 Average rate of hydrogen production by *Rhodobacter capsulatus* DSM1710 immobilized in 4% agar and fed with 60, 80, 100 mM of acetate

Data extracted from Figure 4.19 and Figure 4.20 shown that for 60 mM acetate total hydrogen production was 4.3 liter hydrogen per liter liquid while rate of hydrogen production was 19.5 ml of hydrogen per liter of liquid per hour. For 80 mM acetate total hydrogen production was 3.95 liter hydrogen per liter liquid while rate of hydrogen production was 18.6 ml of hydrogen per liter of liquid per hour. For 80 mM acetate total hydrogen production was 3.9 liter hydrogen per liter liquid while rate of hydrogen production was 14.3 ml of hydrogen per liter of liquid per hour.

As observed in work done with DSM1710 calculated data indicates that 60 mM acetate was preferred over other two concentrations in terms of total hydrogen production and rate of hydrogen production. Comparing this result with 40 mM acetate concentration result which was 3.6 liter of total hydrogen produced per liter of liquid and 22.4 ml of hydrogen per liter per hour, it is possible to conclude that 60 mM acetate has given 1.2 time hydrogen, on the base of average of six values, more than 40 mM which shows slightly higher rate.

If 40 mM results being compared with individual rounds as first and second round of first experiment it is possible to see larger difference were total hydrogen production in first round was 4.96 liter of hydrogen per liter of liquid and the rate of hydrogen production was 26.3 ml of hydrogen per liter of liquid per hour.

Looking over second round of first experiment it is observed that situation is more improved as total hydrogen production reached 6.4 liter of hydrogen per liter of liquid and rate of hydrogen production was around 25 ml of hydrogen per liter of liquid culture per hour.

Such conclusion can give advantage of 60 mM of acetate over 40 mM by about 1.2 to 1.8 times. Those results suggested 60 mM acetate as a good candidate concentration to be applied with immobilized *Rhodobacter capsulatus* YO3 under the present work conditions.

HPLC analysis data for final organic acids concentrations at the end of each round in all processes when 60 mM of acetate is used are shown in Figure 4.21.

Following final concentrations of acetate with DSM strain, it is observed that during the first experiment at the end of first round DSM-R1-1 and second round DSM-R2-1, no acetate was detected. At the end of the third round DSM-R3-1, very low amount of acetate was detected.

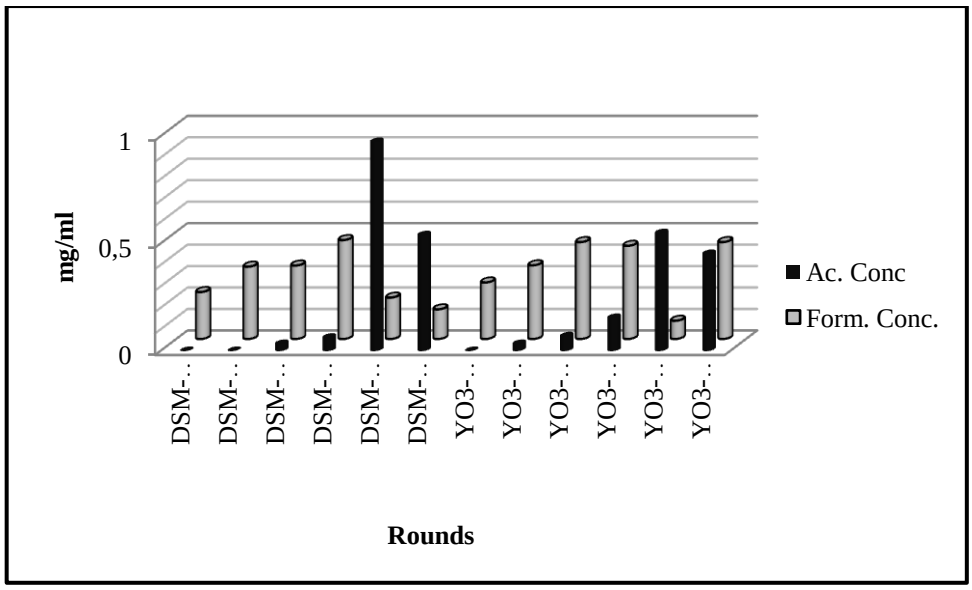


Figure 4.21 Final acetate and formate concentrations at the end of the three rounds of hydrogen production for both strains DSM and YO3 throughout two experiments for each strain when acetate concentration was 60 mM

Those results reflecting the active hydrogen production during the first round which represented by total hydrogen produced ranged between 3.14 – 5.00 liter of pure hydrogen per liter of liquid.

When second experiment of DSM strain is taken in account, data indicates increased values of final acetate concentrations, Figure 4.21, reflected by low hydrogen production, see Figure 3.17.

Regarding data of final acetate concentration in case of using YO3 strain fed with 60 mM acetate. It was observed that the remains of acetate at the end of the three rounds of the first experiment were undetected or low ones (0.0324 and 0.0682 mg/ml at the end of second and third rounds, YO3-R2-1 and YO3-R3-1). During the second experiment it was observed that remains of acetate at the end of the three rounds were still high comparing to opposite values from first experiment. This result also reflects lower hydrogen production during the second experiment of YO3 if compared to the first experiment, see Figure 3.23.

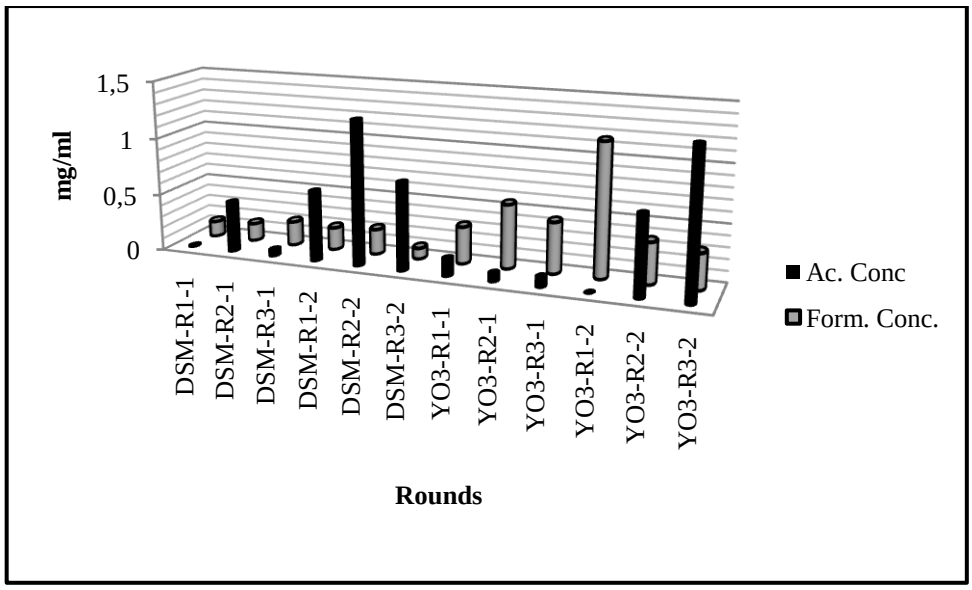


Figure 4.22 Final acetate and formate concentrations at the end of the three rounds of hydrogen production for both strains DSM and YO3 throughout two experiments for each strain when acetate concentration was 80 mM

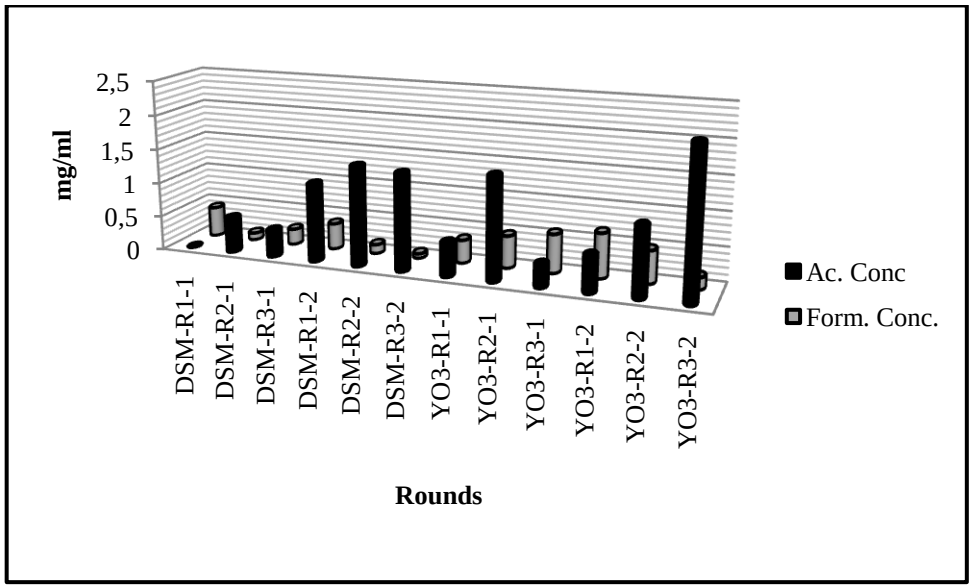


Figure 4.23 Final acetate and formate concentrations at the end of the three rounds of hydrogen production for both strains DSM 1710 and YO3 throughout two experiments for each strain when acetate concentration was 100 mM

Following HPLC data analysis for final concentrations of organic acids during first and second experiment of DSM1710 strain with 80 mM acetate concentration showed same phenomena like seen during 60 mM acetate feed. It was observed that high remains of acetate reflect lower hydrogen production. Such result was observed in the second round of the first experiment and all the three rounds of the second experiment. Regarding second experiment, the second round produced the lowest amount of hydrogen and registered high amount of acetate remains (1.27 mg/ml).

Exploring HPLC data for YO3 strain fed with 80 mM acetate also gives the same conclusion where high acetate remains at the end of the round indicates low amount of hydrogen produced. This has been clarified by the results of second and third round of second experiment, see Figure 4.22 and Figure3.25.

In Figure 4.23 final concentrations of organic acids at the end of each round during hydrogen production by DSM1710 and YO3 strains are shown. Comparing the data of final acetate concentration for each round and total hydrogen production of the same round with data from opposite case will reveal that less hydrogen produced means more remains of acetate at the end of that round. This conclusion is true for all experiments, see Figure 3.21 and Figure 3.27.

4.3 Effect of bacterial concentration on hydrogen production by immobilized *Rhodobacter capsulatus* fed with different acetate concentration

During this part of work effect of increasing bacterial cell concentration has been investig*ated. Bacterial concen*tration was increased from 2.5 mg DCW/ml agar-gel which has been applied during the previous studies into 5 mg DCW/ ml agar-gel. Increasing of bacterial cell concentration aimed to explore its effect on hydrogen production by both strains. This new parameter was applied with different concentrations of acetate (60, 80 and 100).

4.3.1 Effect of increasing bacterial concentration on hydrogen production by immobilized *Rhodobacter capsulatus* DSM1710 by using different concentrations of acetate

This part of study concerned with exploring effect of increasing bacterial cell concentration of DSM1710 strain on hydrogen production by cultures fed with hydrogen production medium with acetate concentration included 60, 80 and 100 mM of acetate. Bacterial

concentration was increased to 5 mg DCW/ml of agar-gel while glutamate concentration was kept at 4 mM.

On the basis of pure hydrogen calculations 60 mM acetate resulted in 2.71 liter per liter liquid of total hydrogen when cell concentration was 5 mg and 4.185 liter of hydrogen per liter liquid when cell concentration was 2.5 mg. 80 mM of acetate produced about 2.1 liter of total hydrogen per liter of liquid when cell concentration was 5 mg and 3.23 liter of total hydrogen when cell concentration was 2.5 mg. Using 100 mM acetate resulted in 1.72 liter of total hydrogen per liter of liquid when cell concentration was 5 mg and 3.02 liter of total hydrogen when cell concentration was 2.5 mg.

Increasing of cell concentration in case of *Rhodobacter capsulatus* DSM 1710 bacteria has given a negative effect. It contributed to lowering of both rate and total hydrogen production. Such confliction can be explained on the fact that *Rhodobacter capsulatus* DSM 1710 has hydrogen uptake enzyme which is hydrogenase. Presence of this enzyme with high concentration (high cell concentration) may contribute to lowering hydrogen production in terms of rate and total hydrogen produced.

Figure 4.24 shows comparison between total amount of hydrogen production by DSM using 2.5 mg DCW/ml agar-gel and using 5 mg DCW/ml agar-gel. Figure 4.25 shows comparison between rate of hydrogen production by DSM using 2.5 mg DCW/ml agar-gel and using 5 mg DCW/ml agar-gel. All represented values in Figure 4.24 and Figure 4.25 are average of doubled experiment.

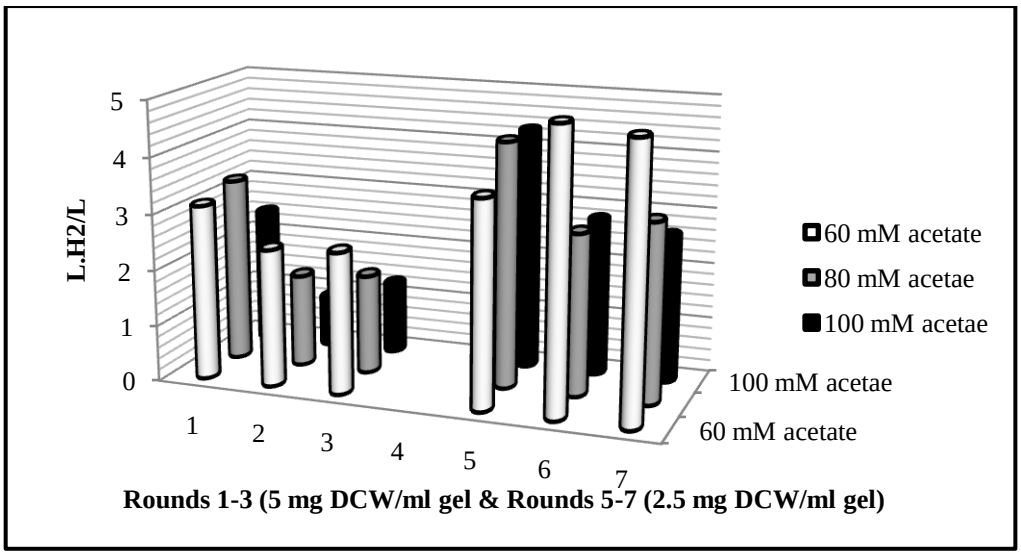


Figure 4.24 Effect of increasing cell concentration (from 2.5-5 mg DCW/ml agar) on total hydrogen produced by immobilized Rhodobacter *capsulatus* DSM1710

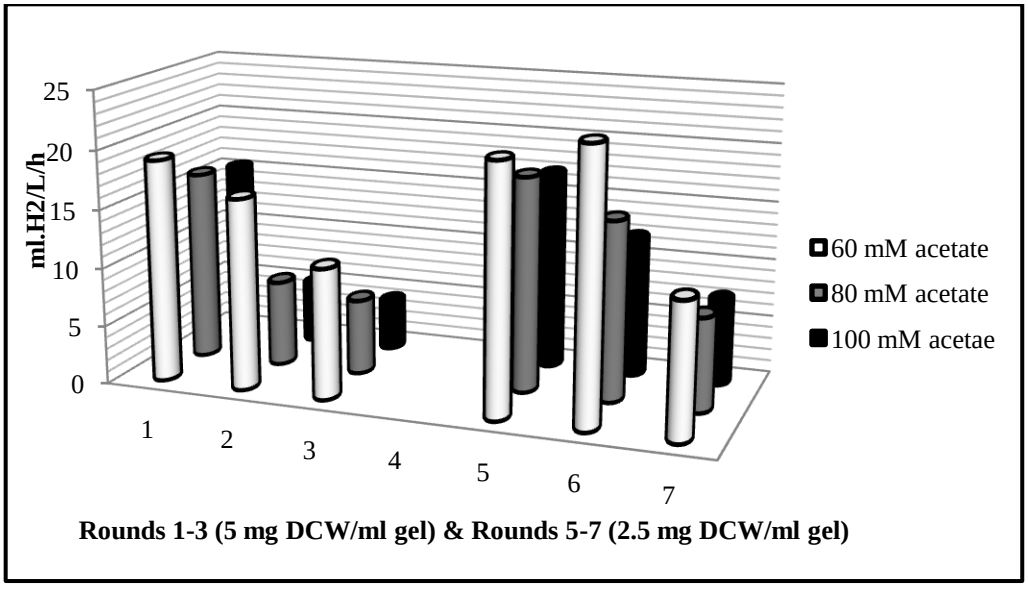


Figure 4.25 Effect of increasing cell concentration (from 2.5-5 mg DCW/ml agar) on rate of hydrogen production by immobilized Rhodobacter *capsulatus* DSM1710

4.3.2 Effect of increasing bacterial concentration on hydrogen production by immobilized *Rhodobacter capsulatus* YO3 by using different concentrations of acetate

This part of study concerned with exploring effect of increasing bacterial cell concentration of YO3 strain on hydrogen production by cultures fed with hydrogen production medium with acetate concentration included 60, 80 and 100 mM of acetate. Bacterial concentration was increased to 5 mg DCW/ml of agar-gel while glutamate concentration was kept at 4 mM.

Increasing of bacterial cell concentration during hydrogen production by YO3 strain contributed an increase of total hydrogen produced and rate of hydrogen production with all concentrations.

On the basis of pure hydrogen ratio in the total gas produced, 60 mM acetate produced 4.56 liter H_2/L when cell concentration was 5 mg and 4.34 L.H_2/L when cell concentration was 2.5 mg. Feeding immobilized bacteria with 80 mM of acetate produced about 4.36 L.H_2/L when cell concentration was 5 mg and 3.95 L.H_2/L when cell concentration was 2.5 mg while 100 mM acetate produced 5.98 L.H_2/L when cell concentration was 5 mg and 3.9 L.H_2/L when cell concentration was 2.5 mg.

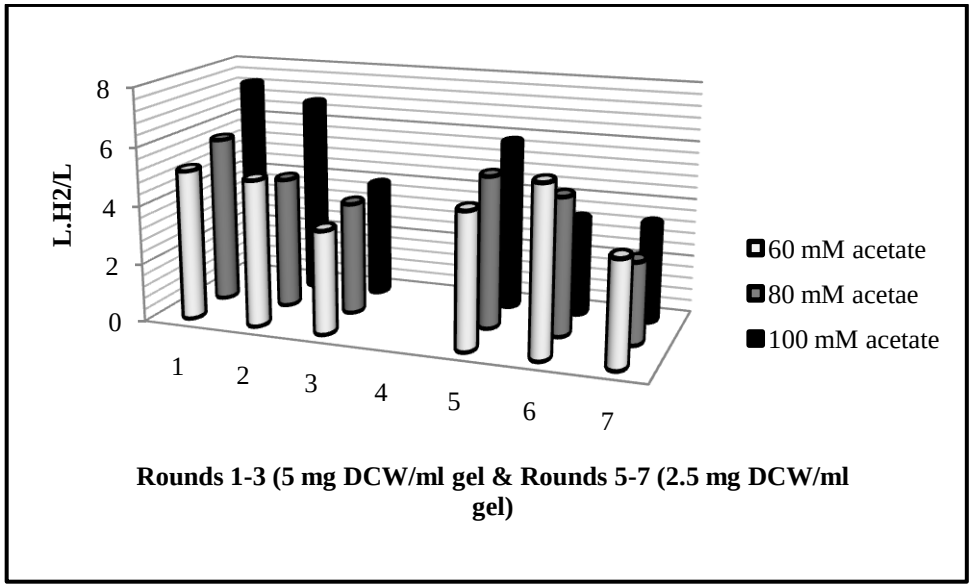


Figure 4.26 Effect of increasing cell concentration (from 2.5-5 mg DCW/ml agar) on total hydrogen produced by immobilized Rhodobacter *capsulatus* YO3

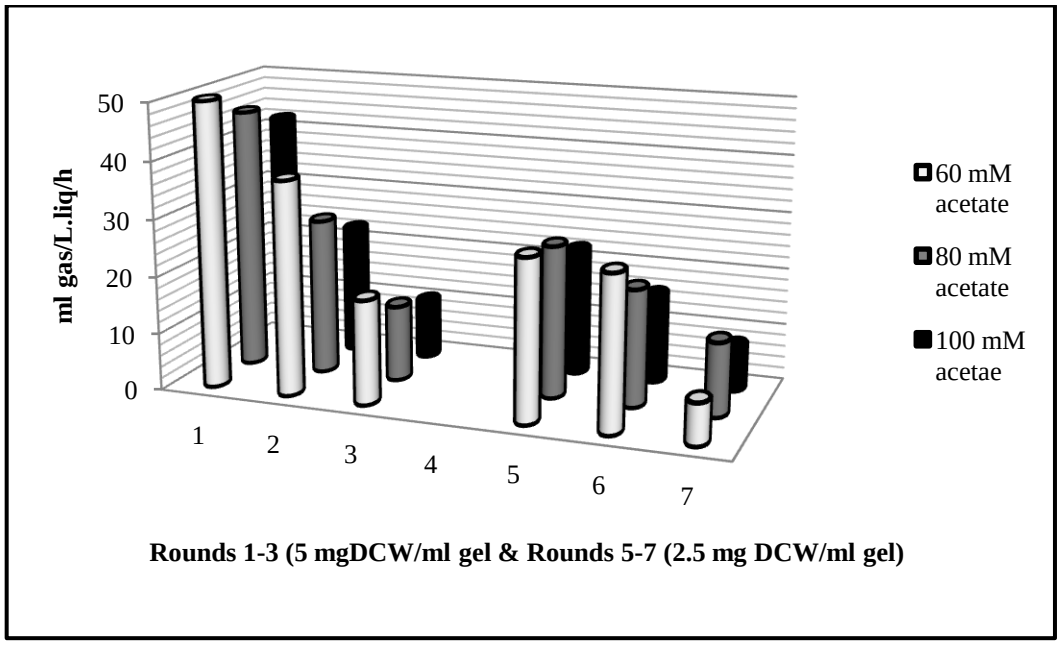


Figure 4.27 Effect of increasing cell concentration (from 2.5-5 mg DCW/ml agar) on rate of hydrogen production by immobilized Rhodobacter *capsulatus* YO3

The results indicated insignificant increase in total hydrogen production by increasing bacterial cell concentration. Figure 4.27 shows significant increase in the rate of hydrogen production in all used concentrations of acetate.

Increasing cell concentration to 5 mg resulted on average rate of hydrogen production about 35.26 ml.H_2/L/h while 2.5 mg cell concentration gives 21 ml H_2/L/h (1.68 times increase). When immobilized bacteria were fed with 80 mM acetate, 5 mg cell concentration produced 28.88 ml.H_2/L/h 2.5 mg cell concentration produced 20 ml.H_2/L/h (1.44 times increase). When immobilized bacteria were fed with 100 mM acetate, 5 mg cell concentration produced 25.1 ml.H_2/L/h while 2.5 mg cell concentration produced 15.35 ml.H_2/L/h (1.64 times increase).

In conclusion, increase cell concentration in case of YO3 strain contributed mainly to increase rate of hydrogen production more than contribution in increase total hydrogen production which was insignificant especially with 60 mM acetate. Increasing of bacterial cell concentration to 5 mg DCW/ml agar-gel is suggested with YO3 strain since it resulted on improving of rate of hydrogen production by YO3 cultures.

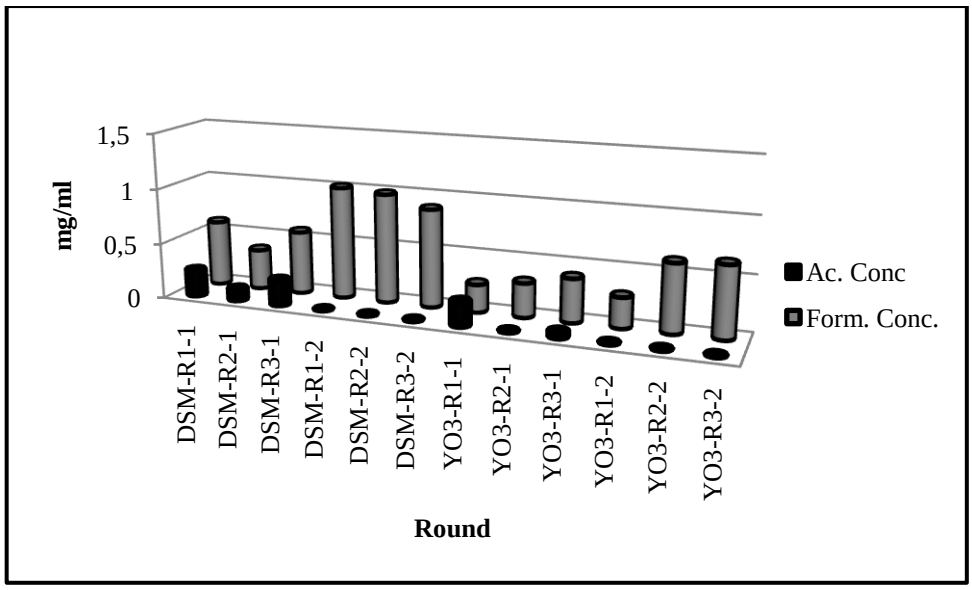


Figure 4.28 Final acetate and formate concentrations at the end of the three rounds of hydrogen production by double experiment of immobilized bacteria of the two strains DSM and YO3 fed with 60 mM when cell concentration was 5mg DCW/ml agar

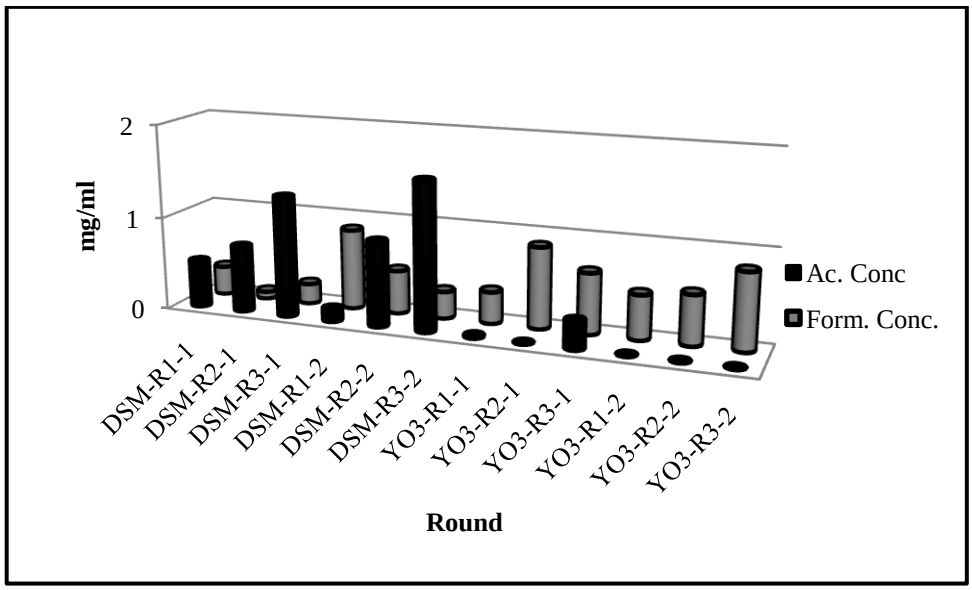


Figure 4.29 Final acetate and formate concentrations at the end of the three rounds of hydrogen production by double experiment of immobilized bacteria of the two strains DSM and YO3 fed with 80 mM when cell concentration was 5mg DCW/ml agar

HPLC analysis data for final concentrations of acetate at the end of each round in first and second experiments of hydrogen production by DSM strain are shown in Figure 4.28. Observations the data indicated undetected acetate remains at the end of the three rounds of second experiment comparing to the first experiment. This finding pointed to higher hydrogen production resulted from second experiment compared to first one.

Figure 4.28 also exhibits HPLC analysis data for 60 mM acetate used with YO3 strain. Data represent double experiments for each round. Observations from data exploration indicating that second experiment resulted in higher hydrogen production than first experiment and this is reflected by exhausting of all acetate by end of each round along the experiment process.

Figure 4.29 show HPLC data for final organic acids concentrations remained at the end of each round of two experiments operated with both strains YO3 and DSM. The data collected from DSM experiments showed high acetate remains in the two experiments. Such findings gave an image about the low production of hydrogen resulted by this strain when fed with 80 mM acetate.

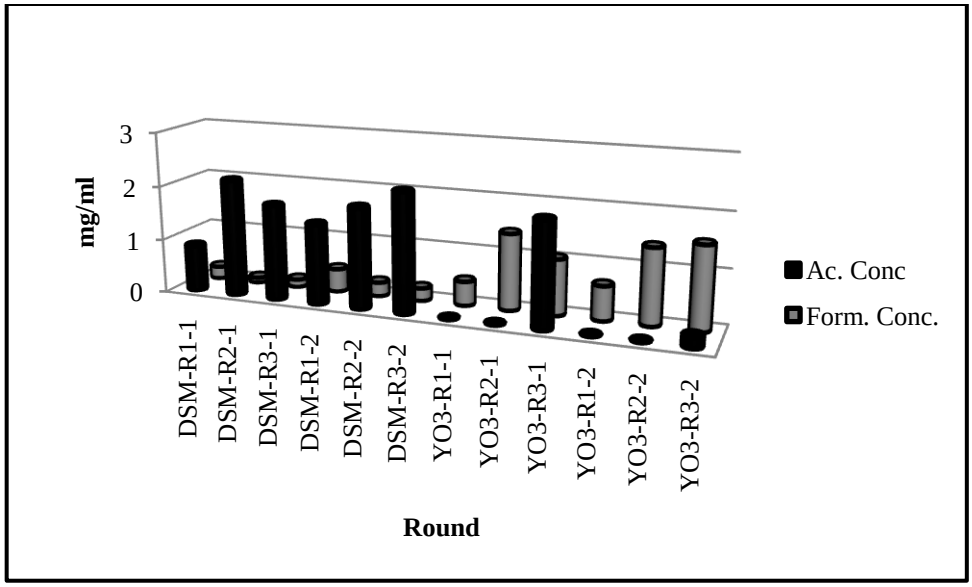


Figure 4.30 Final acetate and formate concentrations at the end of the three rounds of hydrogen production by double experiment of immobilized bacteria of the two strains DSM and YO3 fed with 100 mM when cell concentration was 5mg DCW/ml agar

The data collected from YO3 experiments showed very low or disappearance of acetate remains in the two experiments with exception of third round during first experiment which exhibited little high remains.

HPLC data collected after feeding bacteria with 100 mM are shown in Figure 4.30. Final acetate concentrations related to DSM two experiments exhibiting high acetate remains at the end of each round. Such results supporting the idea that DSM strain will not produce hydrogen in a satisfactory manner when being fed with medium containing high acetate concentrations. The previous HPLC data and the present one supporting 60 mM acetate as strong candidate concentration for hydrogen production by DSM1710 strain.

Regarding YO3 strain, HPLC analysis data for acetate remains shown in Figure 4.30 revealed no acetate or low remained concentrations of acetate. This finding is true for first and second rounds of the two experiments and with some extension is true for third round of the second experiment. Third round of the first experiment exhibited high remains of acetate and reflecting low hydrogen production during this round. Those results suggested that utilization of acetate for hydrogen production by feeding 100 mM acetate concentration is a potential choice.

In conclusion it is possible to suggest that 60 mM acetate concentration is preferred for hydrogen production by DSM1710 strain and higher concentrations are not good choice. This conclusion is supported by results from the previous study concerning using of different concentration of acetate when bacterial cell concentration was 2.5 mg DCW/ml agar-gel was in use.

The results also introduced 60mM acetate as most acceptable concentration for feeding YO3 strain for hydrogen production and at the same time they introduced 80 and 100 mM acetate as candidates for hydrogen production by this strain.

4.4 Effect of manipulation of immobilized *Rhodobacter capsulatus* with 2.5% and 5% glycerol on hydrogen production

Glycerol is a well-known cryoprotectant and it is used to preserve bacteria during lypholyzation. During this part of work effect of using glycerol on hydrogen production by photosynthetic bacteria was investigated. Glycerol was added during preparation of bacteria-gel complex. As described in materials and methods glycerol was added to the suspended bacteria and the new mixture then added to the molded agar and mixed together to form the appropriate glycerol concentration.

According to Nasif *et al*. (2002), glycerol provides protection for cell membrane during immobilization process. They used 10% glycerol in buffer medium to entrap E. coli within silica gel for examining glucose consumption and metabolites production. They concluded that gel with glycerol has kept 65% of bacteria cultivable for two weeks & 40% of bacteria cultivable for four weeks comparing to 15% & 10% without using glycerol with silica gel. They found that ability of bacteria to incorporate glucose was kept by 65% after two weeks and 55% after four weeks comparing to 25% and 5% when silica gel was used without glycerol.

Pena-Vazquez, *et al*. (2009), used entrapped microalgae to build fiber optic biosensors for herbicide monitoring. They entrapped microalgae within silica gel and studied effect of glycerol on membrane stability. They recommend 2.5% glycerol concentration for that purpose as they found that fluorescence intensity was maximized & showed a good reproducibility.

Yang X., *et al*. (2008) recommended using of glycerol in preparation of polyvinyl-alcohol/water soluble chitosan hydro-gel. They concluded that glycerol improves the swelling capacity of hydro-gels.

Using of glycerol during this part of work aimed to provide protection for cell membrane during manipulation of bacteria with hot molded agar and to improve swelling capacity which may decrease mechanical stress exerted on bacteria by concentrated agar.

Syrtsorva L. A., *et al*. (2004), studied influence of glycerol on nitrogenase reactions. They investigated influence of glycerol on ATPase reactions of nitrogenase and reduction of acetylene. They found that glycerol inhibits ATPase nitrogenase reactions dependent on an electron donor. They found that the reaction rate is halved at glycerol concentration of 11% in the medium. The electron donor-independent (decoupled) ATPase reaction of nitrogenase is inhibited to a lesser extent. The inhibition effect of glycerol can be attributed to its influence on protein globule of nitrogenase, for example to change the structure of hydrogen bonds near the active site.

4.4.1 Effect of glycerol on hydrogen production by immobilized *Rhodobacter capsulatus* DSM 1710

Figure 4.31 shows total hydrogen produced and Figure 32 shows rates of hydrogen production by immobilized *Rhodobacter capsulatus* DSM 1710 of manipulated with glycerol. In Figure 4.31 it is observed that average total hydrogen produced at first round by glycerol-manipulated cultures is higher than cultures without glycerol manipulation. This result may be

attributed to utilization of glycerol or to protection provided to bacteria after exposed to hot molded agar before starting the process.

At the same time cultures manipulated with 5% glycerol produced less hydrogen than cultures manipulated with 2.5% glycerol and non-glycerol manipulated cultures during first round. This can be attributed to starting of inhibitory effect of glycerol at such concentration.

Comparing average total hydrogen production along the whole process it is observed that non-glycerol cultures produced 2.7 L.H/L and glycerol-manipulated bacteria produced 3.00 L.H/L (about 10% more). Observed total rate of hydrogen production showed that non-glycerol cultures showed higher rates (19, 16.33 and 11.15) with total average for the whole process about 15.5 ml/L/hr.

Rates of hydrogen production by glycerol-manipulated immobilized bacteria exhibited lower values. For 2.5% glycerol used, rates where (17, 10.72 and 11) ml/liter/hr for the three rounds in double and for 5% glycerol used they were (10.6, 8.5 and 16.18) with total average rate of hydrogen produced about 11.76 ml/L.liq/hr. On the basis of total average ratios of the whole process throughout the three rounds it was observed that using 2.5% glycerol caused decrease by 17% on hydrogen production rate while using 5% glycerol caused more decrease which was around 24%.

Higher amounts of total hydrogen produced when using 2.5% glycerol comparing to non-glycerol cultures can be attributed to the protection provided to the cell membrane (Nasif *et al*. 2002; Yang *et al.* 2008 and Pena-Vazquez, *et al.* 2009) or could be glycerol used for hydrogen production. The lower rate of hydrogen production resulted from glycerol manipulated cultures can be attributed to partial suppression exerted by glycerol on nitrogenase (Syrtsorva *et al.* 2004).

HPLC data shown in Figure4-35 illustrated that when hydrogen production is plenty no or little remains of acetate can be detected at the end of each round, this is observed during first experiment using 2.5% glycerol. During first and second rounds of second experiment with 2.5% glycerol hydrogen production was low this observation was manifested by considerable remains of acetate at the end of each round while at the end of the third round no acetate remains detected reflecting that bacteria accumulated more hydrogen than previous two rounds.

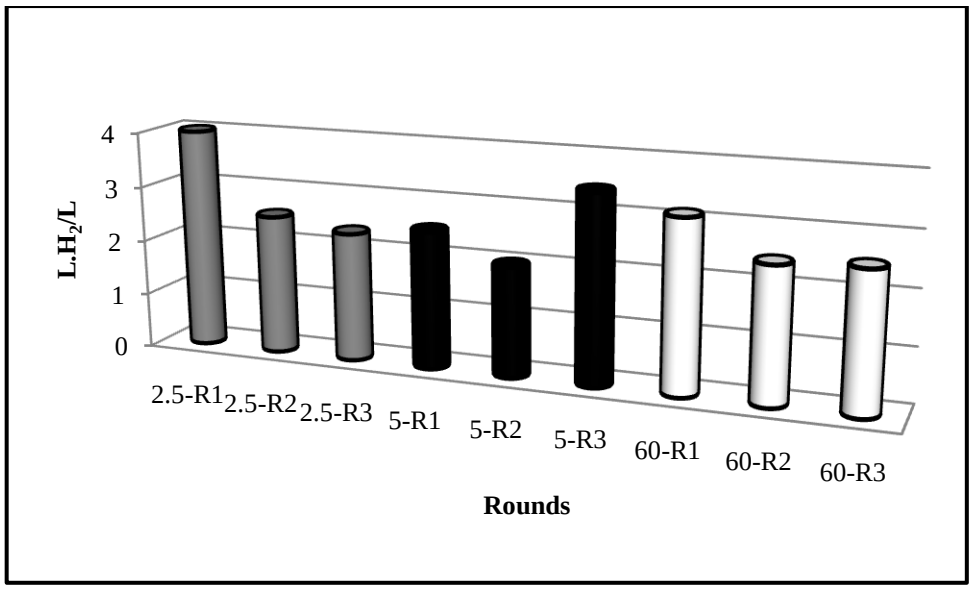


Figure 4.31 Comparing average total hydrogen production by *R. capsulatus* DSM1710with and without glycerol manipulation

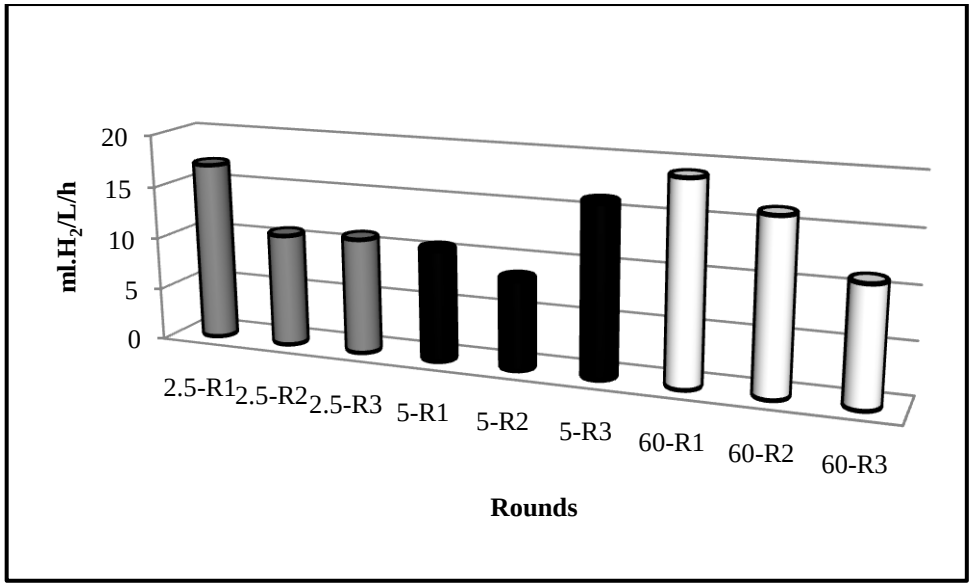


Figure 4.32 Comparing average rates of hydrogen production by *R. capsulatus* DSM1710with and without glycerol manipulation

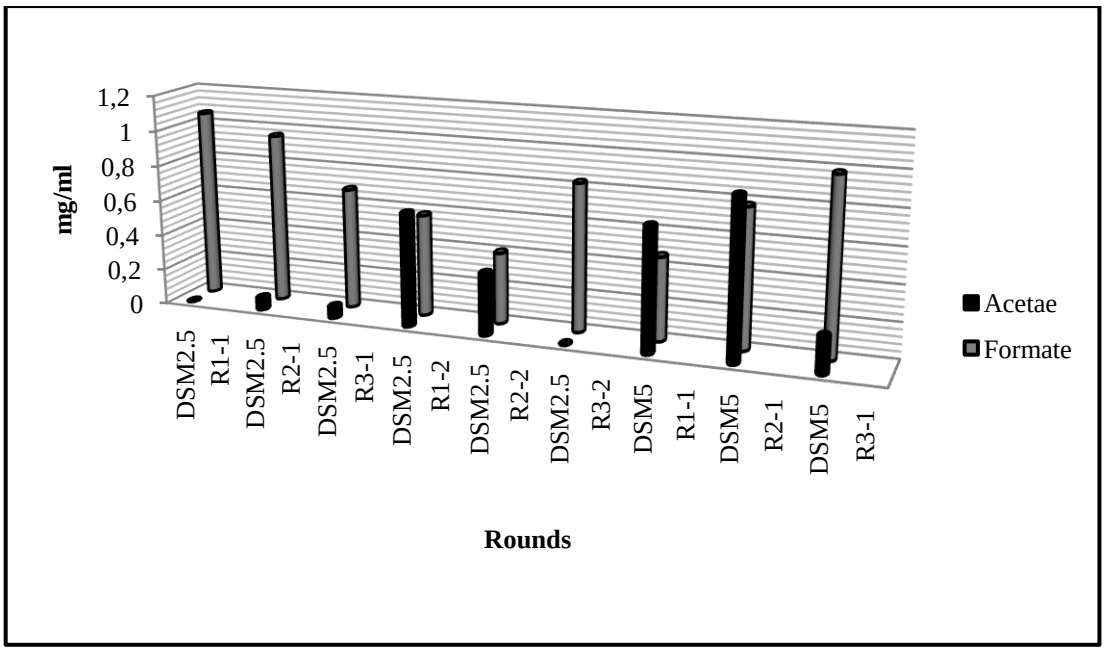


Figure 4.33 Final acetate and formate concentrations at the end of the three rounds of hydrogen production by *R. capsulatus* DSM1710 manipulated by 2.5% and 5% glycerol throughout two experiments

Low hydrogen production by immobilized *R. capsulatus* DSM 1710 manipulated by 5% glycerol, has been reflected by high remains of acetate at the end of first and second round while the third round exhibited less amounts of acetate remains. This observation is reflecting higher accumulation of hydrogen comparing to first and second rounds. This is may be attributed to glycerol consumption and washing out since the bottles were flushed by basal medium before e refreshing the medium.

4.4.2 Effect of glycerol on hydrogen production by immobilized *Rhodobacter capsulatus* YO3

Observations from Figure 4.33 and Figure 4.34 can easily show that glycerol manipulation caused decrease in both total hydrogen production and rate of hydrogen production. Decreasing of total hydrogen production mainly manifested during first and second rounds were using 5% glycerol caused slightly more decrease than using 2.5% glycerol while differences between rates of hydrogen production were insignificance.

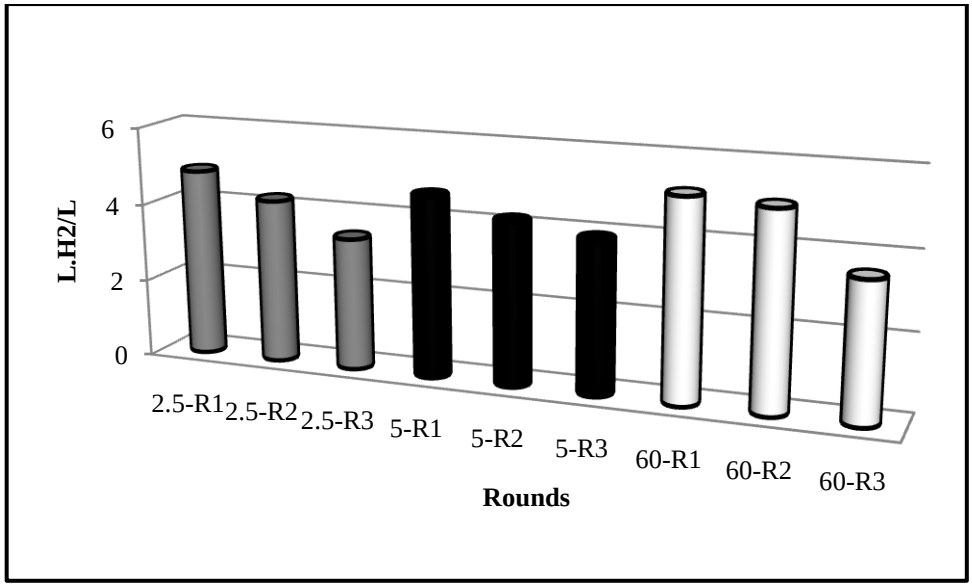


Figure 4.34 Comparing average total hydrogen production by *R. capsulatus* YO3 with and without glycerol manipulation

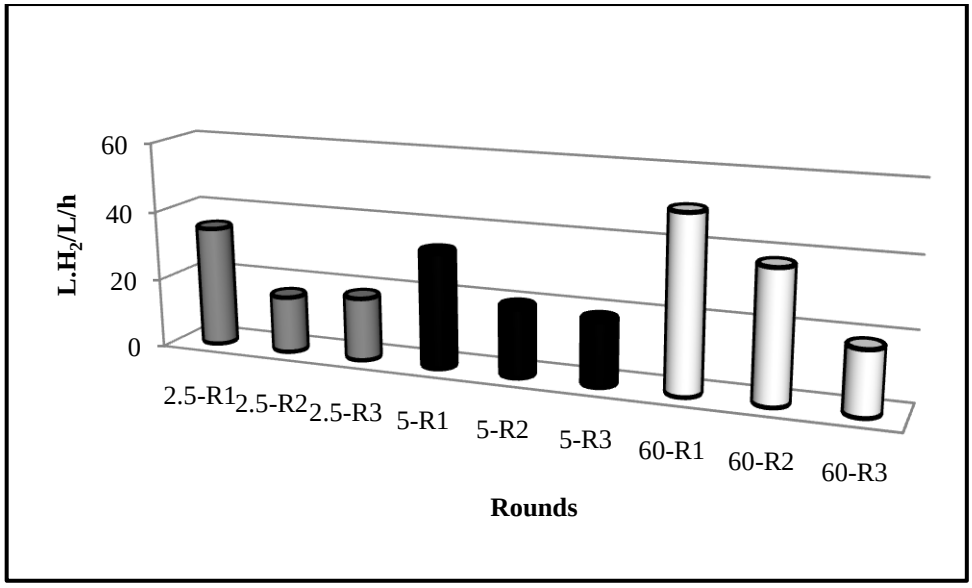


Figure 4.35Comparing average rates of hydrogen production by *R. capsulatus* YO3 with and without glycerol manipulation

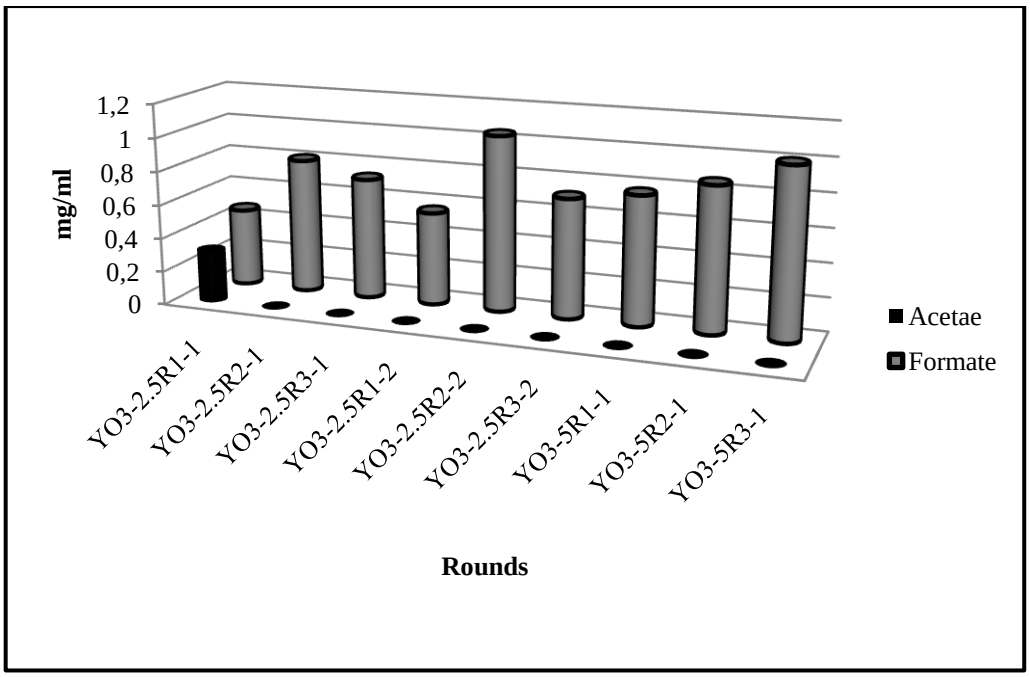


Figure 4.36 Final acetate and formate concentrations at the end of the three rounds of hydrogen production by *R. capsulatus* YO3 manipulated by 2.5% and 5% glycerol throughout two experiments

Decreasing of hydrogen production, rate and total hydrogen production, is mainly supposed resulted from inhibition effect of glycerol on nitrogenase (Syrtsorva *et al.* 2004). Decreasing of rate of hydrogen production was prominent. Considering the total average of whole processes we can find the using glycerol caused around 35% decrease in rate of hydrogen production.

HPLC data shown in Figure 4.36 concerning final acetate concentrations at the end of each round illustrated almost no acetate remains were detected at the end of each round throughout experiments with 2.5% and 5% glycerol with exception of first round YO3-2.5-R1-1 corresponding to first experiment with 2.5% glycerol. The amount of acetate remains at the end of this round was about 0.3113 mg/ml which is not that much high to indicate low hydrogen production taking in account that this round accumulated about 935 ml of hydrogen.

4.5 Effect of sodium dithionite on hydrogen production by *Rhodobacter capsulatus*

Sodium dithionite used in vitro as electron donor to reduce nitrogenase enzyme of nitrogen-fixing *Klebsiella pneumonia* (Roberts *et al*., 1978). Sodium dithionite is used in vitro to reduce and activate nitrogenase enzyme of *Klebsiella pneumonia* mutant strains unable to fix nitrogen. It has been used as electron donor to bypass the natural electron donating system (St. Jhon *et al*. 1975).

Bagai R. and Madamwar (1998) used reducing agents including sodium dithionite, sodium sulfide and sodium acetate with immobilized combined system to induce hydrogen production through reducing the nitrogenase complex. They concluded that using of reducing agents protects nitrogenase from inhibition by oxygen liberated during hydrogen production. They used reducing agents as oxygen scavengers. They added sodium dithionite and other reducing agents separately and continuously with a flow rate 1ml min-1. Concentration of sodium dithionite was 2mM.

During the present work adding of sodium dithionite conducted using different concentrations including 0.5, 1.0 and 1.5 mM. Addition sodium dithionite was applied with same concentrations to the agar gel during preparation. Each concentration was conducted in double experiment for both bacterial strains.

Before using the previous three concentrations, a scanning survey by using higher concentrations including 1.5, 3, 4.5 mM of acetate was conducted with *R. capsulatus* YO3 strain. Results were unsatisfactory with 3 and 4.5 mM and agar gel suffered cracking and loosing integrity. Bacteria produce low amounts of hydrogen and the cultures showed black colored precipitations with bad rotten-egg smell (Data are not) shown. Because of that lower concentrations were scanned during later experiments.

Wayman and Lem (1969) studied the behavior of sodium dithionite in aqueous solutions. In their work they studied the kinetics of decomposition of sodium dithionite and they suggested formation of intermediates and final compounds resulting from decomposition. They illustrated the formation of thiosulfate and sulfite as products. Decomposition process with intermediates and products are illustrated in the following equations.

$$S_2O_4^{2-} + H^+ \rightleftharpoons H\,S_2O^{4-}$$

$$H\,S_2O^{4-} + H_2O \longrightarrow H\,SO^{2-} + H\,SO^{3-} + H^+$$

$$H S_2O^{4-} + H SO^{2-} \longrightarrow S_2O_3^{2-} + H SO_3^{-} + H^{+}$$

Bakels *et al*., (1996) studied effect of sulfite on ATP hydrolysis and synthesis activities in chloroplasts and cyanobacterial membrane vesicles. They concluded that sulfite as the active ion species HSO3- (bisulfate) inhibits cyclic photophosphorylation in chloroplasts and in cyanobacterial membranes. The inhibition is attributed to inhibition of electron transfer or to uncoupling by sulfate. They estimated that in cyanobacterial membranes sulfite can replace a proton gradient as activator of ATP hydrolysis in the same way as chloroplast.

Cappellini *et al*., (1997) studied effect of sulfite on ATP synthase of *Rhodobacter capsulatus*. They found that sulfite stimulates rate of ATP hydrolysis by ATP synthase. Sulfite affects the capacity of the enzyme in translocation the protons. They demonstrated that hydrolytic reaction becomes gradually uncoupled from the process of proton translocation when sulfite concentration is greater than 10 mM. This is found to be accompanied by inhibition of ATP synthesis, either driven by light or by artificially induced ionic gradients. At concentrations of at least 80 mM ATP synthesis will completely inhibited. The researchers demonstrated that low concentrations of this anion (≤ 2mM) prevent the activation by trans-membrane difference in proton electrochemical potential. They estimated that at millimolar concentrations of phosphate inhibition by sulfate can be reversed.

4.5.1 Effect of sodium dithionite on hydrogen production by *Rhodobacter capsulatus* DSM1710

During this part of study effect of the mentioned concentrations of sodium dithionite was investigated and the results of total hydrogen production and rate of hydrogen production are illustrated in Figure 4.37 and Figure 4.38 respectively.

Observations from Figure 4.37 indicate very low total hydrogen production during the first two rounds for each experiment. The Figure shows that average total hydrogen production during first round was around 0.313 liter H_2/liter and 1.043 liter H_2/liter at the end of second round when 1.5 mM sodium dithionite was applied.

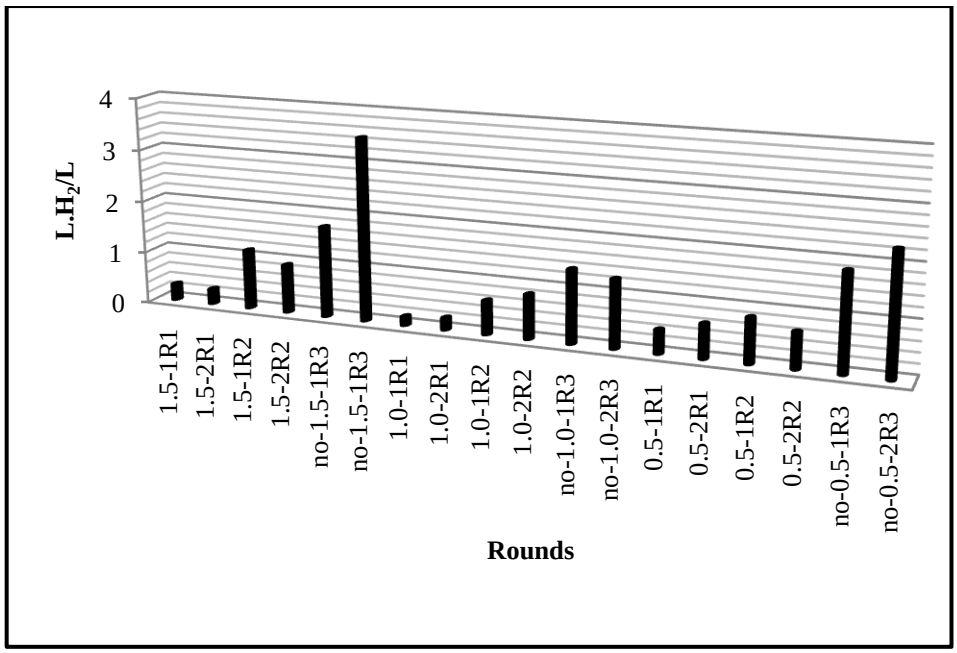


Figure 4.37 Total hydrogen produced by immobilized *Rhodobacter capsulatus* DSM1710 provided with sodium dithionite

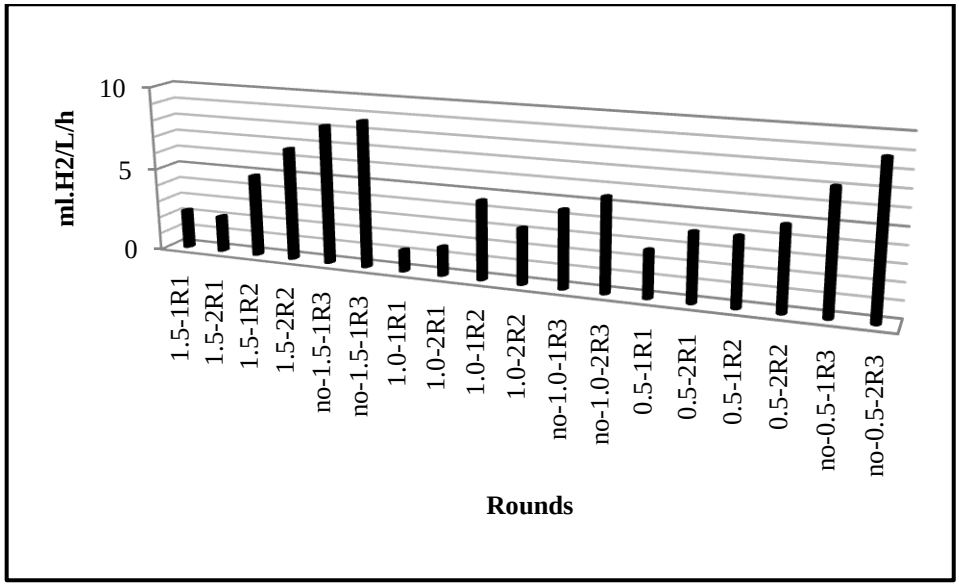


Figure 4.38 Rate of hydrogen production by immobilized *Rhodobacter capsulatus* DSM1710 manipulated with sodium dithionite

Comparing those values with round three when the cultures provided with fresh medium free of sodium dithionite, average of total hydrogen production increased to 2.63 liter which is equivalent to 2.52- 8.4 times of average of total hydrogen produced when immobilized bacteria has been provided with 1.5 mM sodium dithionite was used, Figure, 4.37.

Observations from the rate of hydrogen production revealed that average rate of hydrogen production in the presence of 1.5 mM sodium dithionite have been calculated as 2.23 ml/liter/hr at the end of first rounds and 5.79 ml/liter/hr at the end of second rounds while average rate when using nutrient medium free of 1.5 mM sodium dithionite was 8.5 ml/liter/hr, Figure 4.38.

Regarding using 1.0 mM acetate average of total hydrogen produced was 0.205 liter H_2/liter at the end of first round and 0.75 liter H_2/liter at the end of second round while average rates were calculated as 1.66 ml H_2/liter/hr. When immobilized bacteria provided with nutrient medium free of sodium dithionite average total hydrogen production goes up to 1.35 liter H_2/liter and average rate observed as 5.03 ml H_2/liter/hr, Figure 4.38.

Observations from Figure 4.37 and Figure 4.38 show that using of 0.5 mM of acetate cause average of total hydrogen production by about 0.55 liter H_2/liter at the end of first rounds and average rate of total hydrogen production by around 3 ml H_2/liter/hr. At the end of second round average total hydrogen production was 0.77 liter H_2/liter and average rate of hydrogen production was 4.43 ml.H_2/L//h.

At the end of the third round were no sodium dithionite found within the medium average total hydrogen production goes up to 2.075 liter H_2/liter and average of hydrogen production was around 8.00 ml H_2/liter/hr. Those values represent 2.7 times increase in total hydrogen production and 1.81 times increase in average rate of hydrogen production.

Comparing the above results with results of hydrogen production immobilized *Rhodobacter capsulatus* DSM1710 without exposing to sodium dithionite, see Figure 4.24 and Figure 4.25, it could be observed that average total hydrogen production of three rounds of double experiment was 4.5 L.H/L.liq and average rate of hydrogen production can be calculated around 18.56 ml H/L.liq/h. Those results represent several folds of results achieved by using sodium dithionite.

Black precipitations were observed and they may be attributed to formation of metal sulfides. Intensity of the black precipitations was much more less than observed during experiments with YO3 strain. Bad odor characterized to sewage was observed indicating to formation of hydrogen sulfide.

Thiosulfate and bisulfate species resulted from dissociation of sodium dithionite (Wayman and Lem., 1969) will be exposed to the action of thiosulfate oxidase enzyme and sulfite reductase enzyme of the bacteria. Thiosulfate oxidase will produce sulfate and hydrogen sulfide. Hydrogen sulfide caused the rotten-egg smell appeared from the culture.

Also hydrogen sulfide reacted with metal ions available in the medium leading to form the black precipitation in the culture. By this way metal ions provided to the medium including iron and molybdenum and other trace elements are scavenged from the medium and being unavailable for bacteria causing low nitrogenase activity.

The amount of black precipitation of metal sulfides was not large as was found with YO3 strain. This is may be due to low activity of thiosulfate oxidase and sulfite reductase. Low activity of sulfite reductase left higher amount of sulfite to exert its inhibition effect on ATP synthase and inducing ATP hydrolysis effects, (Cappellini *et al*., 1997).

Such assumption may explain why hydrogen production during second rounds was higher than first rounds were sulfite reductase became more active by time and reducing more sulfite bringing less inhibition to ATP synthase so the ATP-dependent nitrogenase can work better. But thiosulfate oxidase still working and producing more hydrogen sulfide which scavenges more metal ions.

4.5.2 Effect of sodium dithionite on hydrogen production by *Rhodobacter capsulatus* YO3

During this part of study effect of the mentioned concentrations of sodium dithionite was investigated and the results of total hydrogen production and rate of hydrogen production are illustrated in Figure 4.39 and Figure 4.40 respectively.

In spite of that, using of sodium dithionite did not exert negative inhibition on hydrogen production by YO3 strain as much as it did when used with DSM1710 strain, it was clear that the negative effect was exerted through decreasing total hydrogen production and also rate of hydrogen production. The negative effect extends to the stability and integrity of the gel-bacteria complex as seen at the end of first round of experiment two and during second round when agar gel started to crack completely into smaller parts and at the end of the second round were color of the culture converted completely into dark due to black precipitations inside agar and liquid. Black precipitations may be attributed to formation of metal sulfides. The observed bad odor characterized to rotten-egg was indicating to formation of hydrogen sulfide.

The black precipitation was also observed by the end of second rounds when 1.0 sodium dithionite used. Same precipitation was observed during the third round of first experiment with 1.5 mM acetate while during the third round of the second experiment 1.5 mM sodium dithionite was eliminated and the results shown that an increase of hydrogen production equivalent to 2.56 times as the opposite round in first experiment where sodium dithionite kept on 1.5 mM concentration. This finding is supported by same behavior seen by DSM 1710 strain.

Taking in account the first two rounds during hydrogen production in the presence of sodium dithionite and comparing them with results from cultures did not exposed to sodium dithionite, see Figure 4.26 and Figure 4.27. It is possible to observe that average total hydrogen production by bacteria not exposed to sodium dithionite kept all time higher than those fed with medium containing sodium dithionite, see Table 4.1.

Thiosulfate and bisulfate species resulted from dissociation of sodium dithionite (Wayman and Lem., 1969) will be exposed to the action of thiosulfate oxidase enzyme and sulfite reductase enzyme of the bacteria. Thiosulfate oxidase will produce sulfate and hydrogen sulfide. Hydrogen sulfide caused the rotten-egg smell appeared from the culture.

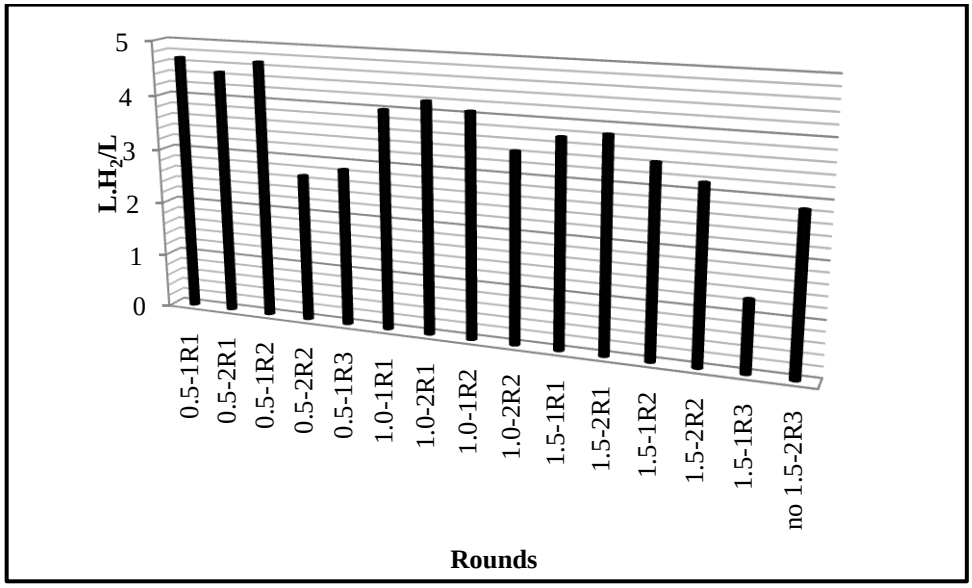


Figure 4.39Total hydrogen produced by immobilized *Rhodobacter capsulatus* YO3 manipulated with sodium dithionite

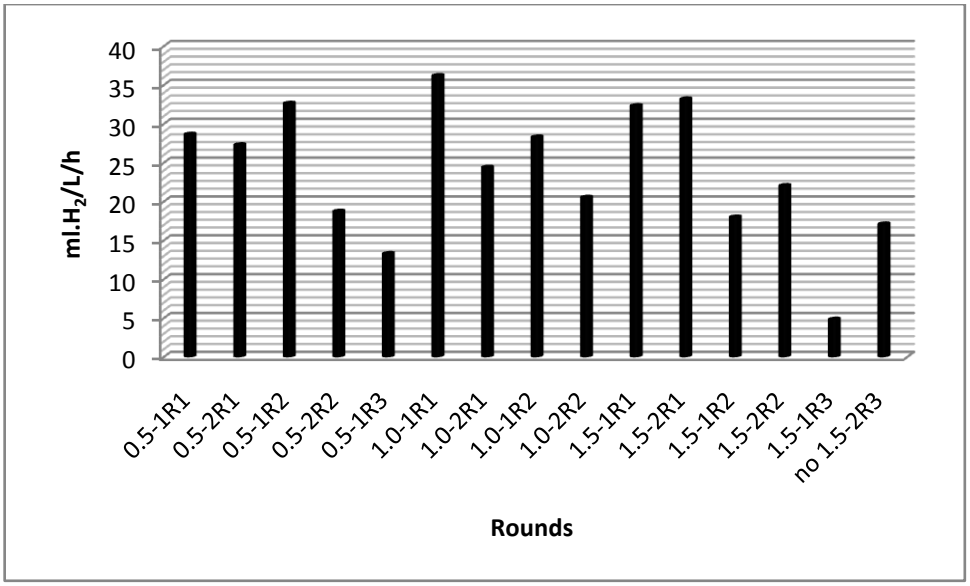


Figure 4.40 Rate of hydrogen production by immobilized *Rhodobacter capsulatus* YO3 manipulated with sodium dithionite

Table 4.1 Comparing average total hydrogen production and average rate of hydrogen production between immobilized *R. capsulatus* YO3 when fed with medium containing sodium dithionite and after fed with medium without sodium dithionite

Round	$L.H_2/L$				$ml.H_2/L/h$			
	0.5 mM SDT	1.0 mM SDT	1.5 mM SDT	No SDT used	0.5 mM SDT	1.0 mM SDT	1.5 mM SDT	No SDT used
First	4.59	4.1	3.8	5.14	28	30.3	32.765	50
Second	3.7	3.76	3.31	5.02	25.7	24.42	20	37.5
Third	*2.875	**	1.3 ***2.875	3.54	*13.3	**	4.85 ***17.11	18.27

(*) Only one bottle remained working, (**) Two bottles stopped working by presence of intense black precipitations, (***) one bottle is isolated and fed with medium free of sodium dithionite

.

This bad smell was more clear and stronger in YO3 strain than DSM1710 strain. The early and then the intensive appearance of black precipitations indicated high hydrogen sulfide production due to thiosulfate oxidation. Those observations mean that thiosulfate oxidase in YO3 strain is more active than in DSM1710

Reaction of hydrogen sulfide with metal ions available in the medium resulted in formation of the black precipitated metal in the cultures. By this way metal ions provided to the medium including iron and molybdenum and other trace elements are scavenged from the medium and being unavailable for bacteria causing low nitrogenase activity.

Results observations in Figure 4.39 and Figure 4.40 indicated the inhibition effect was not drastic like observed with DSM1710 strain. This finding could be attributed to high activity of sulfite reductase which removed sulfite species quickly from the medium leaving ATP synthase in an active mode. The lower hydrogen production observed with bacteria fed with medium containing sodium dithionite comparing to bacteria fed with medium free of sodium dithionite may be attributed also to the chelating action of hydrogen sulfide that precipitated metal ions preventing bacteria from utilizing them.

4.6 Effect of ammonium on hydrogen production by photosynthetic bacteria

As mentioned in material and methods, this experiment was aiming to examine effect of ammonium on hydrogen production by immobilized *R. capsulatus*. For that purpose, three different concentrations of ammonium chloride (2.5, 5 and 7.5 mM) were used as sole nitrogen source to replace glutamate in medium 60/4 which was used to feed the immobilized bacteria during hydrogen production process. The three concentrations were applied in parallel double manner for each of them. Feeding of immobilized bacteria during this experiment employed sequential batch feeding for three rounds as described previously.

4.6.1 Effect of ammonium on hydrogen production by photosynthetic bacteria *Rhodobacter capsulatus* DSM 1710 strain

The observations from Figure 4.41 which describe total hydrogen produced by immobilized DSM 1710 strain indicated that 2.5-1R1 and 2.5-2R1 (both are first round of double experiment) produced 1.75 and 1.72 L.H_2/L. At the end of second round total hydrogen produced decreased to 1.00 L.H_2/L, then it continued decreasing to 0.65 L.H/L.

Comparing those results with results of total hydrogen produced when glutamate was used as sole nitrogen source (2.0, 3.22 and 2.87 L.H_2/L during R1, R2 and R3 respectively), it is possible to conclude that using of ammonium as nitrogen source affected negatively on hydrogen production ability of the immobilized DSM strain.

The results of rate of hydrogen production given by using 2.5 mM ammonium chloride indicating decrease in the rate of hydrogen production. Using 4 mM glutamate as nitrogen source during hydrogen production by immobilized DSM 1710 strain resulted in 11, 12.7 and 9 ml.H_2/L/h for R1, R2 and R3 respectively. Using ammonium chloride produced rates of hydrogen production lower than those given by using 4 mM glutamate.

Application of 2.5 mM of ammonium chloride as nitrogen source caused decrease of hydrogen production at such concentration. This may indicate that immobilization can provide some protection for bacteria to continue producing hydrogen in spite of presence of ammonium at such concentration. The steady decrease in total hydrogen production from R1 to R3 could be attributed to the repression effect of ammonium on synthesis of nitrogenase enzyme itself. This effect was increased steadily indicating that agar is being more saturated with ammonium as exposure time is increased from R1 to R3.

Effect of ammonium on total hydrogen produced also reflected on the rate of hydrogen production by the immobilized bacteria. Following the rate of hydrogen production which could be observed from Figure 4.42 indicated a decrease in rate of hydrogen production by passing from R1, R2 to R3. Such decrease in rate of hydrogen production was observed during hydrogen production when glutamate was used as nitrogen source but the decrease here was more drastic since ammonium is known to cause repression for synthesis of nitrogenase.

Total hydrogen produced by immobilized bacteria provided with nutrient medium containing 5 mM ammonium chloride is illustrated in Figure 4.43. Total hydrogen produced by using 5 mM ammonium chloride started at 1.13 and 1.1 L.H_2/L and decreased to 0.38 and 0.4 L.H_2/L.

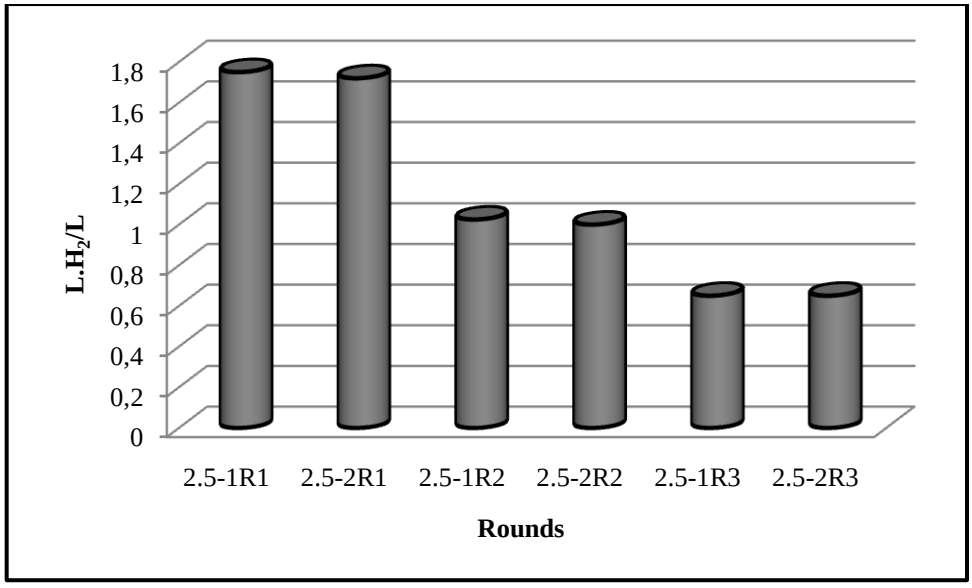


Figure 4.41Total hydrogen produced by immobilized *R. capsulatus* DSM 1710 fed with 2.5 mM ammonium chloride as sole nitrogen source

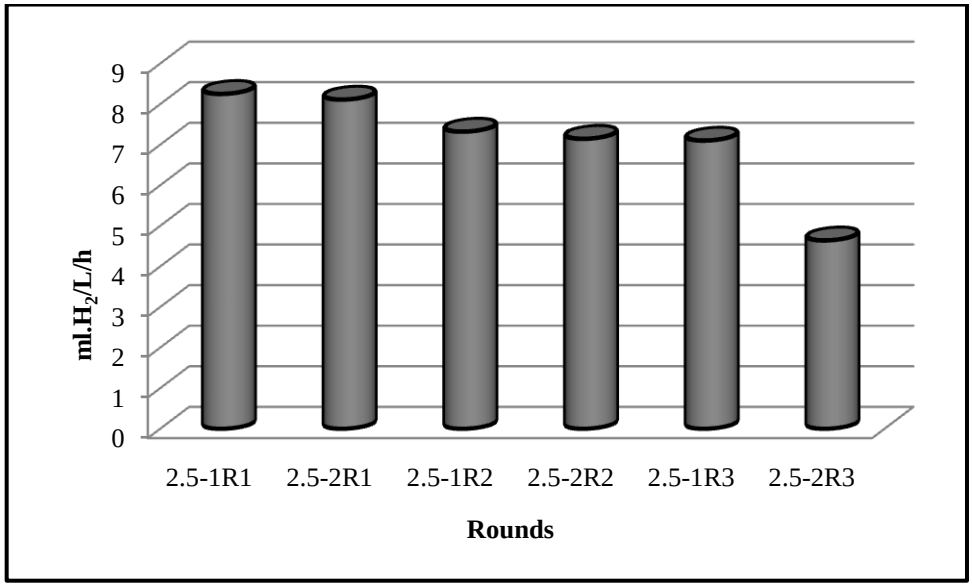


Figure 4.42Rate of hydrogen production by immobilized *R. capsulatus* DSM 1710 fed with 2.5 mM ammonium chloride as sole nitrogen source

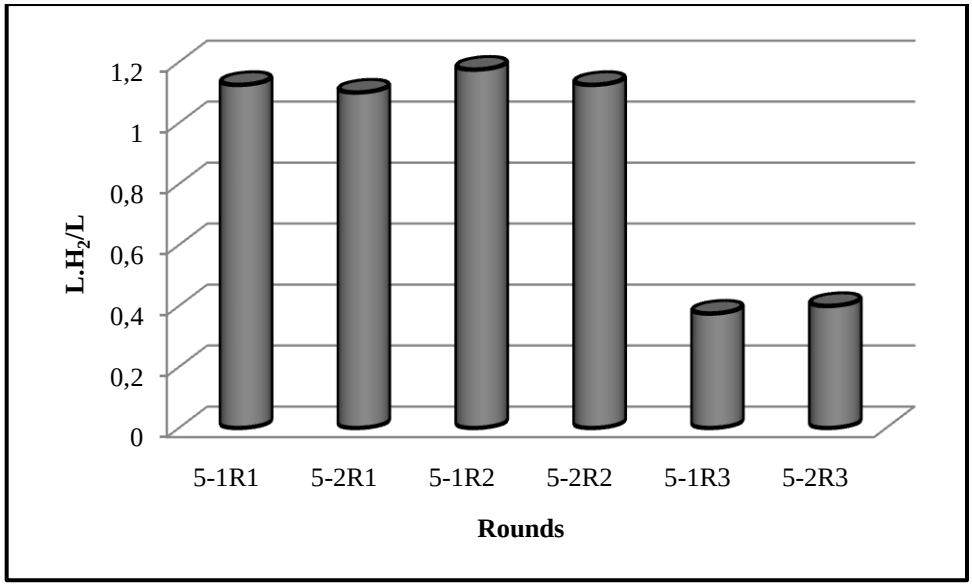


Figure 4.43 Total hydrogen produced by immobilized *R. capsulatus* DSM 1710 fed with 5 mM ammonium chloride as sole nitrogen source

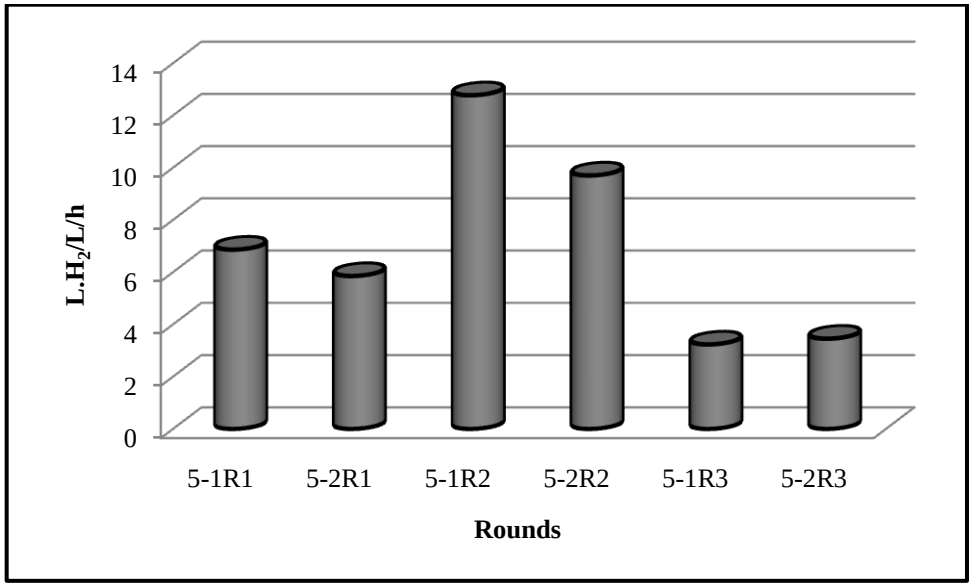


Figure 4.44 Rate of hydrogen production by immobilized *R. capsulatus* DSM 1710 fed with 5 mM ammonium chloride as sole nitrogen source

During the second round total hydrogen produced did not decreased but actually it slightly increased to 1.18 and 1.13 L.H_2/L. Such type of increase during second round was observed during hydrogen production by immobilized DSM 1710 strain when using glutamate as nitrogen source which could be explained as a result of bacterial activation during second round. Bacteria are subjected to relative high temperature, 45°C, during immobilization process and this may cause some repression to bacterial activity.

Rate of hydrogen production by immobilized DSM 1710 strain provided with 5 mM ammonium chloride is illustrated in Figure 4.44. Values of rate of hydrogen produced through the three rounds shown an increase when passing from R1 to R2 where rates of R2 increased to 12.77 and 9.7 ml.H_2/L/h while the rate of R1 were 6.86 and 5.85 ml.H_2/L/h. During R3, rate of hydrogen produced decreased drastically to 3.23 and 3.45 ml.H_2/L/h. The large decrease of rate of hydrogen produced in R3 was reflected by the large decrease in total hydrogen produced. Such finding indicated very low activity of nitrogenase enzyme.

Effect of increasing ammonium concentration on hydrogen production capacity of *R. capsulatus* DSM 1710 strain was also seen more clearly when ammonium concentration was increased to 7.5, (Figure 4.45 and 4.46). In Figure 4.45, it is observed that total hydrogen produced during first round was 0.98 and 1.00 L.H/L and the rate of hydrogen produced was 5.2 and 5.32 ml.H_2/L/h. At the end of second round R2 total hydrogen produced was decreased to 0.8 and 0.75 L.H/L and the rate of hydrogen produced was 8.7 and 11.1 ml.H_2/L/h. The third round R3 exhibited great decrease in total hydrogen produced to 0.28 and 0.38 L.H_2/L and also dramatic decrease in rate of hydrogen production to 2.37 and 3.23 ml.H_2/L/h

The lower hydrogen production capacity by immobilized DSM 1710 bacteria at the first round R1 compared to the second round R2 may be attributed to partial inhibition of bacterial activity by exposure to relatively high temperature during preparing agar gel for immobilizing bacteria.

The results also indicated that immobilized bacteria can work better in term of hydrogen production compared to suspended cultures which stop producing hydrogen completely when ammonium concentration was around 3 mM (data are not shown). The protective capacity of agar against ammonia is not permanent through all the experiment time as it was decreasing by passing from R1 to R3 in all concentration of ammonium applied to the immobilized bacteria. This phenomenon may be attributed to saturation of agar with ammonium when passing from one round to another.

The fact that ammonium has repression effect on nitrogenase synthesis more than its activity may give an explanation why hydrogen production inhibition is increased by passing from R1 to R3 and by increasing concentration of ammonium. Since bacteria is prepared for immobilization when they are at the late growth phase (2-3 days activation), they keep their nitrogenase fresh during first and second rounds and remain active in hydrogen production.

At the third round it is proposed that nitrogenase enzyme is exhausted partially and bacterial growth is very limited in the immobilizing medium so bacteria is in need of synthesizing more nitrogenases but such synthesis is inhibited by the presence of ammonium and so hydrogen production capacity is decreased. This conclusion is supported by the fact that hydrogen production capacity of the immobilized bacteria was decreasing by increasing ammonium concentration while lower concentrations of ammonium (2.5 and 5 mM) were less effective on hydrogen production capacity of the immobilized bacteria.

The protective capacity of agar toward immobilized bacteria was clear during this experiment in spite of using high concentrations of ammonium but hydrogen production capacity of immobilized bacteria fed with nutrient medium containing ammonium was less than hydrogen production capacity of immobilized bacteria fed with 4 mM glutamate as source of nitrogen.

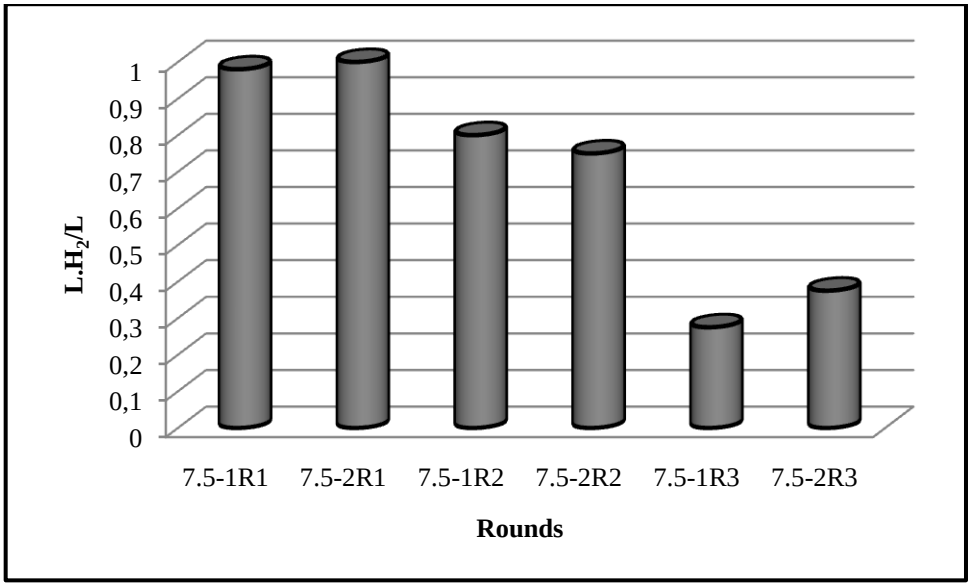


Figure 4.45 Total hydrogen produced by immobilized *R. capsulatus* DSM 1710 fed with 7.5 mM ammonium chloride as sole nitrogen source

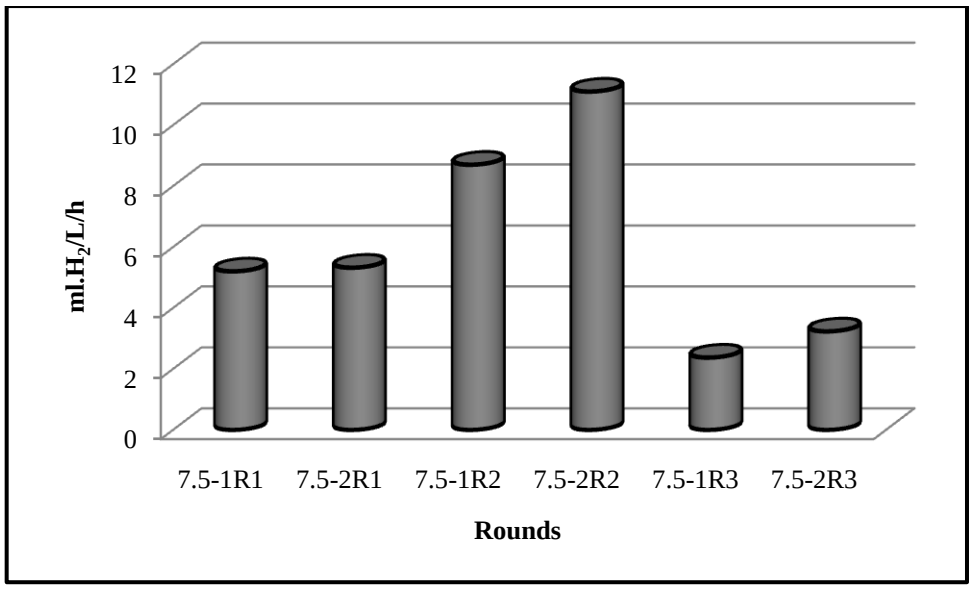


Figure 4.46 Rate of hydrogen production by immobilized *R. capsulatus* DSM 1710 fed with 7.5 mM ammonium chloride as sole nitrogen source

Hydrogen production is very sensitive to ammonium. Inhibition of hydrogen production by *Rb. capsulata* from lactate has been reported in the presence of even 0.1 mM of ammonium (Hilmer & Gest 1977). It was found that immobilization by agar protected bacteria from inhibitory effect of ammonium at 2.3 mM (Zhu *et al*., 1999) for one batch (120 h).

In the present work, protection was manifested at 2.5, 5 & 7.5 mM concentrations for two sequential batches (312-360 h). It was observed that increasing ammonium concentration caused increase pH to more than 8 when ammonium concentarion increased to 5 & 7.5 mM.

The results revealed priority of using glutamate as nitrogen source over using ammonium even if ammonium concentration was as low as 2.5 mM but the results indicating a potential use of immobilized bacteria for hydrogen production by feeding them continuously or by fed-batch method were during continuous feeding process the concentration of ammonium will stay low by the dilution effect.

The effect of ammonium on hydrogen production capacity by immobilized bacteria was also reflected by the results of HPLC analysis of organic acids remains in the spent medium. Acetic acid remains and presence of formic acid were examined at the end of each round through all the experiment period.

Since growth of immobilized bacteria inside agar gel was very limited, it is clear that most of acetic acid will be directed to hydrogen production pathways and the high acetic acid remains will be reflected by low hydrogen production capacity. HPLC analysis indicated such type of correlation. It was observed that acetic acid remains was low at the end of R1 and R2 than at the end of R3 where R1 and R2 were more active in hydrogen production and also acid remains was increasing by increasing ammonium concentration were hydrogen production capacity was decreasing by ammonium increase.

4.6.2 Effect of ammonium on hydrogen production by photosynthetic bacteria *Rhodobacter capsulatus* YO3 strain

The observations from Figure 4.47 which describe total hydrogen produced by immobilized YO3 strain indicated that 2.5-1R1 and 2.5-2R1 (both are first round of double experiment) produced 4.2 and 4.3 L.H_2/L. At the end of second round total hydrogen produced decreased to 2.5 and 2.56 L.H_2/L, then it continued decreasing to 0.55 and 0.58 L.H/L. Comparing those results with results of total hydrogen produced when glutamate was used as sole nitrogen source (5.14, 5.02 and 3.54 L.H_2/L during R1, R2 and R3 respectively), it is possible to conclude that using of ammonium as nitrogen source affected negatively on hydrogen production ability of the immobilized YO3 strain as was seen with DSM strain.

The results of rate of hydrogen production given by using 2.5 mM ammonium chloride indicating decrease in the rate of hydrogen production. Using 4 mM glutamate as nitrogen source during hydrogen production by immobilized YO3 strain resulted in 50, 45.7 and 37.5 ml.H_2/L/h for R1, R2 and R3 respectively. Using ammonium chloride produced rates of hydrogen production lower than those given by using 4 mM glutamate.

Application of 2.5 mM of ammonium chloride as nitrogen source caused decrease of hydrogen production capacity at such concentration. Keeping hydrogen production capacity may indicate that immobilization can provide some protection for bacteria to continue producing hydrogen in spite of presence of ammonium at such concentration.

The steady decrease in total hydrogen production from R1 to R3 could be attributed to the repression effect of ammonium on synthesis of nitrogenase enzyme itself. This effect was increased steadily indicating that agar is being more saturated with ammonium as exposure time is increased from R1 to R3 and by increasing ammonium concentration.

Effect of ammonium on total hydrogen produced also reflected on the rate of hydrogen production by the immobilized bacteria. Following the rate of hydrogen production which could be observed from Figure 4.48 indicated a decrease in rate of hydrogen production by passing from R1, R2 to R3. Such decrease in rate of hydrogen production was observed during hydrogen production when glutamate was used as nitrogen source but the decrease here was more drastic since ammonium is known to cause repression for synthesis of nitrogenase.

Total hydrogen produced by immobilized bacteria provided with nutrient medium containing 5 mM ammonium chloride is illustrated in Figure 4.49. At the end of first round R1 total hydrogen produced by using 5 mM ammonium chloride reached 2.75 and 3.05 $L.H_2/L$ and decreased to 1.63 and 1.8 $L.H_2/L$ at the end of the second round R2. During the third round R3 total hydrogen produced continued decreasing to 0.45 and 0.48 $L.H_2/L/h$. Such mode of decreasing hydrogen production capacity was observed during hydrogen production by immobilized YO3 strain when using glutamate as nitrogen source.

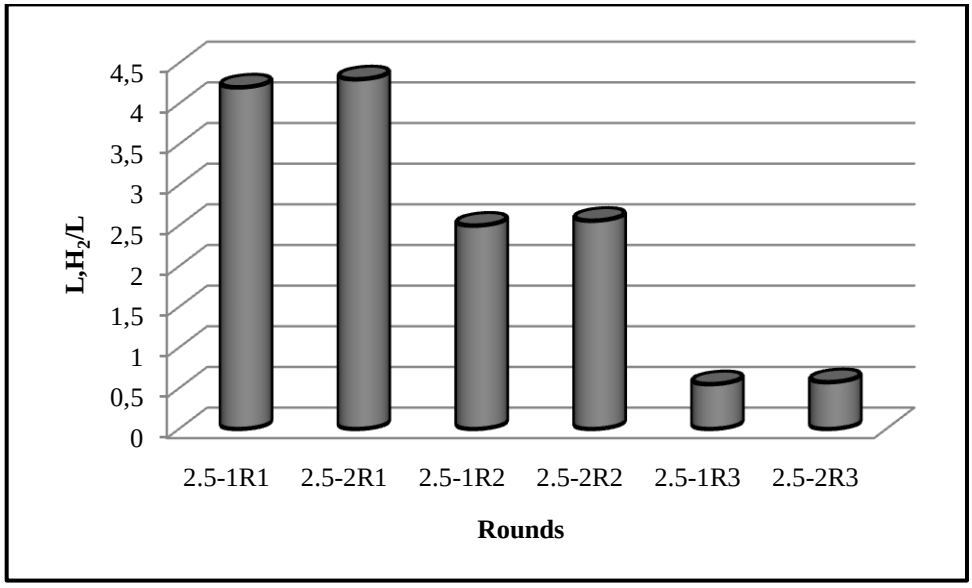


Figure 4.47 Total hydrogen produced by immobilized *R. capsulatus* YO3 fed with 2.5 mM ammonium chloride as sole nitrogen source

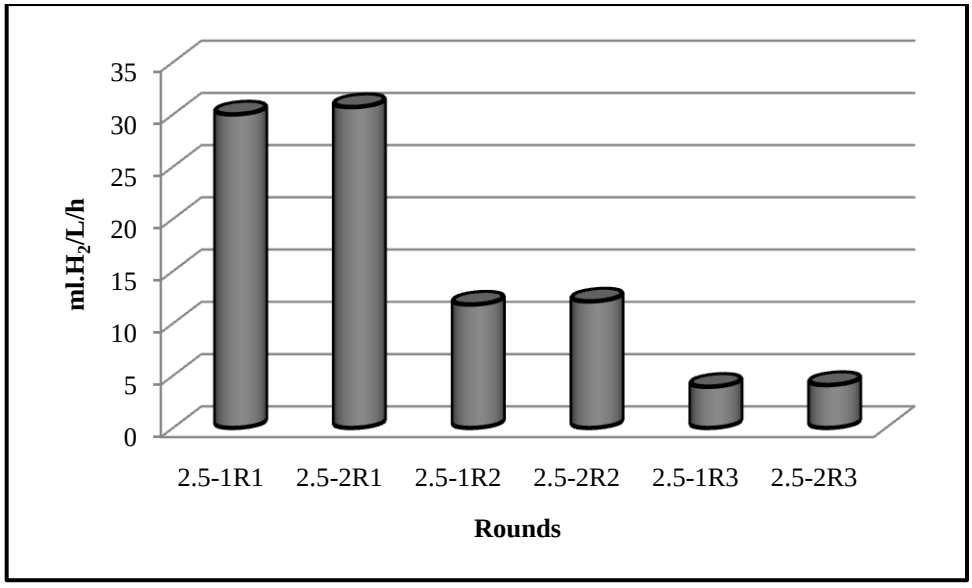


Figure 4.48 Rate of hydrogen production by immobilized *R. capsulatus* YO3 fed with 2.5 mM ammonium chloride as sole nitrogen source

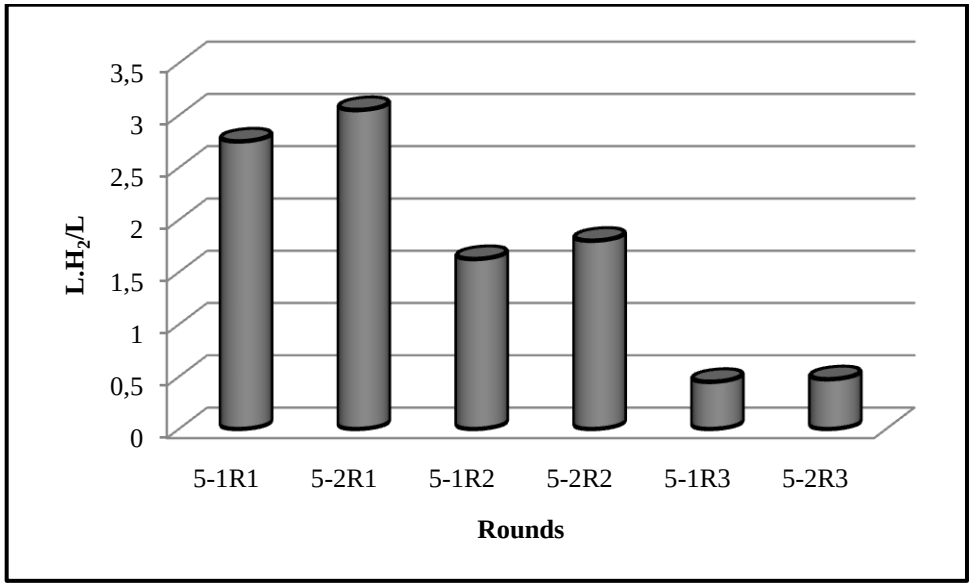


Figure 4.49 Total hydrogen produced by immobilized *R. capsulatus* YO3 fed with 5 mM ammonium chloride as sole nitrogen source

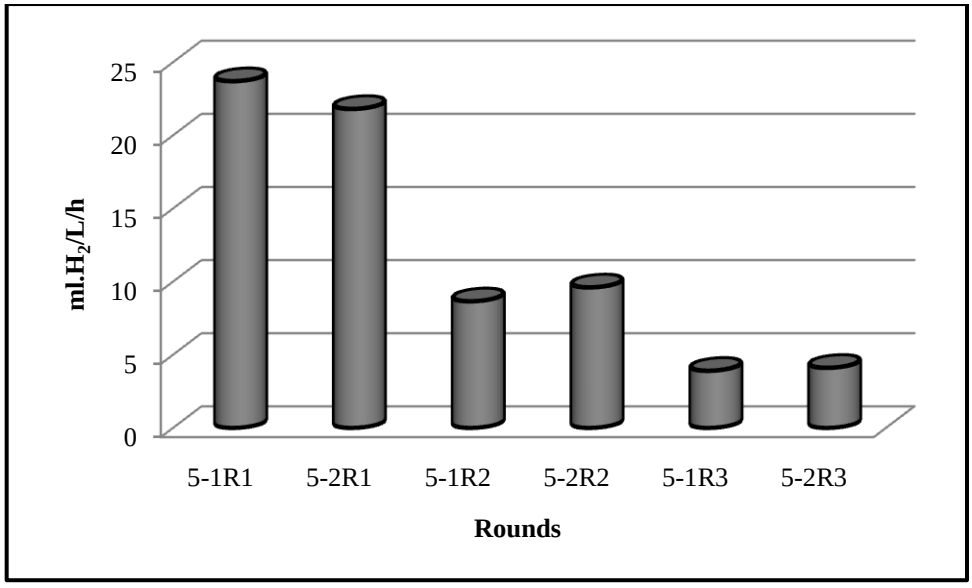


Figure 4.50 Rate of hydrogen production by immobilized *R. capsulatus* YO3 fed with 5 mM ammonium chloride as sole nitrogen source

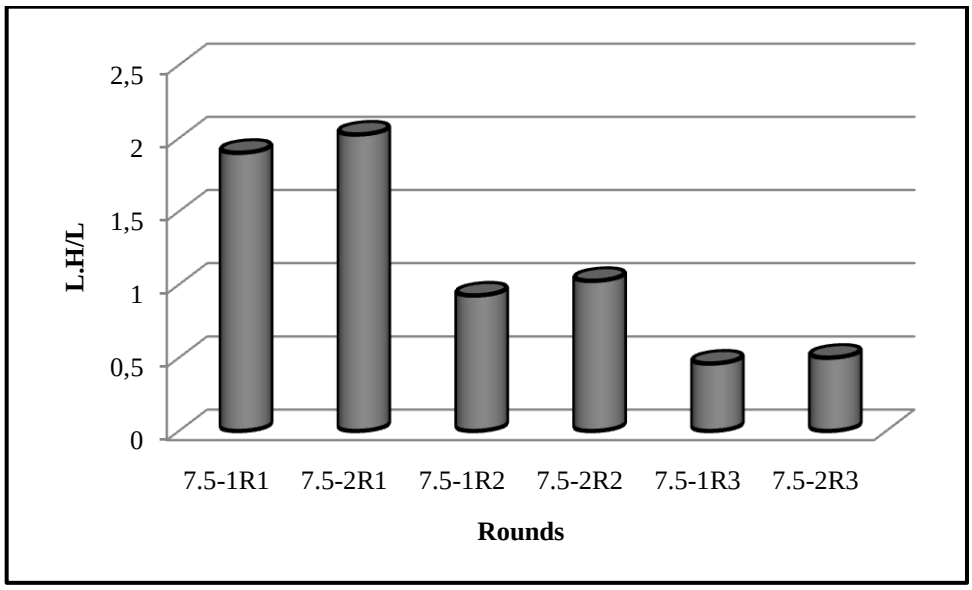


Figure 4.51 Total hydrogen produced by immobilized *R. capsulatus* YO3 fed with 7.5 mM ammonium chloride as sole nitrogen source

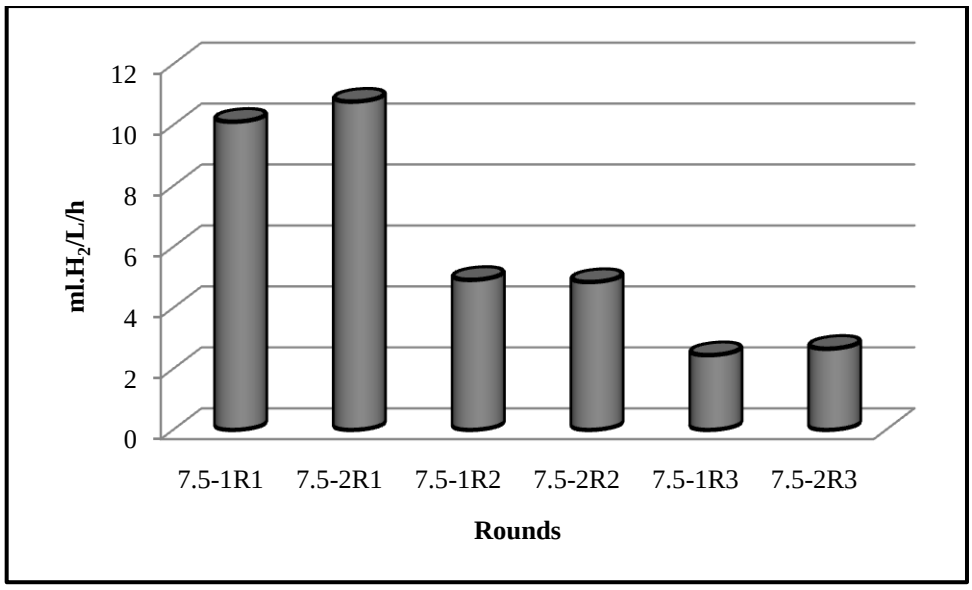


Figure 4.52 Rate of hydrogen production by immobilized *R. capsulatus* YO3 fed with 7.5 mM ammonium chloride as sole nitrogen source

Rate of hydrogen production by immobilized YO3 strain provided with 5 mM ammonium chloride is illustrated in Figure 4.50. Values of rate of hydrogen produced through the three rounds shown decrease when passing from R1, R2 to R3. At the end of R3, rate of hydrogen produced decreased to 3.9 and 4.1 ml.H_2/L/h. The large decrease of rate of hydrogen produced in R3 was reflected by the large decrease in total hydrogen produced, see Figure 4.49. Such finding indicated very low activity of nitrogenase enzyme

Effect of increasing ammonium concentration on hydrogen production capacity of *R. capsulatus* YO3 strain was also seen more clearly when ammonium concentration was increased to 7.5, see Figure 4.51 and Figure 4.52. It is observed that total hydrogen produced at the end of first round was 1.9 and 2.03 L.H_2/L and the rate of hydrogen produced was 10.11 and 10.77 ml.H_2/L/h. At the end of second round R2 total hydrogen produced was decreased to 0.93 and 1.03 L.H_2/L and the rate of hydrogen produced was 4.92 and 4.85 ml.H_2/L/h. The third round R3 exhibited great decrease in total hydrogen produced to 0.46 and 0.5 L.H/L and also dramatic decrease in rate of hydrogen production to 2.45 and 2.66 ml.H_2/L/h.

Decrease in hydrogen production capacity by immobilized YO3 bacteria at the first round R1 compared to the second round R2 was not observed as seen in case of DSM 1710

strain. This may be attributed to bacterial tolerance of harsh conditions during preparing bacteria with agar gel for immobilization.

The results also indicated that immobilized YO3 bacteria can work better in term of hydrogen production compared to DSM 1710. It is also concluded that immobilized bacteria work better than suspended cultures which stop producing hydrogen completely when ammonium concentration was around 3 mM (data are not shown).

The protective capacity of agar against ammonia is not permanent through all the experiment time as was clear from experiments with YO3 and DSM 1710 strains. It was decreasing by passing from R1 to R3 in all concentration of ammonium applied to the immobilized bacteria. This phenomenon may be attributed to saturation of agar with ammonium when passing from one round to another. The fact that ammonium has repression effect on nitrogenase synthesis more than its activity may gives an explanation why hydrogen production inhibition is increased by passing from R1 to R3 and by increasing concentration of ammonium.

Since bacteria is prepared for immobilization when they are at the late growth phase (2-3 days activation), they keep their nitrogenase fresh during first rounds and with less extent during second rounds and remain active in hydrogen production.

At the third round it is proposed that nitrogenase enzyme is exhausted partially and bacterial growth is very limited in the immobilizing medium so bacteria is in need to synthesize more nitrogenases but such synthesis is inhibited by the presence of ammonium and so hydrogen production capacity is decreased. This conclusion is supported by the fact that hydrogen production capacity of the immobilized bacteria was decreasing by increasing ammonium concentration while lower concentrations of ammonium (2.5 and 5 mM) were less effective on hydrogen production capacity of the immobilized bacteria.

Again as was seen with immobilized DSM 1701 strain, immobilizing YO3 in agar provided protection for bacteria against ammonium in nutrition medium. This was clear during this experiment in spite of using high concentrations of ammonium. It was observed that hydrogen production capacity of immobilized bacteria fed with nutrient medium containing ammonium was less than hydrogen production capacity of immobilized bacteria fed with 4 mM glutamate as source of nitrogen.

The results revealed priority of using glutamate as nitrogen source over using ammonium even if ammonium concentration was as low as 2.5 mM. According to the results from experiments with both used strains, it is possible to suggest a potential use of immobilized bacteria for hydrogen production by feeding them defined or undefined nutrient medium for

hydrogen production continuously or by fed-batch method. During continuous feeding process the concentration of ammonium will stay low by the dilution effect and it is expected that the immobilized bacteria will work for longer period than performed by sequential batch method applied in this work.

The effect of ammonium on hydrogen production capacity by immobilized bacteria was also reflected by the results of HPLC analysis of acetic remains in the spent medium. Acetic acid remains and presence of formic acid were examined at the end of each round through all the experiment period.

Since growth of immobilized bacteria inside agar gel was very limited, is clear that most of acetic acid will be directed to hydrogen production pathways and the high acetic acid remains will be reflected by low hydrogen production capacity. HPLC analysis indicated such type of correlation. It was observed that acetic acid remains was low at the end of R1 and R2 than at the end of R3 where R1 and R2 were more active in hydrogen production and also acid remains was increasing by increasing ammonium concentration were hydrogen production capacity was decreasing by ammonium increase.

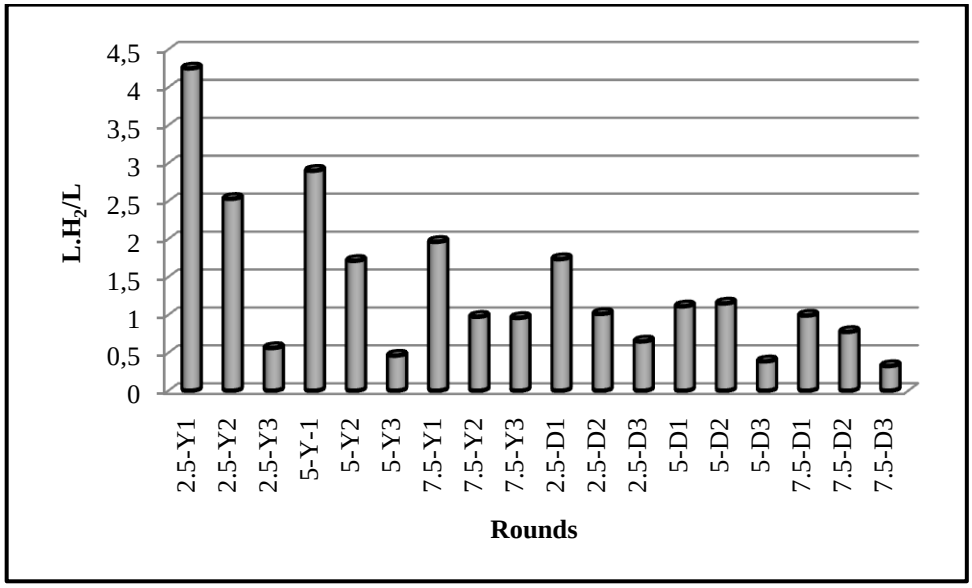


Figure 4.53 Comparison between averages of total hydrogen produced by immobilized *R. capsulatus* YO3 and DSM 1710 fed with different concentrations of ammonium chloride as sole nitrogen source

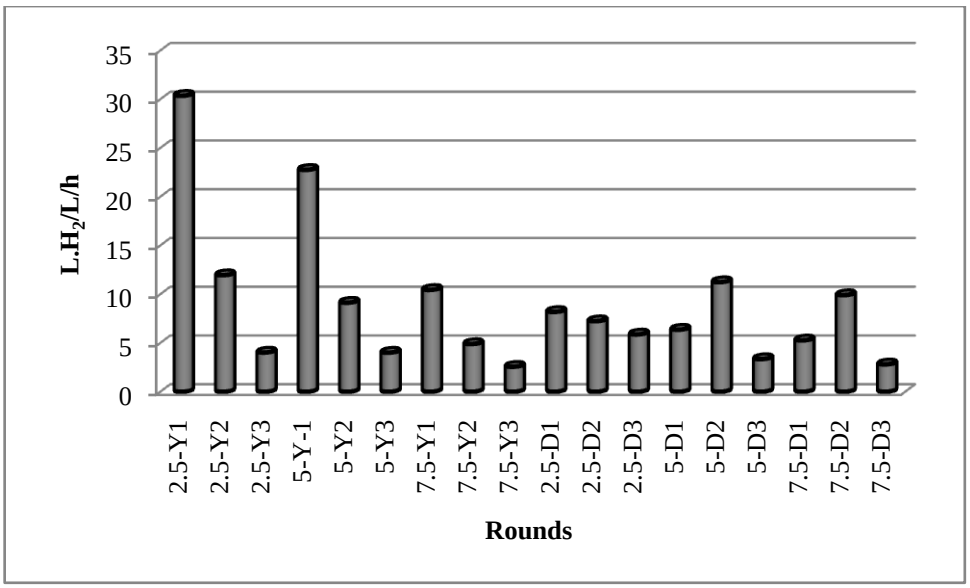


Figure 4.54 Comparison between averages of rates of hydrogen production by immobilized *R. capsulatus* YO3 and DSM 1710 fed with different concentrations of ammonium chloride as sole nitrogen source

Results from both strains of bacteria reveled priority of YO3 strain during first and second rounds, R1 and R2 in term of total hydrogen produced and rate of hydrogen production, see Figure 4.53 and Figure 4.54. At the third round it is clear that both strains suffered from dramatic repression of their hydrogen capacity and the rate and total hydrogen produced at the end of R3 were close to each other.

This result may introduce supporting for the idea concluding that bacteria at first round was keeping its synthesized nitrogenase which gained it during growth before immobilization procedure was start up. That ready synthesized enzyme worked actively for during first round R1 and to less extent through second round until its activity is partially exhausted. At this point bacteria could not regenerate nitrogenase enzyme by synthesizing it as a result of repression effect of ammonium found in the medium.

4.7 Effect of co-immobilizing *Rhodobacter capsulatus* and packed cells of *Halobacterium salinarium* S-9 on hydrogen production

In this part of work *R. capsulatus* bacteria were co-immobilized with packed cells of *H. salinarium* S-9. Packed cells of *Halobacterium salinarium* were prepared as describe in

materials and methods. Amount of bacteriorhodopsin in the packed cells was adjusted as 2.4 μmol.

4.7.1 Hydrogen production by co-immobilizing *R. capsulatus* DSM 1710 strain and halophilic bacteria *Halobacterium salinarium* S-9

In this part of work *Rhodobacter capsulatus* DSM 1710 strain was co-immobilized with packed cells of *Halobacterium salinarium* S-9. Figure 4.55 illustrates total hydrogen produced by co-immobilized system. In this experiment a control cell culture bottle with immobilized *R. capsulatus* DSM 1710 strain only and designed as D. Cell culture bottles containing co-immobilized bacteria were designed D-HS1 and D-HS2 since co-immobilization experiment was carried out in double. This experiment was performed in sequential batch mode as done during all experiments in this study. The experiment covered three rounds designed as R1, R2 and R3.

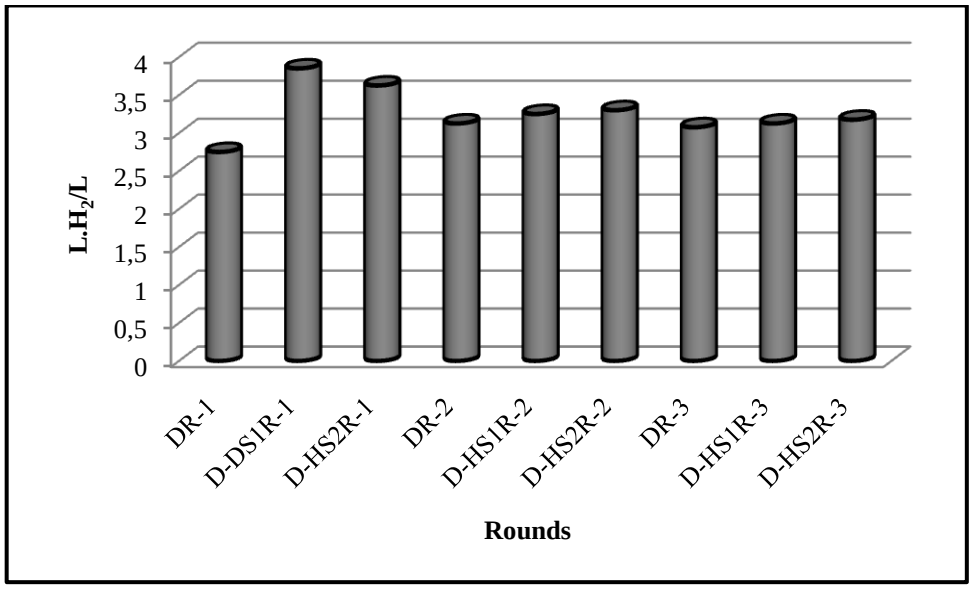


Figure 4.55 Total Hydrogen produced by *R. capsulatus* DSM 1710 strain co-immobilized with packed cells of *H. salinarium* S-9

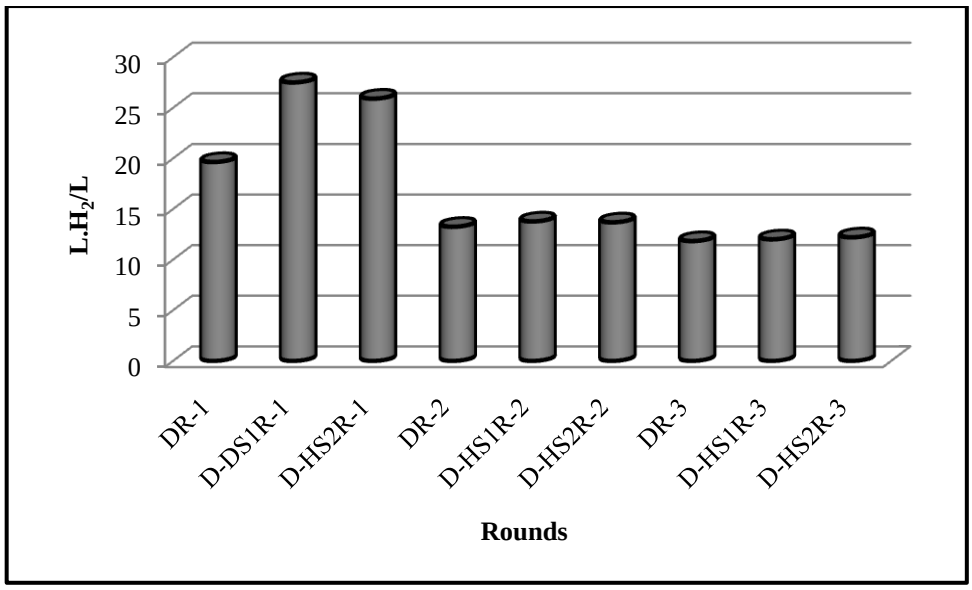


Figure 4.56 Rate of hydrogen production by *R. capsulatus* DSM 1710 strain co-immobilized with packed cells of *Halobacterium salinarium* S-9

Observations from Figure 4.55 shown increase of total hydrogen produced when *R. capsulatus* DSM 1710 strain co-immobilized packed cells of Halobacterium salinarium. At the end of the first round R1, total hydrogen produced from D-HS1 and D-HS2 were 3.85 and 3.63 L.H_2/L compared to single immobilized DSM 1710 bacteria which produced around 2.75 L.H_2/L. Improvement in total hydrogen produced at the end of the first round R1 was about 1.32 to 1.4 times as compared with the single immobilized DSM 1710 strain.

Figure 4.56 illustrates rate of hydrogen production by co-immobilized DSM 1710 with *H. salinarium* compared to single immobilized DSM 1710 bacteria. Observation of first round R1indicated improvement in rate of hydrogen production by co-immobilized bacteria comparing to single immobilized DSM 1710.

At the end of R1 rate of hydrogen produced by D-HS1 was 27.5 ml.H_2/L/h while rate of hydrogen produced by D-HS2 was 25.9 ml.H_2/L/h and rate of hydrogen produced by the single immobilized bacteria was 19.64 ml.H_2/L/h. Improvement in rate of hydrogen production ranged from 1.32 to 1.4 times as compared to single immobilized DSM 1710 bacteria.

Regarding second round R2 and third round R3, the difference in hydrogen production capacity between co-immobilized and single immobilized culture in term of total hydrogen produced and rate of hydrogen production was insignificant, see Figure 4.56.

Decreasing improvement of hydrogen production capacity during second and third rounds R2 and R3 of co-immobilized bacteria may be attributed to the leakage of packed cells from agar to the liquid medium during running of the first round D-HSR1 and lose of those packed cells during subsequent feeding with fresh medium.

As described in materials and methods, feeding with fresh medium was carried out by removing the spent liquid medium and then washing the immobilizing system with basal medium. Leakage of packed cells of *H. salinarium* was indicated by changing the color of the nutrient medium in the cell into red color. Measurement of the optical density of the spent medium by using 570 nanometer light wave (wavelength used for quantifying bacteriorodopsin in the medium) at the end of R1 indicated the presence of bacteriorhodopsin in the spent medium.

4.7.2 Hydrogen production by co-immobilizing photosynthetic bacteria *R. capsulatus* YO3 strain and halophilic bacteria *Halobacterium salinarium* S-9

In this part of work *Rhodobacter capsulatus* YO3 strain was co-immobilized with packed cells of *Halobacterium salinarium* S-9. Figure 4.57 illustrates total hydrogen produced by the co-immobilized system. In this experiment a control cell culture bottle with immobilized *R. capsulatus* YO3 strain only and designed as Y. Cell culture bottles containing co-immobilized bacteria were designed YHS1 and YHS2 since co-immobilization experiment was carried out in double. This experiment was performed in sequential batch mode as done during all experiments in this study. The experiment covered three rounds designed as R1, R2 and R3.

Observations from Figure 4.55 shown increase of total hydrogen produced when R. capsulatus YO3 strain co-immobilized packed cells of Halobacterium salinarium. At the end of the first round R1, total hydrogen produced from Y-HS1 and Y-HS2 were 6.3 and 6.38 L.H_2/L compared to single immobilized YO3 bacteria which produced around 4.53 L.H/L. At the end of the second round R2 Total hydrogen produced by co-immobilized YO3 bacteria was 6.2 and 5.52 L.H/L while the total hydrogen produced by single immobilized YO3 bacteria was 4 L.H_2/L.

Improvement in total hydrogen produced at the end of the first round R1 was about 1.39 to 1.41 times as compared with the single immobilized YO3 strain. At the end of the second round R2 improvement of total hydrogen produced was 1.38 to 1.55 times as compared to single immobilized YO3 strain.

Third round during this experiment co-immobilization shows some improvement in hydrogen production capacity as total hydrogen production was 4.2 and 4.13 L.H_2/L compared

to single immobilized YO3 bacteria which was 3.63 L.H_2/L. Improvement at the end of the third round in term of total hydrogen production was around 1.138 to 1.159 times compared to single immobilized YO3 bacteria.

Figure 4.58 illustrates rate of hydrogen production by co-immobilized YO3 with *H. salinarium* compared to single immobilized YO3 bacteria. Observation of first round R1indicated improvement in rate of hydrogen production by co-immobilized bacteria comparing to single immobilized YO3.

At the end of R1 rate of hydrogen produced by Y-HS1 was 45 ml.H_2/L/h while rate of hydrogen produced by Y-HS2 was 45.53 ml.H_2/L/h and rate of hydrogen produced by the single immobilized bacteria was 32.32 ml.H_2/L/h. Improvement in rate of hydrogen production at the end of first round R1 ranged from 1.39 to 1.41 times as compared to single immobilized YO3 bacteria.

Regarding second round R2 and third round R3, the difference in hydrogen production capacity between co-immobilized and single immobilized culture in term of rate of hydrogen production was still considerable, see Figure 4.58. At the end of R2 rate of hydrogen production of co-immobilized bacteria summed as 37.8 and 33.66 ml.H_2/L/h while rate of hydrogen produced by single immobilized YO3 strain was 24.4 ml.H_2/L/h.

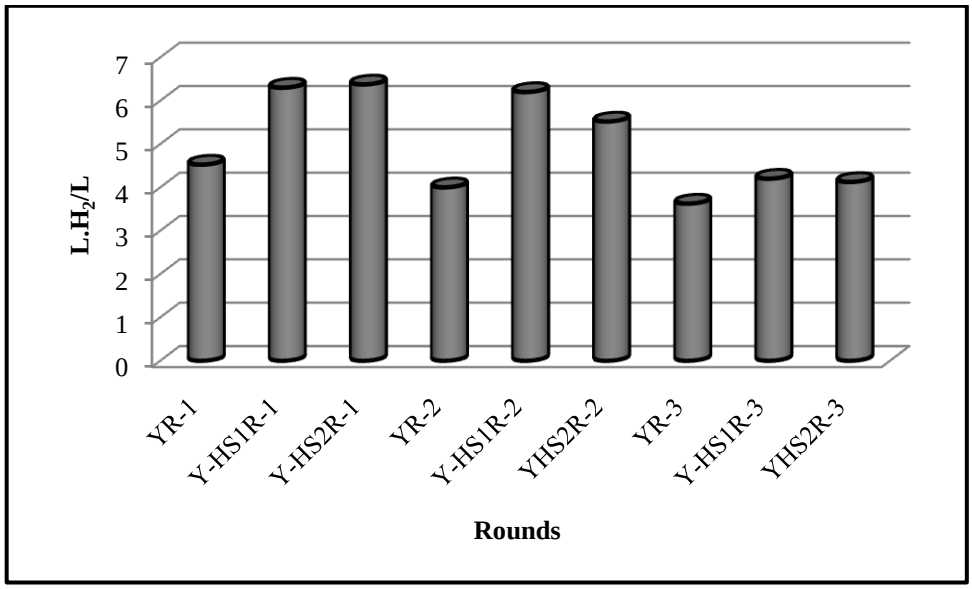


Figure 4.57 Total Hydrogen produced by *R. capsulatus* YO3 strain co-immobilized with packed cells of *Halobacterium salinarium* S-9

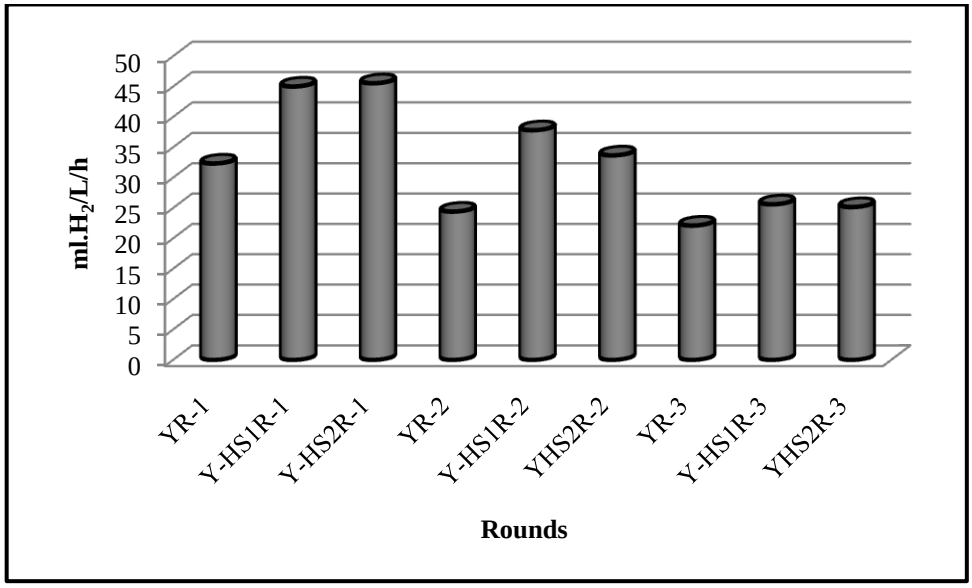


Figure 4.58 Rate of hydrogen production by *R. capsulatus* YO3 strain co-immobilized with packed cells of *Halobacterium salinarium* S-9

Such result pointed improvement in total hydrogen produced by rate of hydrogen production by about 1.55 to 1.8 compared to the second round R2 of single immobilized YO3 strain.

Third round R3 also exhibited improvement in rate of hydrogen production by co-immobilized bacteria. It was found that rate of hydrogen production of co-immobilized YO3 bacteria at the end of R3 was 25.6 and 25.15 ml.H_2/L/h while it was 22.1 ml.H_2/L/h for single immobilized YO3 strain bacteria. Improvement in rate of hydrogen production during third round R3 was around 1.14 to 1.16.

The observed decrease in hydrogen production capacity by immobilized bacteria whether they are co-immobilized or singly immobilized when using YO3 or DSM 1710 strains was observed during all experiments and this was attributed to the aging factor of bacteria and also to nitrogenase activity which became less active as immobilized bacteria have restricted ability to grow and regenerate themselves inside agar.

Other factor which is believed to contribute in reducing hydrogen production capacity of co-immobilized bacteria is the leakage of packed cells from agar to the liquid medium during

running of the experiment and losing of those packed cells during subsequent replacing of spent medium with fresh one.

As described in materials and methods, feeding with fresh medium was carried out by removing the spent liquid medium and then washing the immobilizing system with basal medium. Leakage of packed cells of *H. salinarium* was indicated by changing the color of the nutrient medium in the cell into red color. Measurement of the optical density of the spent medium by using 570 nanometer light wave (wavelength used for quantifying bacteriorodopsin in the medium) at the end of R1 indicated the presence of bacteriorhodopsin in the spent medium.

Observation of results shown in Figures 4.55 to 4.58 concluded that co-immobilization of YO3 strain with *H. salinarium* was more successful than co-immobilization of DSM 1710 strain with *H. salinarium*. The co-immobilizing system containing YO3 strain kept its ability in improving hydrogen production capacity comparing to the single immobilized YO3 strain for three rounds of the process. Co-immobilizing system containing DSM 1710 strain lost its improving ability during second and third round of the process comparing to the single immobilized DSM 1710 strain.

4.8 Hydrogen production by *Rhodobacter capsulatus* immobilized in Panel Photobioreactor

Scaling up hydrogen production by immobilized *Rhodobacter capsulatus* DSM 1710 and YO3 strains was conducted in one liter volume panel reactors described in materials and methods. Experiment with panel reactors were performed indoor under continuous illumination. Temperature was controlled between 30-32 °C through using fans.

4.8.1 Hydrogen production by photosynthetic bacteria immobilized by agar fixed within wells on the inner surface of the panel reactor

After operation of reactor A for 576 hours (24 days), it produced 6.57 liter of hydrogen while reactor B produced about 7.25 liter of hydrogen gas. Rate of hydrogen produced by Reactor A was 11.4 ml.H_2/L/h while rate of hydrogen produced by reactor B was 12.6 ml.H_2/L/h.

Operation of these two reactors was performed to examine validity of wells as place for positioning of agar with immobilizing bacteria. The two reactors were stopped after agar cracking was more dominant and many parts of agar were lost to the liquid medium. The

continuous fed-batch mode feeding followed during this experiment in contrast to the sequential batch mode followed during this work was applied as a result of cracking of agar which started two days after starting the experiment.

Cracking of agar during this experiment as a result of gas pressure required a new design confirmed for long stability and allowing easy escape of gas to the surrounding liquid medium. Presence of agar inside the wells which were made on the inner surface of the reactor was in need for supporting external barrier to prevent falling of agar sheath outside the wells. Such type of barrier was made from plexiglass.

In spite of fixing agar inside the wells and presence of the barrier frame in front of agar sheaths to prevent them from falling into the reactor space, gas pressure caused detaching of the agar sheaths from their position and cracking them as a result of barrier resistance.

4.8.2 Hydrogen production by photosynthetic bacteria immobilized in agar supported by framed-network cloth

Improper performance of immobilizing bacteria in agar sheaths placed in wells fabricated inside the body of the inner surface of the reactor lead to tailor a new design.

The new design has to fill the requirements relating to long period stability, non-cracking and allowing easy escape of gas to the surrounding medium. Such design as described in materials and methods was in the form of rectangle double glass frame sandwiching in between them a network cloth made from Tulle.

This type of cloth is a lightweight, tinny netting fabric made from rayon, nylon or other fibers. The color of Tulle used in this design was white to prevent any interrupting with light. This fabric committed for transparency with tiny textile fibers and continuous opening, see Figure 4.59. The presence of tinny fibers acted to enforce agar gel sheath while the glass frame performed as supporting scaffold for agar sheath enforced with the network cloth.

To keep the newly framed and network enforced agar sheath stable inside the reactor space and at the same time keeping it at suitable distance from the inner surface of reactor two glass clamps were fit at the bottom and one glass clamp on the top of the frame. All described system including frame and its accessories were hand-made.

Figure 4.59 A photograph of a piece of Tulle fabric used for supporting and reinforcement of agar for bacterial immobilization in panel reactor

Hydrogen production by *R. capsulatus* DSM 1710 immobilized in agar supported by framed-network cloth

Experiment operation by immobilized DSM 1710 strain in panel reactor was operated in two reactors as mentioned in materials and methods, reactor 1(D1) and reactor 2 (D2). Feeding reactors with new fresh medium followed sequential batch mode as described in materials and methods. Reactor 1(D1) was operated for about 1608 hours (67 days) and still working for further period of time. The period covered four rounds (R1-R4) depending sequential batch mode feeding. Reactor 2 (D2) was operated for 1968 hours (82 days) covering five rounds designated R1-R5.

Figure 4.60 illustrates total hydrogen produced by each of the two reactors D1 and D2. Reactor D1 operated for four rounds produced 11.16 L.H_2 as total hydrogen produced during the four rounds with average total hydrogen produced as 2.79 L.H_2/round. The second reactor D2

produced about 12.2 L.H_2 as total hydrogen produced through period time of reactor operation. The average total hydrogen produced per round achieved from reactor D2 was 2.44 L.H_2/round.

It was observed that illumination of the reactor from one side of the reactor as has been observed during second round R-2 in both reactors caused decrease in total hydrogen produced from 3.9 L.H at the end of R-1 in reactor D1 to 2.15 L.H_2 and also decreased rate of hydrogen production from 16.25 to 5.97 ml.H_2/L/h, see Figure 4.61. In reactor D2 illumination of the reactor from one side caused also a decrease in total hydrogen produced from 2.6 L.H_2 at the end of R-1 to 1.65 L.H_2 and caused decrease in rate of hydrogen production from 8.3 ml.H_2/L/h in R-1 to 3.74 ml.H_2/L/h.

Turning on illumination from both sides recovered hydrogen production capacity in both reactors in term of total hydrogen produced and rate of hydrogen production, see Figures 4.60 and 4.61. Such result concluded the importance of illumination from both sides of the panel reactors during hydrogen production by immobilized DSM 1710.

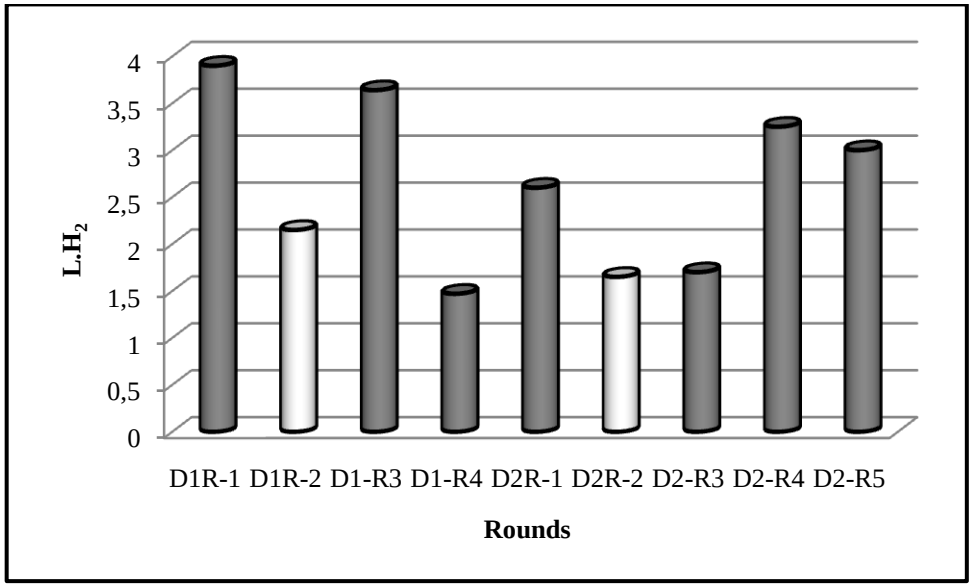


Figure 4.60 Total hydrogen produced by immobilized *R. capsulatus* DSM 1710 strain in panel reactors D1 and D2

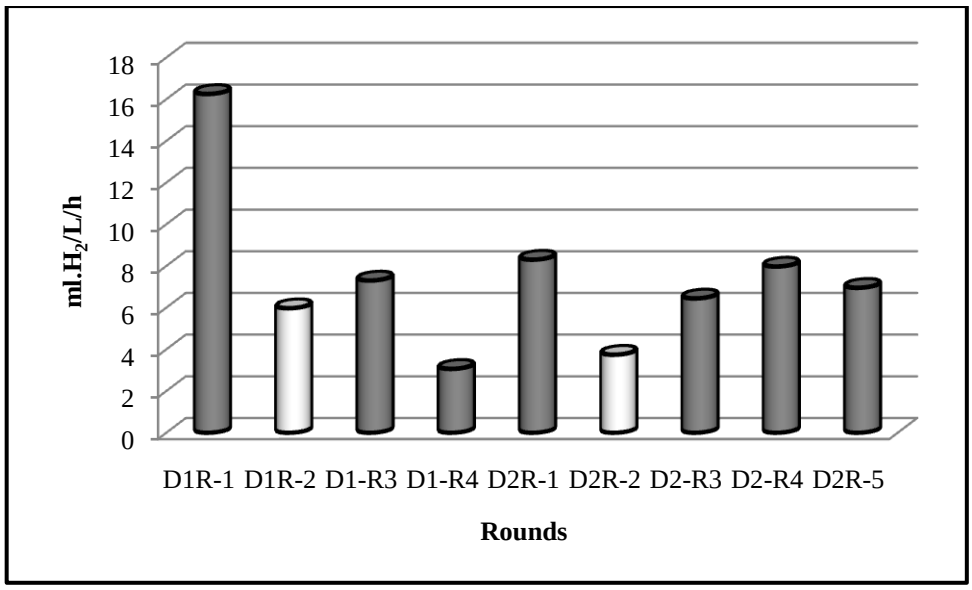


Figure 4.61 Rate of hydrogen production by immobilized *R. capsulatus* DSM 1710 strain in panel reactors D1 and D2

As mentioned in materials and methods and in results, reactor D2 was introduced into 48 hours activation period for immobilized bacteria. Activation was carried out by feeding of the immobilized bacteria inside the reactor with growth medium 20/10 containing 20 mM acetate and 10 mM glutamate. This activation was carried out at the end of third round R-3. Activation was proposed to enhance hydrogen production capacity of the immobilized bacteria which was lower than the opposite reactor D1.

It was proposed that feeding bacteria with nutrient medium enriched with glutamate will achieve such goal. Results from fourth and fifth rounds R-4 and R-5 supported this assumption and hydrogen production capacity has improved even better than first round R-1.

Activation with nutrient medium enriched with glutamate was improved hydrogen production capacity in reactor D2 better than reactor D1 which was started more active than reactor D1 itself and this finding was clear if we compared fourth rounds in the two reactor where D2-R4 produced 3.250 L.H with while D1-R4 produced 1.47 L.H. Such improvement in hydrogen production capacity after activation with growth medium was reflected on rate of hydrogen production which was increased from 6.44 ml.H_2/L/h in D2-R3 to 7.97 ml.H_2/L/h in D2-R4, see Figure 4.61.

Hydrogen production by *R. capsulatus* YO3 immobilized in agar supported by framed-network cloth

Experiment operation by immobilized DSM 1710 strain in panel reactor was operated in two reactors as mentioned in materials and methods, reactor 1(Y1) and reactor 2 (Y2). Feeding reactors with new fresh medium followed sequential batch mode as described in materials and methods. Reactor 1(Y1) was operated for about 1644 hours (69 days). The period covered seven rounds (R1-R7) depending sequential batch mode feeding. Reactor 2 (Y2) was operated for 1720 hours (72 days) covering seven rounds designated R1-R7.

Figure 4.62 illustrates total hydrogen produced by each of the two reactors y1 and Y2. Calculations of reactor Y1 for six rounds produced 24.95 L.H_2 as total hydrogen produced with average total hydrogen produced as 4.158 L.H/round while the seventh round produced only 1.22 L.H_2. The second reactor Y2 produced about 33.1 L.H_2 as total hydrogen produced through period time of reactor operation. The average total hydrogen produced per round achieved from reactor Y2 was 4.73 L.H_2/round.

It was observed that illumination of the reactor from one side of the reactor as has been observed during second round R-2 in both reactors caused decrease in total hydrogen produced from 4.4 L.H_2 at the end of R-1 in reactor Y1 to 3.85 L.H_2 and also decreased rate of hydrogen production from 26.83 to 10.7 ml.H_2/L/h, see Figure 4.63. It has been observed that illumination of reactor Y2from only one side caused decrease in total hydrogen produced from 5 L.H_2 at the end of R-1 to 4 L.H_2 at the end of R-2 and also caused decrease in rate of hydrogen production from 20.83 to 12.52 ml.H_2/L/h.

Turning on illumination from both sides recovered hydrogen production capacity in reactor Y2 in term of total hydrogen produced and rate of hydrogen production but total hydrogen produced in Y1 at the end of R-3 was the same as at the end of R-2 in spite that rate of hydrogen production was improved, see Figure 4.60 and Figure 4.61. Such result concluded the importance of illumination from both sides of the panel reactors during hydrogen production by immobilized YO3.

As mentioned in materials and methods, reactor Y1 was introduced into 48 hours activation period for immobilized bacteria. Activation was proposed to encourage hydrogen production capacity of immobilized bacteria in reactor Y1 which was lower than the opposite reactor Y2. Activation was carried out by feeding of the immobilized bacteria inside the reactor

with growth medium 20/10 containing 20 mM acetate and 10 mM glutamate. This activation was carried out at the end of third round R-3.

It was proposed that feeding bacteria with nutrient medium enriched with glutamate will achieve such goal. Results from fourth, fifth and sixth rounds R-4, R-5 and R-6 supported this assumption and hydrogen production capacity has improved better than third round R-3. Activation with nutrient medium enriched with glutamate was improved hydrogen production capacity in reactor Y1 but it stayed lower than found in reactor Y2 which was started more active than reactor Y1 itself and this finding is clear if we compared total hydrogen produced and rate of hydrogen production in two opposite reactors..

Such improvement in hydrogen production capacity after activation with growth medium was reflected on rate of hydrogen production which was increased from 16 ml.H_2/L/h in R3 to 19.2 ml.H_2/L/h in R-4, see Figure 4.62.

Hydrogen production capacity of immobilized YO3 bacteria in reactor Y2 started to decrease accompanied with accumulation of black precipitations by the end of fourth round R-4 and such black precipitations were increasing steadily through fifth and sixth rounds. The appearance of such precipitations was accompanied by very bad rotten-egg smell characterizing hydrogen sulfide formation.

During seventh round R-7 the precipitations were accumulated in a heavy manner until the black color stained all the gel area were bacteria are immobilized and also attached to the inner surfaces of the reactor. At the end of seventh round R-7 total hydrogen produced was 1.22 L.H_2 and rate of hydrogen production was only 5.1 ml.H_2/L/h.

These results mean that immobilized bacteria lost more than 73% of their hydrogen production capacity. Such results obtained from Y1 reactor may not encourage using activation of YO3 strain in immobilizing system by growth medium. As seen in this work hydrogen production capacity was encouraged for more three rounds and at the same time there were signs indicating that bacteria started to change its metabolic behavior until hydrogen production capacity was nearly lost. Such type of metabolic behavior was not seen after activation of immobilized DSM 1710 strain in reactor D2.

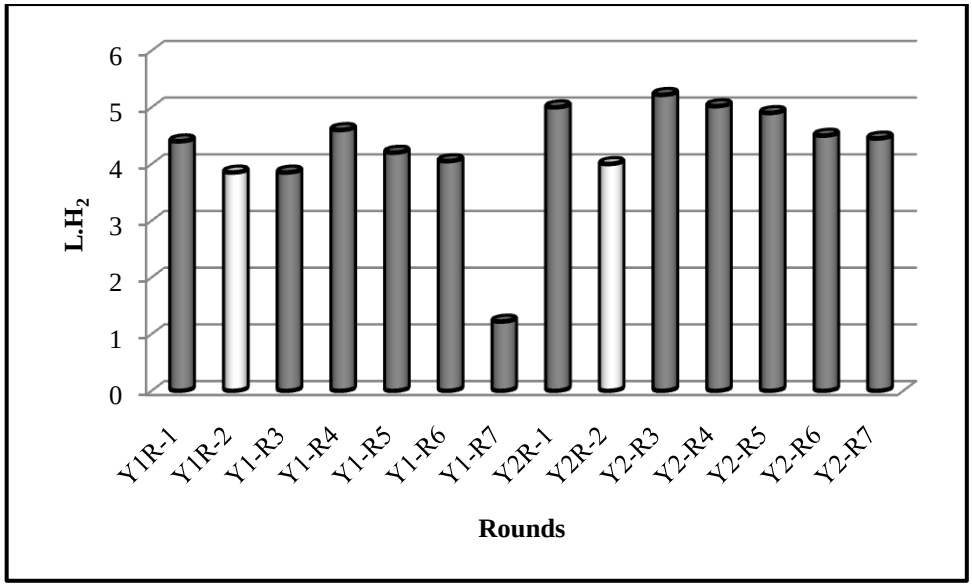


Figure 4.62 Total hydrogen produced by immobilized *R. capsulatus* YO3 strain in panel reactors Y1 and Y2

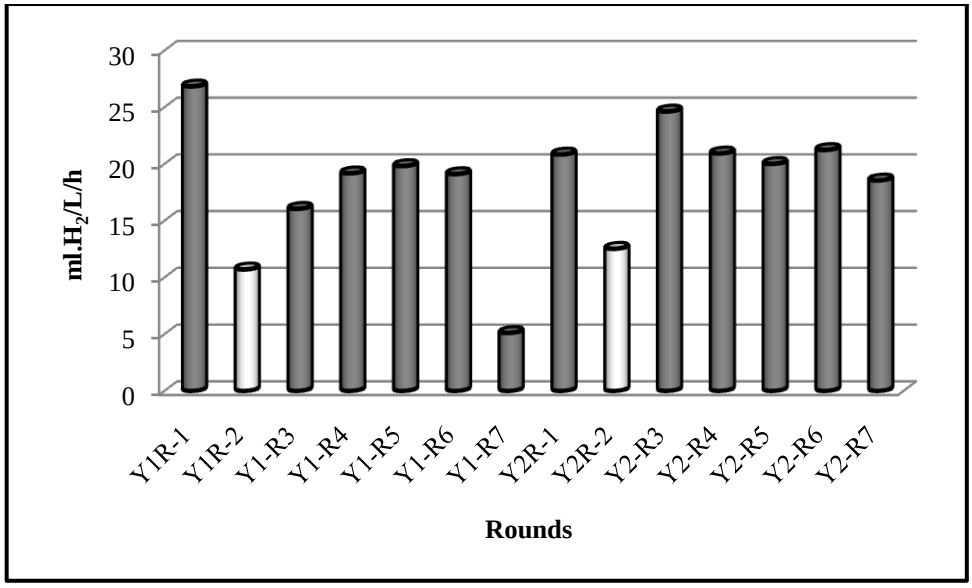


Figure 4.63 Rate of hydrogen production by immobilized *R. capsulatus* YO3 strain in panel reactors Y1 and Y2

The following tables summarize the results of total hydrogen produced, rate of hydrogen production and yields during hydrogen production by immobilized bacteria through the experiments under the different parameters applied during the work.

Table 4.2 Effect of different agar concentrations & glutamate concentrations on total and rate of hydrogen produced at. 2.5 mg DCW/ml.agar while feeding bacteria with 40 mM acetate

Agar conc. %	*R. capsulatus* DSM 1710				*R. capsulatus* YO3			
	40/2 (R1-R4)		40/4 (R5-R7)		40/2 (R1-R4)		40/4 (R5)	
	Total H_2 $L.H_2/L$	Rate of H_2 $ml.H_2/L/h$	Total H_2 $L.H_2/L$	Rate of H_2 $ml.H_2/L/h$	Total H_2 $L.H_2/L$	Rate of H_2 $ml.H_2/L/h$	Total H_2 $L.H_2/L$	Rate of H_2 $ml.H_2/L/h$
3	1.7	7.8	2.7	14.9	1.8	14.6	3.8	23.7
4	1.7	10	2.8	17	2	15.4	3.6	22.4
5	1.5	7.4	2.5	16.3	1.7	13.	2.6	18.4
6	1.7	8.4	2	12.7	1.7	12.6	3	18

Table 4.3 Substrate yield (mmol H_2/mol acetate) during hydrogen production by bacteria immobilized in different agar concentration and fed with 40/2-4 (acetate/glutamate) Ratios are based on theoritical Yeild (4 mmol Hydrogen/mmol acetate)

Agar con. %	*R. capsulatus* DSM 1710		*R. capsulatus* YO3	
	40/2 (R1-R4)	40/4 (R5-R7)	40/2 (R1-R4)	40/4 (R5)
3	1.6	2.7	1.8	3.8
4	1.7	2.8	2	3.6
5	1.5	2.6	1.8	2.6
6	1.8	2	1.7	3

Table 4.4 Effect of increasing acetate concentration & cell concentration on rate and total hydrogen produced by immobilized bacteria

Acetate concentration	*R. capsulatus* DSM 1710				*R. capsulatus* YO3			
	2.5 mg DCW/ml agar		5 mg DCW/ml agar		2.5 mg DCW/ml agar		5 mg DCW/ml agar	
	Total H_2 $L.H_2/L$	Rate of H_2 $ml.H_2/L/h$	Total H_2 $L.H_2/L$	Rate of H_2 $ml.H_2/L/h$	Total H_2 $L.H_2/L$	Rate of H_2 $ml.H_2/L/h$	Total H_2 $L.H_2/L$	Rate of H_2 $ml.H_2/L/h$
60	4.2	17	2.7	15.5	4.3	19.4	4.6	35.3
80	3.2	13	2.2	10	4.1	18.6	4.7	28.8
100	3	11	1.5	8.3	3.9	14.6	5.7	25

Table 4.5 Effect of increasing acetate concentration & cell concentration on substrate yield during hydrogen production by immobilized bacteria
Ratios are based on theoritical Yeild (4 mmol Hydrogen/mmol acetate)

Cell concentration mg DCW/ml	60 mM			80 mM			100 mM		
	R1	R2	R3	R1	R2	R3	R1	R2	R3
2.5 mg DSM 1710	2.3	3.1	3	2	1.4	1.5	1.8	1.12	1
5 mg DSM 1710	2.1	1.6	1.7	1.6	0.8	0.9	1	0.4	0.5
2.5 mg YO3	2.9	3.6	2.2	2.4	2.2	1.3	2.2	1.2	1.3
5 mg YO3	3.4	3.4	2.4	2.9	3	1.9	2.9	2.7	1.2

Table 4.6 Comparing average total hydrogen production and average rate of hydrogen production between immobilized *R. capsulatus* DSM 1710 when fed with medium containing sodium dithionite and after fed with medium without sodium dithionite

Round	Total Hydrogen produced ($L.H_2/L$)				Rate of Hydrogen Production (ml H_2/L/h)			
	0.5 mM SDT	1.0 mM SDT	1.5 mM SDT	No SDT used	0.5 mM SDT	1.0 mM SDT	1.5 mM SDT	No SDT used
First	0.6	0.2	0.3	3.12	3.4	1.5	2.2	19
Second	0.8	0.723	1.00	2.5	4.5	4	5.8	16.3
*Third	2	1.4	2.6	2.5	8	5	8.5	11

(*) Immobilized bacteria were fed with medium free of sodium dithionite

Table 4.7 Comparing average total hydrogen produced and average rate of hydrogen production between immobilized *R. capsulatus* DSM 1710 & YO3 when fed with medium containing different conc. Of ammonium and immobilized bacteria fed with medium containing 4 mM glutamate

Rhodobacter capsulatus DSM 1710								
Round	Total Hydrogen produced $L.H_2/L$				Rate of Hydrogen Production ml $H_2/L/h$			
	2.5 mM	5.0 mM	7.5 mM	4 mM gl.	2.5 mM	5.0 mM	7.5 mM	4 mM gl.
First	1.7	1.1	1	3.1	8.2	6.4	5.3	19
Second	1	1.2	0.8	2.5	7.2	11.2	9.9	16.3
Third	0.7	0.4	0.3	2.5	5.9	3.3	2.8	11.2
Rhodobacter capsulatus YO3								
Round	Total Hydrogen produced $L.H_2/L$				Rate of Hydrogen Production ml $H_2/L/h$			
	2.5 mM	5.0 mM	7.5 mM	4 mM gl.	2.5 mM	5.0 mM	7.5 mM	4 mM gl.
First	4.3	2.9	2	5.1	30.4	22.8	10.4	50
Second	2.5	1.7	1	5	12	9.1	4.9	37.5
Third	0.56	0.5	0.5	3.5	4	4	2.6	18.3

Table 4.8 Improvement ratios (folds) of total hydrogen produced & rate of hydrogen production by co-immobilized bacteria compared to single immobilized ones

Rhodobacter capsulatus DSM 1710			*Rhodobacter capsulatus* YO3	
Round	Total H_2 produced (folds)	Rate of H_2 production (folds)	Total H_2 produced (folds)	Rate of H_2 production (folds)
First	1.3-1.4	1.3-1.4	1.4	1.3-1.4
Second	IS *	IS *	1.4-1.6	1.6-1.8
Third	IS *	IS *	1.14-1.16	1.14-1.16

(*) IS, Isignificant

CHAPTER 5

CONCLUSION AND RECOMENDATIONS

As a result of optimization study, it is clear that 3% and 4% of agar produced the most acceptable results. Selection of 4% agar to be employed as the optimized agar concentration was taking in account the need for stable immobilizing system and the high performance in hydrogen production capacity. Higher concentrations of agar may cause diffusion limitations for both nutrients and products.

Comparisons between results including rate of hydrogen production, operating periods for each culture and the cumulative hydrogen production indicates the need of bacteria for nitrogen source more than is usually used with hydrogen production medium (2 mM of glutamate). The amount of glutamate was duplicated up to 4 mM and the results were in agrees with the assumption.

The long operating time (420 to 1428 hours) indicates stability of the process in all agar concentrations were in use. The stability of pH was clear within a narrow range values (6.82 – 7.5). Such values are suitable for the activity of nitrogenase complex without adding extra buffer through the experiment period except the one added at the start of each round with the medium.

Studying effect of different concentrations of acetate on hydrogen production including 60, 80 and 100 mM was conducted. Results pointed that application of 60 mM acetate with DSM1710 strain was accepted in term of total hydrogen produced and rate of hydrogen production while using higher concentrations was not promising.

Application of the three concentration of acetate with YO3 strain was promising with all concentrations but 60 mM concentration was the most acceptable one.

Doubling bacterial cell concentration was examined with both strains. Doubling bacterial concentration with DSM1710 was insignificant in term of rate of hydrogen production and gave slightly negative effect in term of hydrogen production.

Doubling bacterial concentration with YO3 experiments enhanced rate of hydrogen production in all concentrations and increased total hydrogen produced significantly with 80 and 100 mM acetate.

Studying effect of glycerol on hydrogen production by both strains was examined. Glycerol was expected to provide protection for cell membrane during manipulation with hot molded agar to prepare immobilized bacteria.

DSM 1710 strain produced more total hydrogen production when manipulated with 2.5% glycerol. This result may due to partial utilization of glycerol for hydrogen production by that strain or to probable protection effect provided to the cell membrane. Rate of hydrogen production was much more less when compared with cultures free of glycerol. This could be due to inhibition effect of glycerol on nitrogenase.

Manipulation of YO3 strain with glycerol showed decrease in both total hydrogen produced and in rate of hydrogen production comparing to glycerol-free cultures. This may attributed to the inhibition effect of glycerol on nitrogenase.

Studying effect of reducing agent, sodium dithionite, on hydrogen production by both strains showed clear inhibition effect in both strains which was much prominent with DSM 1710 strain. The more adverse effect with YO3 was shortening the time working of the culture through intense precipitation of black colored metal sulfides and disturbing stability and integrity of agar gel-bacteria complex.

Immobilization of bacteria in agar offered some protection to bacteria against inhibition by ammonium when cultures were provided with hydrogen production medium containing ammonium chloride as sole nitrogen source. It was found that increasing time of exposure to ammonium increased its inhibition effect during the three rounds of the process. Increasing of ammonium concentration caused increasing inhibition effect.

Comparing ammonium to glutamate as nitrogen source, it was clear that glutamate is preferred over ammonium as it supported hydrogen production stability for long time.

Co-immobilization of photosynthetic bacteria *R. capsulatus* with halophilic bacteria *H. salinarium* resulted in encouraging outputs, but the main problem was to keep the halophilic bacteria entrapped inside agar during the process of hydrogen production since the halophilic bacteria are not intact cells during immobilization process but they are in the form of packed

fragmented cells. To achieve this goal it is important to increase agar concentration and such solution contradicts with the optimization results of the present work concluding that increasing agar concentration will cause decrease in hydrogen production capacity of immobilized *R. capsulatus*.

Scaling up hydrogen production system to immobilize bacteria inside panel reactor improved the stability of agar immobilization for long period of time as the design developed during this work provided stability and integrity for agar gel entrapping bacteria. The system permitted contacts of the hydrogen production medium with agar-bacteria complex form both sides and it allowed two side illumination of the reactor. Illumination the reactor from both sides was found to encourage hydrogen production capacity of immobilized bacteria in both involved strains.

Stability extended for long time reached more than 85 days working. It was observed in major experiments of hydrogen production by immobilized bacteria that pH stability was common between 6.7 (initial pH) and 7.5 with little less and more. Such pH levels are suitable for activity of nitrogenase for hydrogen production.

According to this work immobilization of photosynthetic bacteria inside agar gel improved to be convenient and reproducible technique. The new developed system adapted to be used for immobilizing bacteria in panel photoreactor proofed its stability and reproducibility. It is proposed that this system is suitable to be used as scaffold with any type of gelling material used for cell or even enzyme immobilization.

Such system is recommended for further improvements for easier manipulation during large scale work. The improvements are mostly restricted to construction material and to more than the principle idea and technical design. Using of stainless steel frame instead of the used glass frame will give better control for the thickness of agar layer and provide better mechanical stability during preparation and setting up the system. Changing the textile material of the Tule network might be recommended for large scale work and this is subjected to the properties of the materials employed in manufacturing the new network.

The panel reactor used in this work is recommended for developments. The present construction design of the reactor constituted three parts and this make difficulty during manipulation and setting up process. It is recommended to use two parts for construction including one piece rectangular vessel and its lid. Panel reactor of two parts will permit development of the present immobilizing frame to be constructed as Honey-comb structure which will make easy manipulation and setting up of the system.

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APPENDIX A

A. EXPERIMENTAL DATA OF CUMULATIVE HYDROGEN PRODUCTION AND MODE OF PH CHANGE DURING HYDROGEN PRODUCTION

Table A. 1 Total hydrogen production by *Rhodobacter capsulatus* DSM (wild strain) fed with 40/2 hydrogen production nutrient medium immobilized in 3% agar, R1-R4

R1		R2		R3		R4	
Time (h)	ml.H_2	Time (h)	ml.H_2	Time (h)	ml.H_2	Time (h)	ml.H_2
0	0	0	0	0	0	0	0
20	38	20	33	20	4	22	3
42	106	24	40	45	28	27	5
64	136	44	73	68	63	46	53
72	142	48	77	96	80	50	60
89	155	68	103	116	106	70	95
114	163	72	107	140	125	75	103
137	173	96	125	164	140	96	120
164	178	117	140	172	150	99	123
170	180	141	170	193	160	120	145
		146	180	212	175	144	165
		168	193	234	205	168	185
		189	195	260	210	192	195
						216	215
						236	218
						260	220

Table A. 2 Total hydrogen production by Rhodobacter capsulatus DSM (wild strain) fed with 40/4 hydrogen production nutrient medium immobilized in 3% agar, R5-R7

R5		R6		R7	
Time (h)	ml.H_2	Time (h)	ml.H_2	Time (h)	ml.H_2
0	0	0	0	0	0
24	5	18	10	20	25
44	65	24	19	27	50
48	70	42	45	45	115
52	110	65	105	48	120
66	150	72	120	50	135
78	200	89	150	69	180
92	255	94	170	89	185
97	265	113	195	95	215
116	305	120	235	119	240
123	320	144	250	144	260
144	340	161	278		
168		167	285		

Table A. 3 pH change during hydrogen production by *Rhodobacter capsulatus* DSM (wild strain) fed with 40/2 (3%R1-3%R4) and fed with 40/4 (3%R5-3%R7) hydrogen production nutrient medium immobilized in 3% agar gelled by growth medium 20/10

Time (h)	3%R1	3%R2	3%R3	3%R4	3%R5	3%R6	3%R7
0	6.87	6.87	6.82	6.82	6.82	6.82	6.82
24	7.28	7.1	7	6.92	6.84	6.96	7.25
48	7.4	7.29	7.254	6.956	6.869	7.184	7.493
72	7.37	7.2	7.26	7.2	6.96	7.012	7.34
96	7.3	7.123	7.272	7.292	7.003	7.088	7.244
120	7.24	7.18	7.16	7.18	7.08	7.38	7.39
144	7.165	7.231	7.078	6.975		7.44	7.44
168		7.21	7.055	6.95		7	
192		7.208	7.022	6.975		6.987	
216			7.033	6.98			
236			7.066	7			

Table A. 4 Total hydrogen production by *Rhodobacter capsulatus* DSM (wild strain) fed with 40/2 hydrogen production nutrient medium immobilized in 4% agar gelled by growth medium 20/10

R1		R2		R3		R4	
Time (h)	H_2 ml	Time (h)	H_2 ml	Time (h)	H_2 ml	Time (h)	H_2 ml
0	0	0	0	0	0	0	0
20	20	24	25	20	3	22	5
42	82	48	60	45	33	27	10
64	105	68	80	68	72	46	72
72	112	72	85	96	95	50	82
89	122	92	97	116	135	70	112
114	132	96	105	140	162	75	122
137	142	117	187	164	177	96	142
164	144	141	207	172	192	99	150
170	144	146	227	193	202	120	182
		168	237	212	222	144	192
		189	237	234	234	168	202
				260	234	192	207
						216	212

Table A. 5 Total hydrogen production by *Rhodobacter capsulatus* DSM (wild strain) fed with 40/4 hydrogen production nutrient medium immobilized in 4% agar gelled by growth medium 20/10

R5		R6		R7	
Time (h)	H_2 ml	Time (h)	H_2 ml	Time (h)	H_2 ml
0	0	0	0	0	0
24	10	24	8	20	58
44	128	42	53	24	78
48	139	48	90	27	98
52	144	67	140	45	178
70	214	89	200	53	213
78	242	96	215	68	281
92	282	113	245	72	303
104	312	118	255	90	325
118	320	137	285	96	333
123	322	144	295	115	335
148	337	168	305	120	337
		185	315	144	337
		191	325		

Table A. 6 pH change during hydrogen production by *Rhodobacter capsulatus* DSM (wild strain) fed with 40/2 (4%R1-4%R4) and fed with 40/4 (4%R5-4%R7) hydrogen production nutrient medium immobilized in 3% agar gelled by growth medium 20/10

Time (h)	4%R1	4%R2	4%R3	4%R4	4%R5	4%R6	4%R7
0	6.87	6.87	6.82	6.82	6.82	6.82	6.82
24	7.16	7.2	7	6.94	7	6.94	7.25
48	7.34	7.385	7.157	6.998	7.197	7.027	7.424
72	7.28	7.35	7.18	7.25	7.051	7	7.37
96	7.237	7.373	7.235	7.348	7.12	6.98	7.332
120	7.12	7.24	7.16	7.26	7.196	7.012	7.229
144	7.06	7.179	7.021	7.168		7.096	7.188
168		7.11		7.09		7.089	
192		7.03		7.013		7.11	

Table A. 7 Total hydrogen production by *Rhodobacter capsulatus* DSM (wild strain) fed with 40/2 hydrogen production nutrient medium immobilized in 5% agar gelled by growth medium 20/10

R1		R2		R3		R4	
Time (h)	H_2 ml	Time (h)	H_2 ml	Time (h)	H_2 ml	Time (h)	H_2 ml
0	0	0	0	0	0	0	0
20	30	20	25	20	3	22	5
42	65	24	30	45	42	27	10
64	90	44	62	68	92	46	78
72	97	48	70	96	111	50	90
89	110	68	90	116	146	70	120
114	120	72	100	140	181	75	125
137	127	92	110	164		96	150
164	130	96	120	172		99	158
170	130	117	135	193		120	185
		141	145	212		144	215
		168	150	234		168	230
		189	155	260		192	240
						216	265

Table A. 8 Total hydrogen production by *Rhodobacter capsulatus* DSM (wild strain) fed with 40/4 hydrogen production nutrient medium immobilized in 5% agar gelled by growth medium 20/10

R5		R6		R7	
Time (h)	H_2 ml	Time (h)	H_2 ml	Time (h)	H_2 ml
0	0	0	0	0	0
24	10	24	10	20	48
44	125	42	70	24	62
48	135	48	90	27	80
52	145	67	140	45	150
66	212	89	190	53	178
78	235	96	205	68	228
92	245	113	235	72	248
104	267	118	245	90	288
118	292	137	265	96	298
123	295	144	275	115	308
145	312	168	280	120	308
		185	285	144	308
		191	295		
		216	295		

Table A. 9 pH change during hydrogen production by *Rhodobacter capsulatus* DSM (wild strain) fed with 40/2 (5%R1-5%R4) and fed with 40/4 (5%R5-5%R7) hydrogen production nutrient medium immobilized in 3% agar gelled by growth medium 20/10

Time (h)	5%R1	5%R2	5%R3	5%R4	5%R5	5%R6	5%R7
0	6.87	6.87	6.82	6.82	6.82	6.82	6.82
20	7.2	7.21	7.12	6.96	7.12	6.88	6.94
48	7.38	7.35	7.2	7.18	7.204	6.93	7.096
72	7.32	7.34	7.16	7.28	7.17	7.189	7.189
96	7.286	7.338	7.148	7.366	7.21	7.229	7.271
120	7.14	7.29	7.1	7.22	7.24	7.2	7.18
144	7	7.257	7.052	7.18		7.109	7.22
168	7.08	7.23	7	7.22		7	
192	7.04	7.22	7.06	7.332		7.083	

Table A. 10 Total hydrogen production by *Rhodobacter capsulatus* DSM (wild strain) fed with 40/2 hydrogen production nutrient medium immobilized in 6% agar gelled by growth medium 20/10

R1		R2		R3		R4	
Time (h)	ml.H_2	Time (h)	ml.H_2	Time (h)	ml.H_2	Time (h)	ml.H_2
0	0	0	0	0	0	0	0
20	20	24	25	20	5	22	0
42	65	44	50	45	35	27	8
64	93	48	60	68	63	46	55
72	97	68	82	96	85	50	65
89	110	72	85	116	115	70	95
114	125	92	100	140	143	75	100
137	137	96	105	164	157	96	120
164	140	117	150	172	173	99	125
170	143	141	191	193	195	120	160
		146	211	212	210	144	172
		168	231	234	237	168	185
		189	241	260	247	192	195
				264	253	216	202

Tabe A. 11 Total hydrogen production by *Rhodobacter capsulatus* DSM (wild strain) fed with 40/4 hydrogen production nutrient medium immobilized in 6% agar gelled by growth medium 20/10

5th run		6th run		7th run	
Time (h)	ml.H_2	Time (h)	ml.H_2	Time (h)	ml.H_2
0	0	0	0	0	0
24	8	24	6	20	45
44	110	42	25	24	60
48	115	48	52	27	72
52	120	67	81	45	112
66	180	89	110	53	147
78	208	96	120	68	180
92	238	113	130	72	195
97	258	118	135	90	210
116	273	137	142	96	225
123	278	144	160	115	229
144	288	168	165	120	235
		185	172	144	237
		191	175		
		216	175		

Table A. 12 pH change during hydrogen production by *Rhodobacter capsulatus* DSM (wild strain) fed with 40/2 (6%R1-6%R4) and fed with 40/4 (6%R5-6%R7) hydrogen production nutrient medium immobilized in 3% agar gelled by growth medium 20/10

Time (h)	6%R1	6%R2	6%R3	6%R4	6%R5	6%R6	6%R7
0	6.87	6.87	6.82	6.82	6.82	6.82	6.82
24	7.2	7.22	7.1	6.96	7.2	7.12	7.28
48	7.318	7.318	7.243	7.062	7.339	7.257	7.461
72	7.38	7.3	7.16	7.26	6.743	7.32	7.34
96	7.4	7.326	7.137	7.382	6.96	7.39	7.251
120	7.28	7.26	7.08	7.28	7.092	7.36	7.18
144	7.177	7.206	7.049	7.079		7.357	7.044
168	7.1	7.2	7	7		7.28	
192		7.18	6.955	7.089		7.211	
216			7.06	7.06			
240			7.11				

Table A. 13 Total hydrogen production by *Rhodobacter capsulatus* YO3 (hup- mutated strain) fed with 40/2 (3%R1-3%R4) and fed with 40/4 (3%R5) immobilized in 3% agar gelled by distilled water

R1		R2		R3		R4		R5	
Time (h)	ml.H_2	Time (h)	ml.H_2	Time (h)	ml.H_2	Time (h)	ml.H_2	Time (h)	ml.H_2
0	0	0	0	0	0	0	0	0	0
12	34	1	3	4	3	20	10	16	40
17	48	5	12	20	8	44	75	20	58
36	93	6.5	17	39	22	48	80	40	218
48	118	21	67	44	30	70	112	64	303
60	162	26	92	65	90	92	142	68	318
72	180	52	167	72	100	116	172	86	370
84	197	67	202	90	130	120	185	90	378
93	203	87	237	114	145	137	245	112	418
110	208			120	150	161	255	117	423
118	209			140	160			136	430
134	213							141	453
								160	455

Table A. 14 pH change during hydrogen production by *Rhodobacter capsulatus* YO3 (hup- mutated strain) fed with 40/2 (3%R1-3%R4) and fed with 40/4 (3%R5) immobilized in 3% agar gelled by distilled water

Time (h)	3%R1	3%R2	3%R3	3%R4	3%R5
0	6.84	6.84	6.84	6.87	6.82
20	7.28	7.128	6.9	6.96	6.93
48	7.38	7.12	7.41	7.33	7.48
72	7.22	7.09	7.47	7.2	7.4
96	7.14	7.1	7.3	7.15	7.34
120	7.01		7.14	7.1	7.2
144			7.38	7	7.1
168				7.17	7.23

Table A. 15 Total hydrogen production by *Rhodobacter capsulatus* YO3 (hup- mutated strain) fed with 40/2 (4%R1-4%R4) and fed with 40/4 (4%R5) immobilized in 4% agar gelled by distilled water

R1		R2		R3		R4		R5	
Time (h)	ml.H_2	Time (h)	ml.H_2	Time (h)	ml.H_2	Time (h)	ml.H_2	Time (h)	ml.H_2
0	0	0	0	0	0	0	0	0	0
12	34	14	9	20	10	18	15	16	55
17	41	37	106	42	85	44	30	20	100
36	79	66	184	48	107	65	70	40	200
48	94	74	203	68	152	72	85	64	270
60	115	93	242	90	242	89	160	68	280
72	130	113	250	12	262	113	182	86	322
84	152	137	255	137	265	137	246	90	335
93	162							112	380
110	178							117	390
118	181							136	415
134	198							141	422
142	208							160	430

Table A. 16 pH change during hydrogen production by *Rhodobacter capsulatus* YO3 (hup- mutated strain) fed with 40/2 (4%R1-4%R4) and fed with 40/4 (4%R5) immobilized in 4% agar gelled by distilled water

Time (h)	4%R1	4%R2	4%R3	4%R4	4%R5
0	6.84	6.84	6.84	6.87	6.82
20	7.08	6.94	6.96	6.93	7.2
48	7.43	7.15	7.26	7.18	7.472
72	7.32	7.165	7.3	7	7.4
96	7.16	7.12	7.38	6.95	7.38
120	7.03	7.4	7.43	7.1	7.25
144		7.22	7.5	7.104	7.151
160					7.28

Table A. 17 Total hydrogen production by *Rhodobacter capsulatus* YO3 (hup- mutated strain) fed with 40/2 (5%R1-5%R4) and fed with 40/4 (5%R5) immobilized in 5% agar gelled by distilled water

5%R-1		5%R-2		5%R-3		5%R-4		5%R-5	
Time (h)	ml.H_2	Time (h)	ml.H_2	Time (h)	ml.H_2	Time (h)	ml.H_2	Time (h)	ml.H_2
0	0	0	0	0	0	0	0	0	0
12	0	22	30	22	15	22	20	16	0
17	25	27	45	27	27	27	35	20	10
36	65	48	100	48	90	48	98	40	110
48	77	72	130	72	130	72	126	64	185
60	104	96	140	96	160	96	143	68	190
72	116	120	145	120	160	120	153	86	235
84	137	144	150	144	190	144	191	90	250
93	142	168	150	168	200	168	193	112	280
110	157	174	150	174	200	174	193	117	290
118	170							136	300
134	186							141	309
142	195							160	311

Table A. 18 pH change during hydrogen production by *Rhodobacter capsulatus* YO3 (hup- mutated strain) fed with 40/2 (5%R1-5%R4) and fed with 40/4 (5%R5) immobilized in 5% agar gelled by distilled water

Time (h)	5%R1	5%R2	5%R3	5%R4	5%R5
0	6.84	6.82	6.82	6.82	6.82
20	7.28	6.88	7	7	7.2
48	7.43	7.02	7.11	7.17	7.46
72	7.28	7.25	7.38	7.38	7.44
96	7.23	7.31	7.47	7.44	7.49
120	7.22	7.2	7.3	7.35	7.4
144		7.18	7.24	7.15	7.16
160				7.19	7.31

Table A. 19 Total hydrogen production by *Rhodobacter capsulatus* YO3 (hup- mutated strain) fed with 40/2 (6%R1-6%R4) and fed with 40/4 (6%R5) immobilized in 6% agar gelled by distilled water

R1		R2		R3		R4		R5	
Time (h)	ml.H_2	Time (h)	ml.H_2	Time (h)	ml.H_2	Time (h)	ml.H_2	Time (h)	ml.H_2
0	22	0	0	0	0	0	0	0	0
12	28	14	0	20	30	18	40	16	20
17	68	37	97	42	90	44	118	20	38
36	77	66	175	48	110	65	165	40	128
48	91	74	192	68	160	72	180	64	203
60	98	93	242	90	210	92	200	68	218
72	113	113	255	112	217	113	213	86	263
84	121	137	255	137	217	137	230	90	278
93	135					161	255	112	318
110	139							117	326
118	153							136	338
134	156							141	344
								160	351

Table A. 20 pH change during hydrogen production by *Rhodobacter capsulatus* YO3 (hup-mutated strain) fed with 40/2 (6%R1-6%R4) and fed with 40/4 (6%R5) immobilized in 6% agar gelled by distilled water

Time (h)	6%R1	6%R2	6%R3	6%R4	6%R5
0	6.84	6.84	6.84	6.87	6.82
20	7.17	6.98	7	6.96	6.98
48	7.28	7.11	7.29	7.2	7.18
72	7.38	7.2	7.2	7.1	7.4
96	7.21	7.14	7.5	7.07	7.52
120	7.14	7.1	7.47	7.12	7.38
144			7.33	7.15	7.19
168				7.18	7.28

Table A. 21 Hydrogen production by different concentrations of acetate fed to *Rhodobacter capsulatus* DSM1710 immobilized in 4% agar, Experiment 1

	R1				R2				R3			
Time (h)	60/4	80/4	100/4	H	60/4	80/4	100/4	H	60/4	80/4	100/4	
0	0	0	0	0	0	0	0	0	0	0	0	
14	5	5	10	15	38	19	15	19	35	10	5	
19	7	10	15	23	60	29	25	25	60	25	15	
39	15	20	25	40	150	36	80	42	150	45	35	
45	25	25	30	48	190	91	96	49	175	55	41	
64	55	55	55	63	240	125	120	66	225	70	60	
70	75	75	70	72	280	143	138	73	230	77	66	
86	150	125	100	88	310	168	167	91	290	110	85	
92	180	150	125	95	360	180	180	114	345	160	130	
112	250	225	190	112	410	225	200	138	420	195	175	
136	350	315	275	138	470	275	250	162	480	220	225	
140	375	330	300	146	540	280	250	165	500	245	240	
159	400	375	350	161	565	296	266	189	535	245	240	
184	405	400	380	188	570	357	315	213	540	285	265	
189	405	410	380	192	575	360	315	237	590	315	290	
206		445	410	207	590	360	320	260	615	335	300	
214		450	425	213	615	360	320	284	635	375	340	
232		460	440	232	620			308	635	415	390	
239		460	440	238	635			332	635	445	420	
254		465	465	256	645			356	640			
263		470	470					380	640			
279		475	480									

Table A. 22 pH change during hydrogen production by different concentrations of acetate fed to *Rhodobacter capsulatus* DSM1710 immobilized in 4% agar, Experiment 1

R1				R2				R3			
(h)	60/4	80/4	100/4	(h)	60/4	80/4	100/4	(h)	60/4	80/4	100/4
0	6.7	6.7	6.7	0	6.7	6.7	6.7	0	6.7	6.7	6.7
88	6.85	6.9	6.97	48	7.45	7.07	7.14	48	7.33	6.85	6.91
144	7.4	7.48	7.38	96	7.33	7.1	7.13	96	7.27	6.95	6.84
189	7.26	7.59	7.62	144	7.47	7.21	7.18	144	7.12	7.1	7.01
239	-	7.81	7.93	210	7.22	7.27	7.21	192	7.02	7.18	7.14
				256	7.32			240	7.00	7.15	7.14
								288	6.97		

Table A. 23 Hydrogen production by different concentrations of acetate fed to *Rhodobacter capsulatus* DSM1710 immobilized in 4% agar, Experiment 2

R1				R2				R3			
(h)	60/4	80/4	100/4	(h)	60/4	80/4	100/4	(h)	60/4	80/4	100/4
0	0	0	0	0	0	0	0	0	0	0	0
18	25	15	15	20	25	10	20	20	20	20	25
38	60	75	80	40	35	25	30	40	45	35	55
64	160	140	125	64	75	50	75	64	100	40	85
88	200	180	240	88	100	75	120	88	125	50	100
112	220	210	275	112	125	95	150	112	200	70	130
136	245	225	300	136	150	120	175	136	225	85	160
164	255	235	320	160	175	120	190	160	265	130	180
188	270	290	325	184	175	120	200	184	275	140	200
212	270	305	325	208			200	208	325	140	225
								232	345	140	235
								256	400	160	285
								280	410	160	290
								304	425	195	300
								328	455	195	300
								352	435	210	325
								376	460	210	325
								400	470	230	350
								424	485	230	350
								448	510	270	350
								472	535	275	
								496	550	290	
								520	570	300	
								544	575	300	
								568	575		

Table A. 24 pH change during hydrogen production by different concentrations of acetate fed to *Rhodobacter capsulatus* DSM1710 immobilized in 4% agar, Experiment 2

R1				R2				R3			
(h)	60/4	80/4	100/4	(h)	60/4	80/4	100/4	(h)	60/4	80/4	100/4
0	6.8	6.8	6.8	0	6.7	6.7	6.7	0	6.7	6.7	6.7
40	7.63	7.76	7.72	48	6.99	6.93	7.05	48	6.82	6.71	6.83
40	7.63	7.76	7.5	96	7.00	7.1	7.73	96	6.91	6.72	7.2
94	7.51	7.38	7.42	144	7.04	7.176	7.46	144	6.98	6.85	7.28
144	7.4	7.37	7.3	192	7.13	7.19	7.16	192	7.05	7.11	7.19
192	7.32	7.40	7.29	216			7.13	240	7.00	7.13	7.25
240								288	7.08	7.06	
								352	7.17	7.12	

Table A. 25 Hydrogen production by different concentrations of acetate fed to *Rhodobacter capsulatus* YO3 (hup- mutated strain) immobilized in 4% agar, Experiment1

R1				R2				R3			
(h)	60/4	80/4	100/4	(h)	60/4	80/4	100/4	(h)	60/4	80/4	100/4
0	0	0	0	0	0	0	0	0	0	0	0
14	70	50	40	15	50	50	26	19	30	25	10
19	125	100	70	23	95	75	40	25	50	35	15
39	340	295	225	40	210	175	86	42	70	55	30
45	380	330	250	48	300	275	103	49	80	60	41
64	485	435	375	63	385	340	209	66	120	80	45
70	515	460	390	72	430	380	238	73	125	100	50
86	555	510	445	88	465	445	265	91	155	125	70
92	560	530	450	95	590	490	290	114	175	150	90
112	580	565	460	112	600	535	310	138	200	175	115
136	590	610	520	138	615	570	365	162	225	190	145
140	630	640	545	146	645	595	375	165	230	200	148
159	640	670	595	161	685	630	390	186	250	220	160
184	640	685	660	186	715	665	450	213	275	230	180
189	640	685	680	192	753	680	450	237	290	250	200
206			730	207	760	705	450	260	300	270	200
214			735	213	810	750	450	284	305	275	250
232			755	232	825	775		308	305	290	360
239			790	238	830	780		332	325	325	450
254			810	256	830	805		356	325	350	520
263			825					380	330	350	550
279			840					404	330		575
								428			600

Table A. 26 pH change hydrogen production by different concentrations of acetate fed to *Rhodobacter capsulatus* YO3 (hup- mutated strain) immobilized in 4% agar, Experiment1

R1				R2				R3			
(h)	60/4	80/4	100/4	(h)	60/4	80/4	100/4	(h)	60/4	80/4	100/4
0	6.7	6.7	6.7	0	6.7	6.7	6.7	0	6.7	6.7	6.7
88	7.01	7.15	7.28	48	7.65	7.59	7.37	48	7.85	7.5	7.00
142	7.24	7.68	7.45	96	7.47	7.57	7.39	96	7.9	7.55	6.94
239	-	-	7.68	144	7.45	7.59	7.37	144	7.68	7.5	7.05
				210	7.55	7.52	7.46	192	7.53	7.44	7.22
				256	7.73	7.74		240	7.47	7.57	7.14
								288	7.29	7.43	

Table A. 27 Hydrogen production by different concentrations of acetate fed to *Rhodobacter capsulatus* YO3 (hup- mutated strain) immobilized in 4% agar, Experiment2

R1				R2				R3			
(h)	60/4	80/4	100/4	(h)	60/4	80/4	100/4	(h)	60/4	80/4	100/4
0	0	0	0	0	0	0	0	0	0	0	0
18	170	100	85	20	55	25	25	20	30	25	35
38	280	240	235	40	175	100	60	40	75	35	35
64	380	325	315	64	290	200	130	64	125	65	45
88	440	395	350	88	355	250	175	88	155	75	55
112	465	475	405	112	425	275	210	112	200	85	75
136	470	525	450	136	500	310	230	136	225	100	75
164	475	550	480	160	540	320	250	160	250	100	75
188	475	555	500	184	550	330	275	184	270	100	75
212		565	530	208			320	208	280	105	85
234			555	232			340	232	295	110	100
				256			350	256	325	120	140
								280	335	130	150
								304	350	140	150
								328	370	150	160
								352	380	150	180
								376	400	180	190
								400	430	205	200
								424	455	225	210
								448	460	250	
								472	475	275	
								496	490	280	
								520	510	300	
								544	520	320	
								568	520	320	

Table A. 28 pH change hydrogen production by different concentrations of acetate fed to *Rhodobacter capsulatus* YO3 (hup- mutated strain) immobilized in 4% agar, Experiment2

R1				R2				R3			
(h)	60/4	80/4	100/4	(h)	60/4	80/4	100/4	(h)	60/4	80/4	100/4
0	6.8	6.8	6.8	0	6.7	6.7	6.7	0	6.7	6.7	6.7
40	7.32	7.48	7.49	48	7.01	6.98	7.12	48	7.14	6.92	6.82
94	7.15	7.33	7.33	96	7.28	7.1	7.16	96	7.07	7.06	6.92
144	7.05	7.2	7.26	144	7.3	7.14	7.22	144	7.06	6.95	6.94
192	7.0	7.06	7.16	192	7.26	7.22	7.10	192	7.14	6.95	7.08
				208			7.17	240	7.15	6.91	
				256			7.102	288	7.07	6.82	
								352	7.273	6.99	

Table A. 29 Effect of increasing cell concentration to 5 mg DCW/ml agar-gel on hydrogen production by immobilized *Rhodobacter capsulatus* DSM1710 fed with different concentrations of acetate, experiment 1

R1				R2				R3			
(h)	60/4	80/4	100/4	(h)	60/4	80/4	100/4	(h)	60/4	80/4	100/4
0	0	0	0	0	0	0	0	0	0	0	0
20	100	125	65	20	125	10	10	20	80	20	25
44	250	210	200	44	250	15	15	44	140	40	40
68	480	370	280	68	430	30	25	68	225	70	70
92	530	415	345	92	485	45	30	92	250	85	80
116	560	445	380	116	525	65	40	116	370	100	90
140	570	475	400	140	530	90	75	140	465	120	100
164	580	495	410	164		100	100	164	540	150	120
188		500	420	188		120	120	188	560	170	125
								212	570	170	125
								236	580	170	125
								260		180	150
								284		200	155
								308		250	175
								332		250	210
								356		250	220
								380		300	250
								404		325	275
								428		335	285
								452		345	300
								476		355	300
								500		355	300

Table A. 30 Change of pH during hydrogen production by immobilized 5 mg DCW/ml agar-gel of *Rhodobacter capsulatus* DSM1710 fed with different concentrations of acetate, experiment 1

R1				R2				R3			
(h)	60/4	80/4	100/4	(h)	60/4	80/4	100/4	(h)	60/4	80/4	100/4
0	6.8	6.8	6.8	0	6.7	6.7	6.7	0	6.7	6.7	6.7
44	7.4	7.50	7.49	48	7.2	6.87	6.83	48	7.21	6.89	6.84
96	7.32	7.37	7.31	96	7.22	6.93	6.9	96	7.14	7.04	6.94
168	7.12	7.21	7.22	144	7.07	7.2	7.10	144	7.1	6.94	6.91
				192		7.0	7.04	192	7.15	7.0	7.0
								240	6.98	7.00	7.00
								264		7.07	7.02

Table A. 31 Effect of increasing cell concentration to 5 mg DCW/ml agar-gel on hydrogen production by immobilized *Rhodobacter capsulatus* DSM1710 fed with different concentrations of acetate, experiment 2

R1				R2				R3			
(h)	60/4	80/4	100/4	(h)	60/4	80/4	100/4	(h)	60/4	80/4	100/4
0	0	0	0	0	0	0	0	0	0	0	0
20	95	90	55	20	100	25	25	20	40	25	30
44	295	245	205	44	200	50	75	44	135	50	60
68	460	390	375	68	300	160	150	68	225	125	100
92	555	515	405	92	350	300	210	92	300	275	120
116	630	615	530	116	425	375	225	116	335	300	160
140	660	690	550	140	435	425	230	140	360	305	195
164	665	755		164	450	475	240	164	390	325	200
188		795		188	450	500	240	188	400	350	210
212		815		212		530		212	425	350	210
				236		540		236	425		

Table A. 32 Change of pH during hydrogen production by immobilized 5 mg DCW/ml agar-gel of *Rhodobacter capsulatus* DSM1710 fed with different concentrations of acetate, experiment 2

R1				R2				R3			
(h)	60/4	80/4	100/4	(h)	60/4	80/4	100/4	(h)	60/4	80/4	100/4
0	6.7	6.7	6.7	0	6.7	6.7	6.7	0	6.7	6.7	6.7
48	7.1	7.18	7.15	48	7.23	7.02	7.02	48	7.18	7.18	6.77
96	7.18	7.31	7.31	96	7.24	7.05	7.15	96	7.18	7.27	6.96
144	7.0	7.2	7.16	144	7.19	7.24	7.17	144	7.24	7.27	7.07
192		7.24		192	6.95	7.28	7.24	192	7.33	7.38	7.18
240				240		7.15		240	7.14		7.2

Table A. 33 Effect of increasing cell concentration to 5 mg DCW/ml agar-gel on hydrogen production by immobilized *Rhodobacter capsulatus* YO3 fed with different concentrations of acetate, experiment 1

R1				R2				R3			
(h)	60/4	80/4	100/4	(h)	60/4	80/4	100/4	(h)	60/4	80/4	100/4
0	0	0	0	0	0	0	0	0	0	0	0
20	270	240	220	20	230	160	185	20	50	70	60
44	700	600	520	44	550	340	450	44	150	200	100
72	875	850	770	68	650	520	680	68	250	300	160
92	895	990	895	92	730	645	760	92	350	380	210
116	905	1075	1150	116	830	745	850	116	425	450	240
136		1120	1245	140	880	835	940	140	475	510	260
160		1120	1320	164	905	880	1050	164	530	555	300
184			1350	188	905	880	1075	188	565	595	330
				212			1195	212	570	655	420
				260			1315	260	580	710	485
				284			1365	284	590	720	530
				308			1370	308	590	735	580
								332	590	740	625
								354	605	740	675
								378	615		720
								402	615		750
								426			770
								448			780
								472			810
								496			825

Table A. 34 Change of pH during hydrogen production by immobilized 5 mg DCW/ml agar-gel of *Rhodobacter capsulatus* YO3 fed with different concentrations of acetate, experiment 1

R1				R2				R3			
(h)	60/4	80/4	100/4	(h)	60/4	80/4	100/4	(h)	60/4	80/4	100/4
0	6.8	6.8	6.8	0	6.7	6.7	6.7	0	6.7	6.7	6.7
48	7.25	7.29	7.21	48	7.22	6.98	7.18	48	7.13	7.11	6.91
96	7.17	7.33	7.36	96	7.17	7.18	7.16	96	7.12	7.19	6.98
168		7.31	7.27	144	7.17	7.11	7.29	144	7.19	7.23	7.01
				192	7.11	7.15	7.28	192	7.17	7.25	7.11
				240	7.2		7.22	240	7.1	7.33	7.14
				288	7.25			288	7.1	7.29	7.17
								352	7.17	7.35	7.21

Table A. 35 Effect of increasing cell concentration to 5 mg DCW/ml agar-gel on hydrogen production by immobilized *Rhodobacter capsulatus* YO3 fed with different acetate concentrations, experiment 2

R1				R2				R3			
(h)	60/4	80/4	100/4	(h)	60/4	80/4	100/4	(h)	60/4	80/4	100/4
0	0	0	0	0	0	0	0	0	0	0	0
20	375	280	230	20	230	125	180	20	75	35	75
44	800	640	570	44	540	300	390	44	330	150	330
68	1005	905	900	68	750	360	565	68	500	270	500
92	1045	1105	1150	92	915	375	650	92	610	350	610
116		1160	1360	116	1100	475	790	116	725	475	725
140		1165	1460	140	1100	575	910	140	755	580	755
164			1560	164		635	1010	164	800	650	800
188				188		695	1080	188	800	680	800
212				212		725	1140	212		730	
236				236		745	1210	236		770	
				260		750	1300	260		800	
				284			1320	284			

Table A. 36 Change of pH during hydrogen production by immobilized 5 mg DCW/ml agar-gel of *Rhodobacter capsulatus* YO3 fed with different concentrations of acetate, experiment 2

R1				R2				R3			
(h)	60/4	80/4	100/4	(h)	60/4	80/4	100/4	(h)	60/4	80/4	100/4
0	6.7	6.7	6.7	0	6.7	6.7	6.7	0	0	0	0
48	7.16	7.3	7.36	48	7.12	7.04	7.27	48	7.34	7.28	7.19
96	7.24	7.44	7.48	96	7.28	7.32	7.16	96	7.5	7.39	7.28
144		7.34	7.35	144	7.17	7.38	7.18	144	7.08	7.78	7.24
192				192		7.63	7.33	192	7.13	7.48	7.08
240				240		7.56	7.19	240		7.33	
				284			7.17	284			

Table A. 37 Hydrogen production by immobilized *Rhodobacter capsulatus* DSM1710 manipulated with 2.5% and 5% glycerol and fed with 60 mM acetate

R1				R2				R3			
(h)	2.5-1	2.5-2	5-1	(h)	2.5-1	2.5-2	5-1	(h)	2.5-1	2.5-2	5-1
0	0	0	0	0	0	0	0	0	0	0	0
20	113	68	60	20	50	50	25	20	20	40	50
44	358	215	190	44	120	100	75	44	40	125	150
68	520	370	285	68	190	200	175	68	95	210	270
92	650	425	325	92	280	250	210	92	150	320	390
116	800	490	370	116	370	300	250	116	185	375	460
140	880	500	380	140	420	335	285	140	245	450	560
164	935	530	425	164	480	365	305	164	280	500	630
188	970	565	450	188	530	385	345	188	325	535	655
212	980	620	485	212	570	405	375	212	360	575	685
236	980	630	500	236	595	415	400	236			

Table A. 38 Change of pH during hydrogen production by immobilized *Rhodobacter capsulatus* DSM1710 manipulated with 2.5% and 5% glycerol and fed with 60 mM acetate

R1				R2				R3			
(h)	2.5-1	2.5-2	5-1	(h)	2.5-1	2.5-2	5-1	(h)	2.5-1	2.5-2	5-1
0	6.7	6.7	6.7	0	6.7	6.7	6.7	0	6.7	6.7	6.7
48	6.93	7.05	7	48	7.0	6.79	6.63	48	6.98	7.07	7.14
96	7.28	7.27	7.29	96	6.94	7.16	7.06	96	7.08	7.12	7.17
144	7.08	7.01	7.06	144	6.96	7.27	7.12	144	7.13	7.19	7.21
192	7.02	6.94	7.04	192	7.0	6.96	6.81	192	7.13	7.21	7.25
240		7.07	7.0	240	7.02	6.93	7.0	240			

Table A. 39 Hydrogen production by immobilized *Rhodobacter capsulatus* YO3 manipulated with 2.5% and 5% glycerol and fed with 60 mM acetate

R1				R2				R3			
(h)	2.5-1	2.5-2	5-1	(h)	2.5-1	2.5-2	5-1	(h)	2.5-1	2.5-2	5-1
0	245	245	135	0	0	0	0	0	0	0	0
20	505	550	415	20	110	60	200	20	50	25	100
44	700	795	685	44	320	225	460	44	230	150	300
68	835	940	835	68	360	405	540	68	400	250	490
92	920	975	915	92	475	540	660	92	550	340	625
116	935	1000	930	116	590	695	740	116	650	450	725
140				140	680	780	775	140	720	575	745
164				164	745	805	795	164	755	580	765
188				188	775	815	820	188	755	600	780
212				212	805	830	835	212		605	780
236				236	825	850		236			
				260	830	855					

Table A. 40 Change of pH during hydrogen production by immobilized *Rhodobacter capsulatus* YO3 manipulated with 2.5% and 5% glycerol and fed with 60 mM acetate

R1				R2				R3			
(h)	2.5-1	2.5-2	5-1	(h)	2.5-1	2.5-2	5-1	(h)	2.5-1	2.5-2	5-1
0	6.7	6.7	6.7	0	6.7	6.7	6.7	0	6.7	6.7	6.7
48	6.87	6.96	6.92	48	7.12	7.02	7.16	48	7.11	7.23	7.01
96	6.93	7.1	7.03	96	7.15	7.2	7.22	96	7.2	7.28	7.27
144	7.19	7.22	7.08	144	7.18	7.05	7.01	144	7.25	7.3	7.3
192				192	7.33	7.27	7.34	192	7.24	7.28	7.16
240				240	7.278	7.298	7.278	240		7.28	7.16

Table A. 41 Effect of 0.5 mM sodium dithionite (SDT) on hydrogen production by immobilized *Rhodobacter capsulatus* DSM1710

Time (h)	R1		R2		R3 (no SDT in the nutrient medium)	
	0.5-1	0.5-2	0.5-1	0.5-2	0.5-1	0.5-2
0	0	0	0	0	0	0
20	10	10	50	40	50	65
44	35	60	85	100	120	175
68	50	75	115	115	235	275
92	55	90	130	115	285	325
116	65	125	150	145	320	375
140	85	140	150	160	335	400
164	105	150	175	160	360	430
188	105	150	185		385	460
212			200		390	475
236			200		395	485
260					400	500
284					400	500

Table A. 42 Change of pH during hydrogen production by immobilized *Rhodobacter capsulatus* DSM1710 in the presence of 0.5 mM sodium dithionite (SDT)

Time (h)	R1		R2		R3 (no SDT in the nutrient medium)	
	0.5-1	0.5-2	0.5-1	0.5-2	0.5-1	0.5-2
0	6.7	6.7	6.7	6.7	6.7	6.7
44	6.72	6.78	7.0	6.9	7.17	7.19
92	6.76	6.84	7.12	7.287	7.0	7.05
140	6.85	7.23	7.05	7.18	7.49	7.14
188			7.11		7.23	7.3
236			7.18		7.05	7.28
284						7.21

Table A. 43 Effect of 1.0 mM sodium dithionite (SDT) on hydrogen production by immobilized *Rhodobacter capsulatus* DSM1710

Time (h)	R1		R2		R3 (no SDT in the nutrient medium))	
	1.0-1	1.0-2	1.0-1	1.0-2	1.0-1	1.0-2
0	0	0	0	0	0	0
20	15	10	40	50	50	50
44	25	25	50	75	100	115
68	35	40	95	100	150	150
92	35	40	130	110	170	175
116	35	50	145	125	180	200
140	40	55	150	130	195	220
164	40	55	150	150	200	230
188				170	220	250
212				175	220	260
236				180	245	280
260				200	270	280
284				200	275	
308					300	
332					300	

Table A. 44 Change of pH during hydrogen production by immobilized *Rhodobacter capsulatus* DSM1710 in the presence of 1.0 mM sodium dithionite (SDT)

Time (h)	R1		R2		R3 (no SDT in the nutrient medium))	
	1.0-1	1.0-2	1.0-1	1.0-2	1.0-1	1.0-2
0	6.7	6.7	6.7	6.7	6.7	6.7
44	6.65	6.72	7.1	7.15	7.09	7.02
92	6.73	6.75	7.09	7.37	7.14	6.98
140	6.84	6.86	7.22	7.26	7.05	7.33
188				7.32	7.34	7.19
236				7.39	7.35	7.07
284				7.4	7.15	
332					7.28	

Table A. 45 Effect of 1.5 mM sodium dithionite (SDT) on hydrogen production by immobilized *Rhodobacter capsulatus* DSM1710

Time (h)	R1		R2		R3 (no SDT in the nutrient medium))	
	1.5-1	1.5-2	1.5-1	1.5-2	1.5-1	No-1.5-2
0	0	0	0	0	0	0
20	15	10	45	60	70	75
44	35	35	100	100	170	160
68	50	50	125	150	210	200
92	60	55	150	175	285	360
116	60	65	180	200	310	460
140	75	70	200	220	345	530
164	75	70	225	220	365	535
188			240		375	545
212			255		380	
236			270		380	
260			270			

Table A. 46 Change of pH during hydrogen production by immobilized *Rhodobacter capsulatus* DSM1710 in the presence of 1.5 mM sodium dithionite (SDT)

Time (h)	R1		R2		R3 (no SDT in the nutrient medium))	
	1.5-1	1.5-2	1.5-1	1.5-2	1.5-1	1.5-2
0	6.7	6.7	6.7	6.7	6.7	6.7
44	7.0	6.96	7.04	7.14	7.25	7.34
92	6.72	6.69	7.18	7.24	7.21	7.44
140	7.14	7.09	7.08	7.14	7.61	7.27
188					7.35	7.44
236					7.34	7.55
284						7.28
332						7.28
380						7.08

Table A. 47 Effect of 0.5 mM sodium dithionite (SDT) on hydrogen production by immobilized *Rhodobacter capsulatus* YO3

Time	R1		R2		R3	
(h)	0.5-1	0.5-2	0.5-1	0.5-2	0.5-1	0.5-2
0	0	0	0	80	0	
20	230	265	205	250	40	
44	690	665	475	380	185	
68	874	800	635	455	283	
92	940	850	796	505	365	
116	940	875	846	530	426	
140	940	895	940	540	488	
164	940	895	940		533	
188					553	
212					575	

Table A. 48 Change of pH during hydrogen production by immobilized *Rhodobacter capsulatus* YO3 in the presence of 0.5 mM sodium dithionite (SDT)

Time	R1		R2		R3	
(h)	0.5-1	0.5-2	0.5-1	0.5-2	0.5-1	0.5-2
0	6.7	6.7	6.7	6.7	6.7	
44	7.32	7.19	7.24	7.41	7.21	
92	7.16	7.06	7.31	7.44	7.21	
140		7.0	7.11	7.23	7.37	
188					7.31	
236					7.42	

Table A. 49 Effect of 1.0 mM sodium dithionite (SDT) on hydrogen production by immobilized *Rhodobacter capsulatus* YO3

Time	R1		R2		R3	
(h)	1.0-1	1.0-2	1.0-1	1.0-2	1.0-1	1.0-2
0	0	0	0	0		
20	210	160	127	60		
44	635	525	433	235		
68	780	705	603	465		
92	820	775	752	585		
116	840	787	800	656		
140	840	795	815	670		
164		800	815	690		
188				690		

Table A. 50 Change of pH during hydrogen production by immobilized *Rhodobacter capsulatus* YO3 in the presence of 1.0 mM sodium dithionite (SDT)

Time	R1		R2		R3	
(h)	1.0-1	1.0-2	1.0-1	1.0-2	1.0-1	1.0-2
0	6.7	6.7	6.7	6.7		
44	7.19	7.14	7.27	7.23		
92	7.03	7.02	7.59	7.48		
140	6.94	7.02	7.34	7.29		
188			7.33	7.29		

Table A. 51 Effect of 1.5 mM sodium dithionite (SDT) on hydrogen production by immobilized *Rhodobacter capsulatus* YO3

Time	R1		R2		R3	
(h)	1.5-1	1.5-2	1.5-1	1.5-2	1.5-1	No-1.5-2
0	0	0	0	0	0	0
20	233	220	66	45	10	40
44	510	580	265	227	50	175
68	672	700	415	413	110	320
92	745	765	525	520	130	425
116	750	770	640	595	180	500
140	750	770	672	635	200	550
164			680	635	215	575
188			690		230	575
212					250	
236					260	

Table A. 52 Change of pH during hydrogen production by immobilized *Rhodobacter capsulatus* YO3 in the presence of 1.5 mM sodium dithionite (SDT)

Time	R1		R2		R3	
(h)	1.5-1	1.5-2	1.5-1	1.5-2	1.5-1	1.5-2
0	6.7	6.7	6.7	6.7	6.7	6.7
44	7.25	7.2	7.32	7.42	7.51	7.49
92	7.12	7.16	7.62	7.70	7.73	7.31
140	6.87	6.92	7.4	7.45	7.54	7.44
188			7.35	7.38	7.83	7.48
236					7.59	

Table A. 53 Effect of 2.5 mM ammonium chloride on hydrogen production by immobilized *Rhodobacter capsulatus* DSM 1710

Time (h)	2.5-1R1	2.5-2R1	2.5-1R2	2.5-2R2	2.5-1R3	2.5-2R3
0	0	0	0	0	0	0
20	20	19	80	80	55	20
44	20	20	130	125	100	80
68	25	25	155	150	125	120
92	75	73	170	167	130	125
116	175	170	200	195	130	130
140	230	225	205	200	130	130
164	290	285	205	200		
188	325	320				
212	350	344				

Table A. 54 Change of pH during hydrogen production by immobilized *Rhodobacter capsulatus* DSM 1710 in the presence of 2.5 mM ammonium chloride

Time (h)	2.5-1R1	2.5-2R1	2.5-1R2	2.5-2R2	2.5-1R3	2.5-2R3
0	6.7	6.7	6.7	6.7	6.7	6.7
44	7.01	6.78	7.03	7.025	7.0	6.93
92	7.22	7.24	7.1	7.12	7.03	7.08
140	7.24	7.3	7.11	7.21	7.17	7.18
188	7.32	7.34				

Table A. 55 Effect of 5 mM ammonium chloride on hydrogen production by immobilized *Rhodobacter capsulatus* DSM 1710

Time (h)	5-1R1	5-2R1	5-1R2	5-2R2	5-1R3	5-2R3
0	0	0	0	0	0	0
20	10	10	75	75	15	15
44	10	10	200	175	30	40
68	15	40	225	200	45	50
92	70	125	235	220	55	65
116	170	190	235	225	75	80
140	205	205			75	80
164	225	210				
188	225	220				

Table A. 56 Change of pH during hydrogen production by immobilized *Rhodobacter capsulatus* DSM 1710 in the presence of 5 mM ammonium chloride

Time (h)	5-1R1	5-2R1	5-1R2	5-2R2	5-1R3	5-2R3
0	6.7	6.7	6.7	6.7	6.7	6.7
44	6.911	7.792	7.601	7.872	7.75	7.9
92	7.875	8.198	7.56	7.8	8.483	8.528
140	7.8	7.96	7.574	7.814	8.385	8.527
188	7.75	7.919				

Table A. 57 Effect of 7.5 mM ammonium chloride on hydrogen production by immobilized *Rhodobacter capsulatus* DSM 1710

Time (h)	7.5-1R1	7.5-2R1	7.5-1R2	7.5-2R2	7.5-1R3	7.5-2R3
0	0	0	0	0	0	0
20	5	5	55	50	12	15
44	5	5	130	125	25	30
68	10	20	156	150	30	50
92	123	125	160	150	50	70
116	170	175	160	150	55	75
140	186	190			55	75
164	192	195				
188	196	200				

Table A. 58 Change of pH during hydrogen production by immobilized *Rhodobacter capsulatus* DSM 1710 in the presence of 7.5 mM ammonium chloride

Time (h)	7.5-1R1	7.5-2R1	7.5-1R2	7.5-2R2	7.5-1R3	7.5-2R3
0	6.7	6.7	6.7	6.7	6.7	6.7
44	8.18	8.02	7.62	7.92	7.56	7.89
92	7.79	7.90	8.3	7.9	8.0	8.51
140	7.83	7.8	8.21	8.12	7.97	8.51
188	7.75	7.67				

Table A. 59 Effect of 2.5 mM ammonium chloride on hydrogen production by immobilized *Rhodobacter capsulatus* YO3

Time (h)	2.5-1R1	2.5-2R1	2.5-1R2	2.5-2R2	2.5-1R3	2.5-2R3
0	0	0	0	0	0	0
20	180	200	50	52	20	20
44	350	375	140	143	40	40
68	610	635	250	256	70	72
92	790	825	350	358	95	98
116	830	855	400	410	100	103
140	840	860	425	435	110	115
164			460	471	110	115
188			490	500		
212			500	512		

Table A. 60 Change of pH during hydrogen production by immobilized *Rhodobacter capsulatus* YO3 in the presence of 2.5 mM ammonium chloride

Time (h)	2.5-1R1	2.5-2R1	2.5-1R2	2.5-2R2	2.5-1R3	2.5-2R3
0	6.7	6.7	6.7	6.7	6.7	6.7
44	7.2	7.11	7.33	7.38	7.34	7.21
92	7.21	7.13	7.3	7.34	7.35	7.2
140	7.18	7.12	7.15	7.19	7.33	7.29
188			7.08	7.19		

Table A. 61 Effect of 5 mM ammonium chloride on hydrogen production by immobilized *Rhodobacter capsulatus* YO3

Time (h)	5-1R1	5-2R1	5-1R2	5-2R2	5-1R3	5-2R3
0	0	0	0	0	0	0
20	155	155	30	34	15	10
44	300	325	120	133	50	50
68	460	535	200	222	75	75
92	525	600	260	285	85	85
116	530	605	275	305	90	95
140	550	610	290	320	90	95
164			310	344	90	95
188			325	360		
212			325	360		

Table A. 62 Change of pH during hydrogen production by immobilized *Rhodobacter capsulatus* YO3 in the presence of 5 mM ammonium chloride

Time (h)	5-1R1	5-2R1	5-1R2	5-2R2	5-1R3	5-2R3
0	6.7	6.7	6.7	6.7	6.7	6.7
44	7.07	7.08	7.63	7.65	7.65	7.64
92	7.11	7.12	7.49	7.55	7.56	7.5
140	7.13	7.13	7.29	7.26	7.42	7.44
188			7.27	7.31		

Table A. 63 Effect of 7.5 mM ammonium chloride on hydrogen production by immobilized *Rhodobacter capsulatus* YO3

Time (h)	7.5-1R1	7.5-2R1	7.5-1R2	7.5-2R2	7.5-1R3	7.5-2R3
0	0	0	0	0	0	0
20	100	100	17	20	8	10
44	195	210	65	75	32	37
68	300	342	110	125	55	62
92	340	385	146	160	75	80
116	345	387	155	170	77	85
140	360	390	160	180	80	90
164	370	400	172	192	86	96
188	380	405	185	200	92	100
212			185	205	92	100

Table A. 64 Change of pH during hydrogen production by immobilized *Rhodobacter capsulatus* YO3 in the presence of 7.5 mM ammonium chloride

Time (h)	7.5-1R1	7.5-2R1	7.5-1R2	7.5-2R2	7.5-1R3	7.5-2R3
0	6.7	6.7	6.7	6.7	6.7	6.7
44	7.86	7.95	7.74	8.28	9.18	9.66
92	7.97	7.95	8.44	8.76	9.2	9.3
140	8.4	8.2	8.88	8.46	9.05	9.28
188			9.27	9.33		

Table A. 65 Cumulative Hydrogen Production, DSM 1710 co-immobilized with *H. salinarium*

Time (h)	DR-1	D-HS1R-1	D-HS2R-1	DR-2	D-HS1R-2
0	0	0	0	0	0
20	135	55	30	25	20
44	320	277	230	45	60
68	475	525	480	180	140
92	525	740	710	315	285
116	550	770	725	385	355
140	555	770	725	450	450
164				584	565
188				600	620
212				620	640
236				625	650

Time (h)	D-HS2R-2	DR-3	D-HS1R-3	D-HS2R-3
0	0	0	0	0
20	40	20	25	25
44	150	40	45	65
68	248	135	130	160
92	405	215	350	335
116	495	360	480	440
140	570	440	545	500
164	620	510	595	580
188	640	575	620	600
212	655	580	615	620
236	660	600	620	630
260		615	625	635

Table A. 66 Mode of pH change during hydrogen production by DSM 1710 co-immobilized with *H. salinarium*

Time (h)	DR-1	D-HS1R-1	D-HS2R-1	DR-2	D-HS1R-2
0	6.7	6.7	6.7	6.7	6.7
44	7.08	7.092	7.064	7.532	7.504
92	7.42	7.537	7.312	7.351	7.613
140	7.49	7.407	7.406	7.471	7.663
188				7.577	7.412
236				7.376	7.318
284				7.35	7.314

Time (h)	D-HS2R-2	DR-3	D-HS1R-3	D-HS2R-3
0	6.7	6.7	6.7	6.7
44	7.58	7.42	7.54	7.41
92	7.55	7.54	7.49	7.41
140	7.47	7.39	7.44	7.33
188	7.5	7.59	7.43	7.53
236	7.4	7.49	7.36	7.42
284	7.38	7.43	7.38	7.33

Table A. 67 Cumulative Hydrogen Production, YO3 co-immobilized with *H. salinarium*

Time (h)	YR-1	Y-HS1R-1	Y-HS2R-1	YR-2	Y-HS1R-2
0	0	0	0	0	0
20	190	250	250	180	270
44	465	610	615	340	580
68	630	760	765	530	850
92	815	1070	1077	665	1040
116	905	1250	1255	750	1150
140	905	1260	1270	775	1200
164	905	1260	1275	800	1240
188				800	1240

Time (h)	Y-HS2R-2	YR-3	Y-HS1R-3	Y-HS2R-3
0	0	0	0	0
20	275	155	230	50
44	550	365	505	330
68	780	515	680	510
92	1000	605	780	710
116	1065	660	825	815
140	1090	703	835	820
164	1105	725	840	825
188	1110			

Table A. 68 Mode of pH change during hydrogen production by DSM 1710 co-immobilized with *H. salinarium*

Time (h)	YR-1	Y-HS1R-1	Y-HS2R-1	YR-2	Y-HS1R-2
0	6.7	6.7	6.7	6.7	6.7
44	7.271	7.529	7.403	7.174	7.297
92	7.034	6.991	7.052	7.278	7.357
140	7.226	7.131	7.155	7.28	7.351
Time (h)	Y-HS2R-2	YR-3	Y-HS1R-3	Y-HS2R-3	
0	6.7	6.7	6.7	6.7	
44	7.465	7.356	7.32	7.39	
92	7.46	7.443	7.437	7.451	
140	7.377	7.119	7.287	7.341	

Table A. 69 Cumulative hydrogen production by *R. capsulatus* YO3 fed with 40/4 medium and immobilized in panel photobioreactor (first design), Reactor A and Reactor B

Time (h)	Reactor A	Reactor B	Time (h)	Reactor A	Reactor B
0	0	0	312	4140	5160
24	270	115	336	4330	5445
48	740	670	360	4570	5785
72	1025	990	384	4940	5940
96	1190	1775	408	5050	6090
120	1365	2580	432	5395	6335
144	1505	2985	456	5580	6405
168	1975	3150	480	5810	6615
192	2235	3770	504	5995	6675
216	2735	3960	528	6180	6845
240	2995	4575	552	6285	7145
264	3445	4715	576	6570	7250
288	3655	4830			

Table A. 70 Mode of pH change during hydrogen production by *R. capsulatus* YO3 fed with 40/4 medium and immobilized in panel photobioreactor (first design), Reactor A and Reactor B

Time (h)	Reactor A	Reactor B	Time (h)	Reactor A	Reactor B
0	6.82	6.82	312	7.47	7.4
24	7.31	7.52	336	7.47	7.35
48	8.42	7.5	360	7.47	7.43
72	8.51	7.5	384	7.46	7.34
96	8.22	7.21	408	7.41	7.42
120	7.92	7.25	432	7.39	7.34
144	7.64	7.12	456	7.35	7.5
168	7.33	7.38	480	7.45	7.41
192	7.4	7.33	504	7.5	7.55
216	7.456	7.37	528	7.13	7.29
240	7.43	7.29	552	7.37	7.41
264	7.52	7.36	576	7.34	7.49
288	7.42	7.31			

Table A. 71 Cumulative hydrogen production by *R. capsulatus* DSM 1710 immobilized in panel photobioreactor, Reactor D1

Time (h)	D1R-1	Time (h)	D1R-2	Time (h)	D1R-3	Time (h)	D1R-4
0	0	240	0	600	0	1104	0
20	0	264	300	624	200	1128	180
44	750	288	600	648	440	1152	250
68	1650	312	800	672	1000	1176	500
92	2350	336	1000	696	1300	1200	560
116	2800	360	1200	720	1520	1224	660
140	3200	384	1450	744	1700	1248	700
164	3500	408	1600	768	1900	1272	760
188	3800	432	1700	792	2050	1296	810
212	3850	456	1750	816	2200	1320	850
240	3900	480	1800	840	2380	1344	890
		240	0	600	0	1104	0
		504	1900	864	2520	1368	950
		528	1950	888	2670	1392	990
		552	2010	912	2880	1416	1030
		576	2080	936	3020	1440	1060
		600	2150	960	3120	1464	1110
				984	3220	1488	1150
				1008	3300	1512	1210
				1032	3380	1536	1310
				1056	3500	1560	1390
				1080	3560	1584	1470
				1104	3640		

Table A. 72 Mode of pH change during hydrogen production by *R. capsulatus* DSM 1710 immobilized in panel photobioreactor, Reactor D1 (second design)

Time (h)	D1R-1	D1R-2	D1-R3	D2-R-4
0	6.7	6.7	6.7	6.7
44	7.34	7.88	6.94	7.10
92	7.48	7.82	7.52	7.55
140	7.54	7.65	7.72	7.56
188	7.4	7.44	7.56	7.53
240	7.43	7.15	7.37	7.53
288		7.17	7.39	7.66
336		7.18	7.39	7.77
384		7.18	7.35	7.3
432		7.22	7.25	7.18
480		7.26	7.17	7.30
528		7.31		
576		7.27		
624		7.26		
672		7.29		
720		7.39		
768		7.68		

Table A. 73 Cumulative hydrogen production by *R. capsulatus* DSM 1710 immobilized in panel photobioreactor, Reactor D2

Time (h)	D2R-1	Time (h)	D2R-2	Time (h)	D2R-3	Time (h)	D2R-4
0	0	336	0	840	0	1128	0
20	0	360	20	864	200	1152	700
44	240	384	120	888	480	1176	1200
68	530	408	260	912	800	1200	1550
92	820	432	370	936	950	1224	1750
116	1100	456	480	960	1080	1248	1950
140	1390	480	580	984	1200	1272	2050
164	1475	504	710	1008	1320	1296	2150
188	1660	528	810	1032	1450	1320	2200

Table A. 73 contiued

Time (h)	D2R-1	Time (h)	D2R-2	Time (h)	D2R-3	Time (h)	D2R-4
212	2000	552	900	1056	1550	1344	2300
240	2190	576	980	1080	1650	1368	2400
264	2275	600	1040	1104	1700	1392	2500
288	2415	624	1110	1128	1700	1416	2600
312	2600	648	1170			1440	2750
336	2600	672	1230			1464	2950
		696	1300			1488	3100
		720	1380			1512	3200
		744	1460			1536	3250
		768	1520			Time (H)	D2R-5
		792	1580			1536	0
		816	1640			1560	350
		840	1650			1584	750
						1608	950
						1632	1100
						1656	1250
						1680	1400
						1704	1550
						1728	1750
						1752	1900
						1776	2000
						1800	2100
						1824	2250
						1848	2300
						1872	2400
						1896	2550
						1920	2750
						1944	2900
						1968	3000

Table A. 74 Mode of pH change during cumulative hydrogen production by *R. capsulatus* DSM 1710 immobilized in panel photobioreactor, Reactor D2

Time (h)	D2R-1	D2R-2	D2-R3	D2-R4	D2-R5
0	6.7	6.7	6.7	6.7	6.7
44	7.17	7.51	6.98	7.98	7.55
92	7.72	8.24	7.2	8.07	7.58
140	7.64	8.28	7.27	7.98	7.45
188	7.64	8.12	7.29	7.64	7.52
240	7.65	7.95	7.31	7.5	7.36
288	7.55	7.64		7.35	7.32
336		7.57		7.24	7.36
384		7.35		7.44	7.26
432		7.45		7.44	7.38
480		7.48		7.27	7.34

Table A. 75 Cumulative hydrogen production by *R. capsulatus* YO3 immobilized in panel photobioreactor, Reactor Y1 (second design)

Time (h)	Y1-R1	Time (h)	Y1-R2	Time (h)	Y1-R3	Time (h)	Y1-R4
0	0	188	0	552	0	816	0
20	1400	212	120	576	135	840	950
44	2500	240	340	600	470	864	2200
68	3350	264	640	624	1650	888	3250
92	3900	288	1200	648	2550	912	3900
116	3900	312	1680	672	3000	936	4150
140	4300	336	2220	696	3200	960	4350
164	4400	360	2650	720	3550	984	4450
188	4400	384	3050	744	3700	1008	4500
		408	3400	768	3800	1032	4550
		432	3570	792	3850	1056	4600
		456	3680	816	3850		
		480	3760				
		504	3800				

Table A. 75 continued

		528	3840		
		552	3850		
Time (h)	Y1-R5	Time (h)	Y1-R6	Time (h)	Y1-R7
1056	0	1296	0	1536	0
1080	460	1320	700	1560	100
1104	1400	1344	1353	1584	240
1128	2400	1368	2400	1608	400
1152	3000	1392	3200	1632	750
1176	3380	1416	3700	1656	900
1200	3700	1440	3850	1680	1025
1224	3900	1464	3950	1704	1100
1248	4150	1488	4000	1728	1180
1272	4200	1512	4050	1752	1200
1296	4200	1536	4050	1776	1220
				1800	

Table A. 76 Mode of pH change during cumulative hydrogen production by *R. capsulatus* YO3 immobilized in panel photobioreactor, Reactor Y1

Time (h)	Y1-R1	Y1R-2	Y1-R3	Y1R-4	Y1-R5	Y1-R6	Y-R7
0	6.7	6.7	6.7	6.7	6.7	6.7	6.7
44	7.57	7.26	7.6	7.12	7.93	7.27	7.68
92	7.34	7.62	7.8	7.32	7.58	7.16	7.71
140	7.31	7.61	7.61	7.17	7.44	7.18	7.75
188	7.3	7.46	7.52	7.38	7.25	7.32	7.78
240		7.72	7.48	7.56	7.35	7.19	7.41
288		7.34					8.01
336		7.41					

Table A. 77 Cumulative hydrogen production by *R. capsulatus* YO3 immobilized in panel photobioreactor, Reactor Y2 (second design)

Time (h)	Y2R-1	Time (h)	Y2R-2	Time (h)	Y2R-3	Time (h)	Y2R-4
0	0	264	0	600	0	816	0
20	1000	288	300	624	700	840	800
44	2300	312	725	648	2580	864	3000
68	3400	336	1500	672	4030	888	3900
92	4100	360	2200	696	4500	912	4400
116	4350	384	2825	720	4800	936	4560
140	4650	408	3275	744	5000	960	4660
164	4800	432	3550	768	5100	984	4760
188	4900	456	3725	792	5180	1008	4840
212	4950	480	3825	816	5220	1032	4940
240	5000	504	3900			1056	5020
264	5000	528	3950			1080	5020
		552	3975				
		576	4000				
		600	4000				

Time (h)	Y2R-5	Time (h)	Y2R-6	Time (h)	Y2R-7
1080	0	1368	0	1560	0
1104	650	1392	800	1584	1000
1128	2600	1416	2750	1608	3200
1152	3550	1440	3750	1632	3650
1176	4100	1464	4000	1656	3950
1200	4360	1488	4250	1680	4050
1224	4480	1512	4300	1704	4100
1248	4620	1536	4400	1728	4300
1272	4700	1560	4500	1752	4400
1296	4760			1776	4450
1320	4800				
1344	4840				
1368	4900				

Table A. 78 Mode of pH change during cumulative hydrogen production by *R. capsulatus* YO3 immobilized in panel photobioreactor, Reactor Y2

Time (h)	Y2R-1	Y2R-2	Y2-R3	Y2R-4	Y2R-5	Y2R-6	Y2-R7
0	6.7	6.7	6.7	6.7	6.7	6.7	6.7
44	7.55	7.54	7.38	7.59	7.73	7.46	7.38
92	7.4	7.48	7.37	7.33	7.57	7.34	7.37
140	7.21	7.55	7.48	7.33	7.48	7.65	7.72
188	7.31	7.53	7.69	7.26	7.44	7.56	7.46
240	7.35	7.51	7.56	7.24	7.42		7.28
288		7.52					
336		7.41					
384		7.51					

APPENDIX B

B. EXPERIMENTAL DATA OF HPLC ANALYSIS OF ACETATE AND FORMATE DURING HYDROGEN PRODUCTION

3% agar
3% agar R1 (spent medium)
Acetate concentration (nd)
Formate concentration (0.1517 mg/ml)

Table B. 1 Acetate and formate concentrations during hydrogen production by *Rhodobacter capsulatus* YO3 immobilized in 3% agar

(R2) (h)	Acetate mg/ml	Formate mg/ml	(R3) (h)	Acetate mg/ml	Formate mg/ml
0	2.1146	0	0	2.1146	0
24	0.9066	0.1443	48	1.4408	0.0487
52	0.3411	0.1990	90	0.8747	0.0890
75	0.1565	0.2159	120	0.4211	0.1619
96	0.0715	0.2926	144	0.2294	0.2158
(R4) (h)	Acetate mg/ml	Formate mg/ml	(R5) (h)	Acetate mg/ml	Formate mg/ml
0	2.1146	0	0	2.1146	0
48	0	0.2305	48	0.579	0,1035
96	0.2916	0.2799	96	0	0,3052
146	0	0.3658	120	0	0,3983
164	0	0.3319	144	0	0,3369

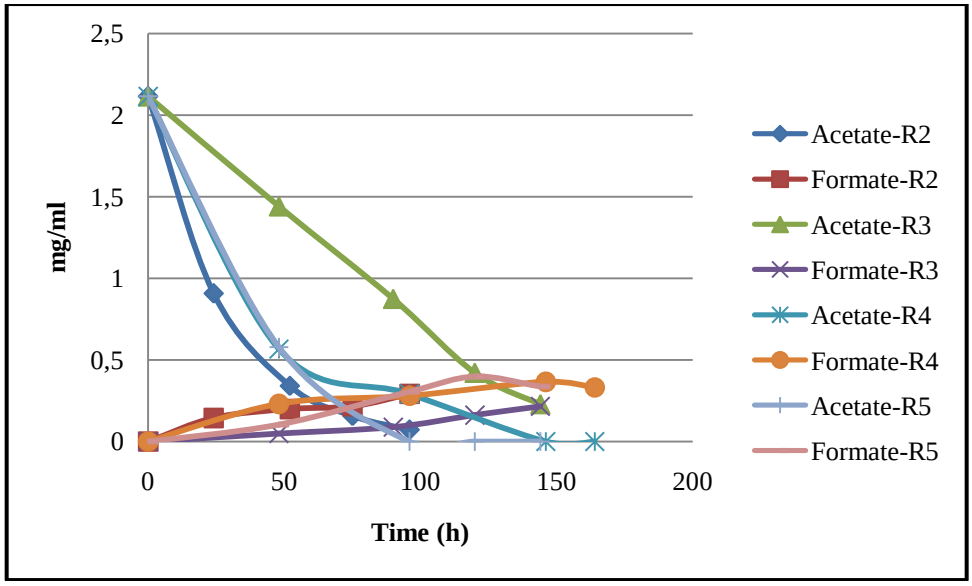

Figure B. 1 Acetate and formate concentrations during hydrogen production by *Rhodobacter capsulatus* YO3 immobilized in 3% agar

4% agar
4% agar R1
Acetate concentration (nd)
Formate concentration (0.2397 mg/ml)

Table B. 2 Acetate and formate concentrations during hydrogen production by *Rhodobacter capsulatus* YO3 immobilized in 4% agar

(R2) Time	Acetate mg/ml	Formate mg/ml	(R3) Time	Acetate mg/ml	Formate mg/ml
0	2.1146	0	0	2.1146	0
24	0.9883	0.1051	48	0.6837	0.1277
52	0.5280	0.1732	90	0.1152	0.2276
72	0.2775	0.2184	144	0	0.3221
96	0.03068	0.2463			
(R4) Time	Acetate mg/ml	Formate mg/ml	(R5) Time	Acetate mg/ml	Formate mg/ml
0	2.1146	0	0	2.1146	0
48	0.633	0.1395	48	0.6683	0,1103
90	0	0.2760	96	0.1664	0,2978
144	0	0.3033	120	0	0,3668
			144	0	0,4235

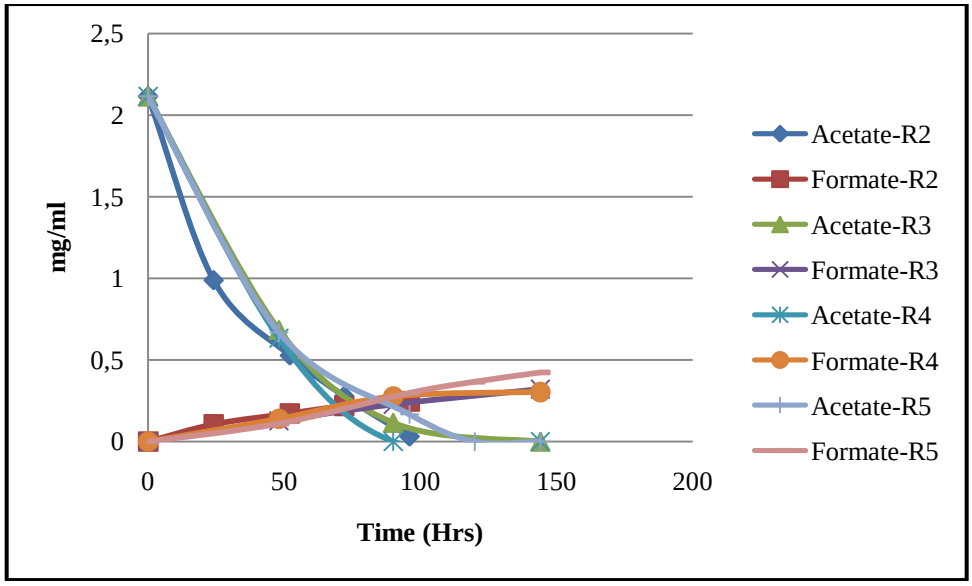

Figure B. 2 Acetate and formate concentrations during hydrogen production by *Rhodobacter capsulatus* YO3 immobilized in 4% agar

5% agar
5% agar R1
Acetate concentration (nd)
Formate concentration (0.2405 mg/ml)

Table B. 3 Acetate and formate concentrations during hydrogen production by *Rhodobacter capsulatus* YO3 immobilized in 5% agar

Time (h)	R2		R3		R4		R5	
	Acetate mg/ml	Formate mg/ml	Acetate mg/ml	Formate mg/ml	Acetate mg/ml	Formate mg/ml	Acetate mg/ml	Formate mg/ml
0	2.1146	0	2.1146	0	2.1146	0	2.1146	0
48	0.8589	0.216	1.0599	0.2114	1.1023	0.1814	0.8612	0.0969
96	0.7371	0.3061	0.7585	0.4374	0.7514	0.3022	0.3101	0.3291
120	0.5155	0.3135	0.5973	0.6365	0.6754	0.6183	0	0.4418
144	0.4935	0.3105	0.3498	0.4253	0.5481	0.3271	0	0.4342

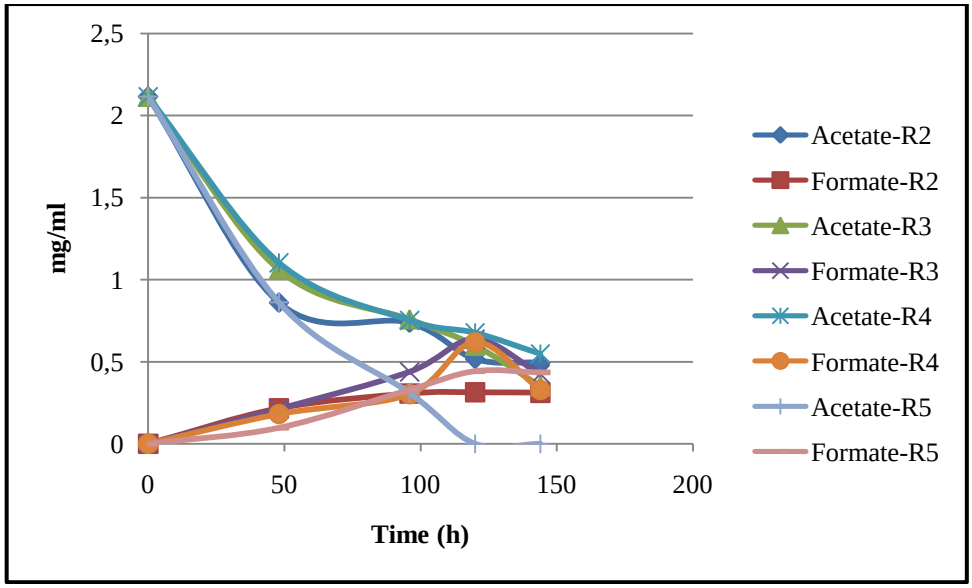

Figure B. 3 Acetate and formate concentrations during hydrogen production by *Rhodobacter capsulatus* YO3 immobilized in 5% agar

6% agar
6% agar R1
Acetate concentration (0.0235 mg/ml)
Formate concentration (0.2667 mg/ml)

Table B. 4 Acetate and formate concentrations during hydrogen production by *Rhodobacter capsulatus* YO3 immobilized in 6% agar

(R2) Time (h)	Acetate mg/ml	Formate mg/ml	(R3) Time (h)	Acetate mg/ml	Formate mg/ml
0	2.1146	0	0	2.1146	0
24	1.0609	0.1288	48	0.5733	0.1186
52	0.3787	0.1874	90	0	0.2086
72	n.d	0.2608	144	0	0.2638
96	n.d	0.2565			
(R4) Time (h)	Acetate mg/ml	Formate mg/ml	(R5) Time (h)	Acetate mg/ml	Formate mg/ml
0	2.1146	0	0	2.1146	0
48	0.579	0.1389	48	0.9828	0,0962
96	0	0.2409	96	0.2453	0,2841
			120	0	0,4019
			144	0	0,4446

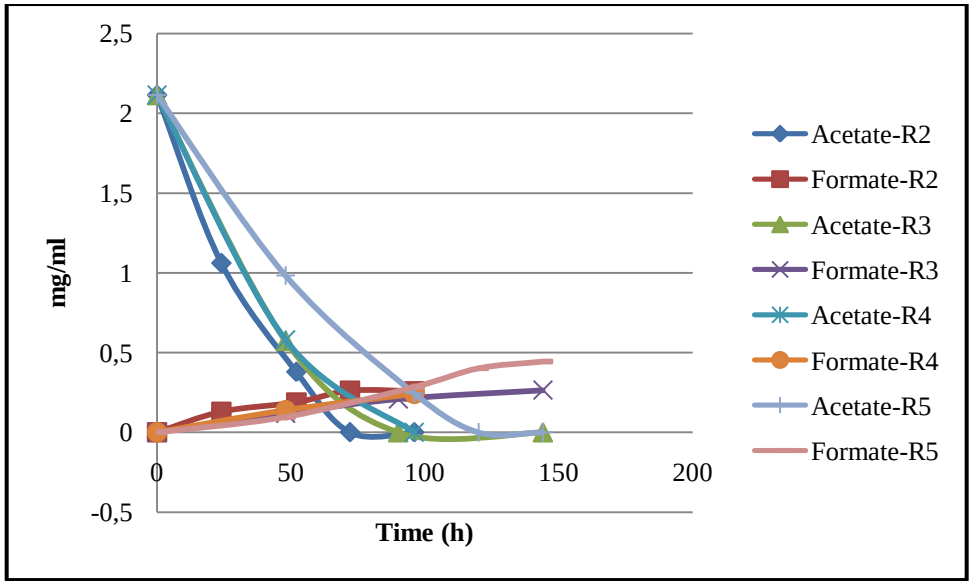

Figure B. 4 Acetate and formate concentrations during hydrogen production by *Rhodobacter capsulatus* YO3 immobilized in 6% agar DSM 1710 experiment, agar gilled with growth medium 3% agar agar

Table B. 5 Acetate and formate concentrations during hydrogen production by *Rhodobacter capsulatus* DSM1710 immobilized in 3% agar

(R1) Time	Acetate mg/ml	Formate mg/ml	(R2) Time	Acetate mg/ml	Formate mg/ml
0	2.1146	0	0	2.1146	0
44	0.9806	0.2983	44	1.1132	0.2622
96	0.3481	0.4201	96	0.7149	0.6022
120	0	0.6243	120	0.4814	0.5465
170	0	0.6295	170	0.1278	0.7289
(R3) Time	Acetate mg/ml	Formate mg/ml	(R4) Time	Acetate mg/ml	Formate mg/ml
0	2.1146	0	0	2.1146	0
48	1.312	0.2091	44	1.6378	0.2910
96	0.9800	0.4121	96	1.2340	0.4723
144	0.8829	0.5182	144	0.8624	0.6472
192	0.6685	0.5985	192	0.7702	0.6745

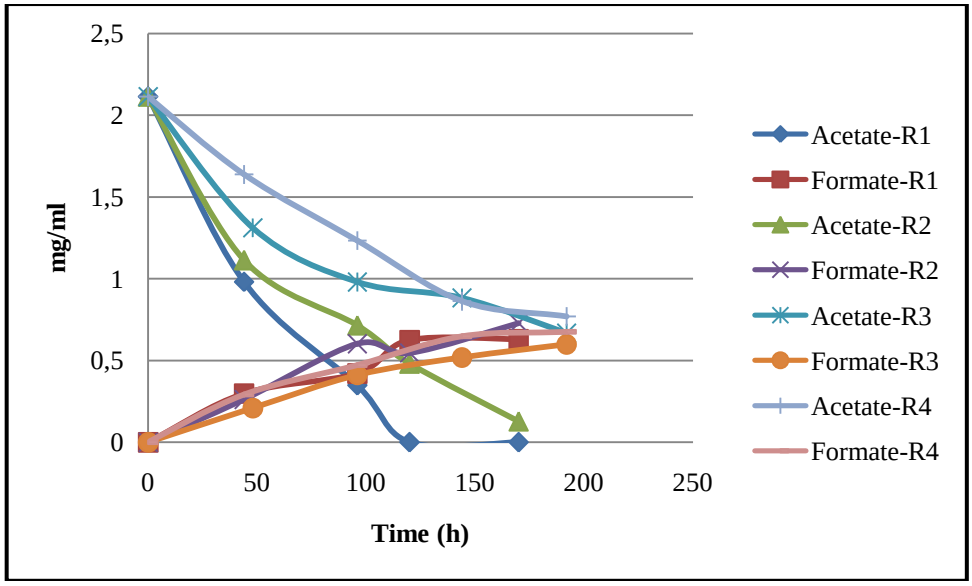

Figure B. 5 Acetate and formate concentrations during hydrogen production by *Rhodobacter capsulatus* DSM1710 immobilized in 3% agar

Table B. 6 Acetate and formate concentrations during hydrogen production by *Rhodobacter capsulatus* DSM1710 immobilized in 3% agar

(R5) Time (h)	Acetate mg/ml	Formate mg/ml	(R6) Time (h)	Acetate mg/ml	Formate mg/ml	(R7) Time (h)	Acetate mg/ml	Formate mg/ml
0	2.1146	0	0	2.1146	0	0	2.1146	0
48	1.2944	0.3202	48	1.3864	0.3080	48	0.7060	0.3607
96	0.3646	0.6305	96	0.7086	0.4947	96	0.0665	0.5764
144	0.0647	0.8323	144	0.1479	0.7051	144	0	0.7683
			192	0.0675	0.8649			

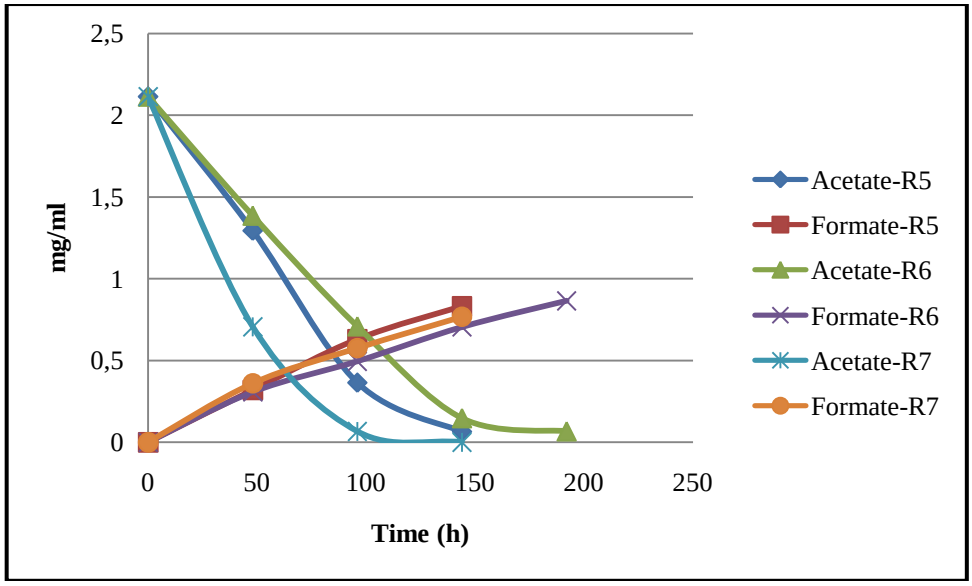

Figure B. 6 Acetate and formate concentrations during hydrogen production by *Rhodobacter capsulatus* DSM1710 immobilized in 3% agar

Table B. 7 Acetate and formate concentrations during hydrogen production by *Rhodobacter capsulatus* DSM1710 immobilized in 4% agar

(R1) Time (h)	Acetate mg/ml	Formate mg/ml	(R2) Time (h)	Acetate mg/ml	Formate mg/ml
0	2.4899	0	0	2.1146	0
44	0.7293	0.2288	44	1.1656	0.3329
96	0.3451	0.5239	96	0.7817	0.4419
120	0	0.7290	120	0.4584	0.7272
170	0	0.7895	170	0.0818	0.6652
(R3) Time (h)	Acetate mg/ml	Formate mg/ml	(R4) Time (h)	Acetate mg/ml	Formate mg/ml
0	2.1146	0	0	2.1146	0
48	1.6788	0.2025	44	1.4568	0.3042
96	1.2127	0.4060	96	0.7941	0.4550
144	0.4844	0.5392	144	0.3193	0.5181
192	0.3419	0.6871	192	0.2291	0.6832
			240	0.2395	0.7353

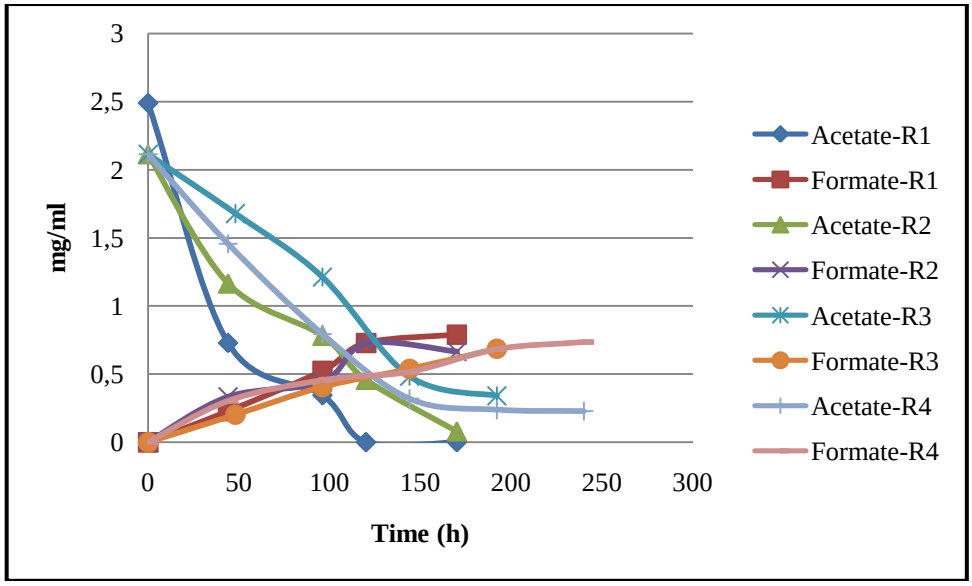

Figure B. 7 Acetate and formate concentrations during hydrogen production by *Rhodobacter capsulatus* DSM1710 immobilized in 4% agar

Table B. 8 Acetate and formate concentrations during hydrogen production by *Rhodobacter capsulatus* DSM1710 immobilized in 4% agar

(R5) Time	Acetate mg/ml	Formate mg/ml	(R6) Time	Acetate mg/ml	Formate mg/ml	(R7) Time	Acetate mg/ml	Formate mg/ml
0	2.1146	0	0	2.1146	0	0	2.1146	0
48	1.0205	0.3298	48	1.0988	0.3129	48	0.6137	0.1555
96	0.3622	0.4626	96	0.3452	0.4649	96	0.0915	0.2708
144	0.0464	0.6758	144	0.0247	0.6419	144	0.0173	0.3641
192	0	0.8123	192	0	0.7582			

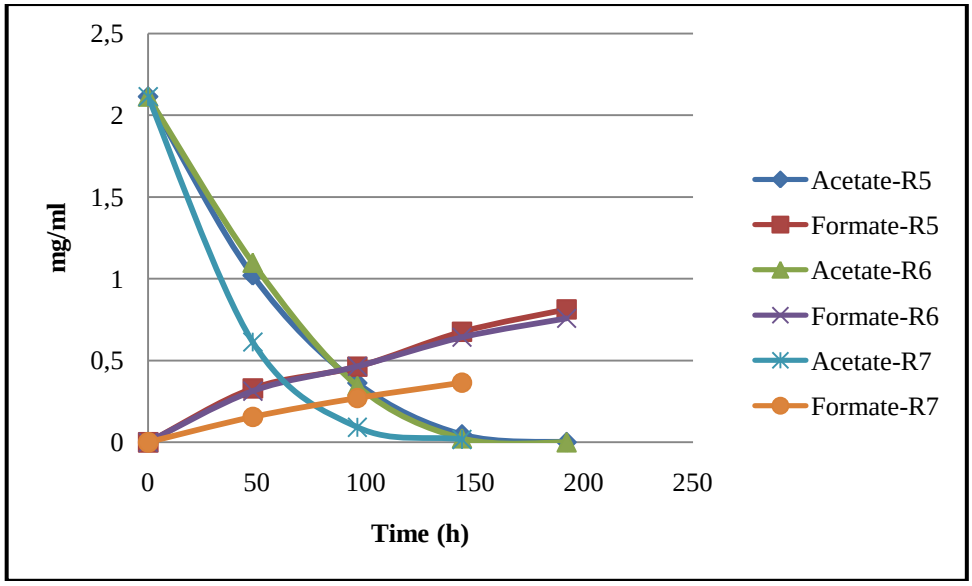

Figure B. 8 Acetate and formate concentrations during hydrogen production by *Rhodobacter capsulatus* DSM1710 immobilized in 4% agar

Table B. 9 Acetate and formate concentrations during hydrogen production by *Rhodobacter capsulatus* DSM1710 immobilized in 5% agar

(R1) Time (h)	Acetate mg/ml	Formate mg/ml	(R2) Time (h)	Acetate mg/ml	Formate mg/ml
0	2.1146	0	0	2.1146	0
44	0.9996	0.1889	44	1.1661	0.2771
96	0.4770	0.4644	96	0.58	0.3988
120	0	0.6294	120	0.2818	0.5570
170	0	0.6874	170	0.0818	0.6666
(R3) Time (h)	Acetate mg/ml	Formate mg/ml	(R4) Time (h)	Acetate mg/ml	Formate mg/ml
0	2.1146	0	0	2.1146	0
48	1.2344	0.2344	44	1.6643	0.3527
96	1.0241	0.4352	96	0.9474	0.5231
144	0.5740	0.5604	144	0.6739	0.7052
192	0.4230	0.8203	192	0.6651	0.7219
			240	0.5374	0.6968

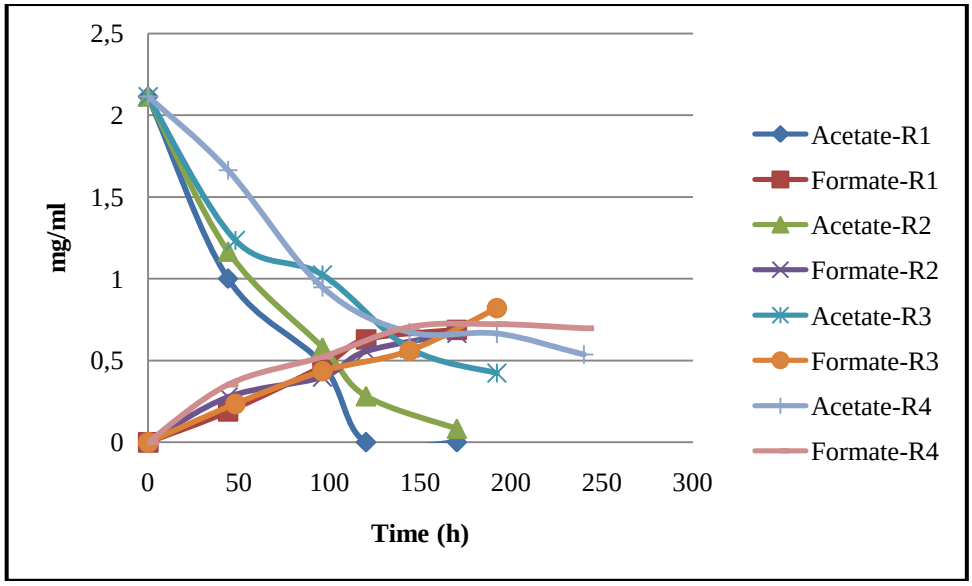

Figure B. 9 Acetate and formate concentrations during hydrogen production by *Rhodobacter capsulatus* DSM1710 immobilized in 5% agar

Table B. 10 Acetate and formate concentrations during hydrogen production by *Rhodobacter capsulatus* DSM1710 immobilized in 5% agar

(R5) Time	Acetate mg/ml	Formate mg/ml	(R6) Time	Acetate mg/ml	Formate mg/ml	(R7) Time	Acetate mg/ml	Formate mg/ml
0	2.1146	0	0	2.1146	0	0	2.1146	0
48	1.0235	0.3396	48	1.4101	0.2602	48	0.6507	0.1413
96	0.3824	0.4425	96	0.5328	0.4400	96	0.069	0.3070
144	0	0.7341	144	0.0395	0.6698	144	0	0.3582
192	0	0.8364	192	0	0.6814			

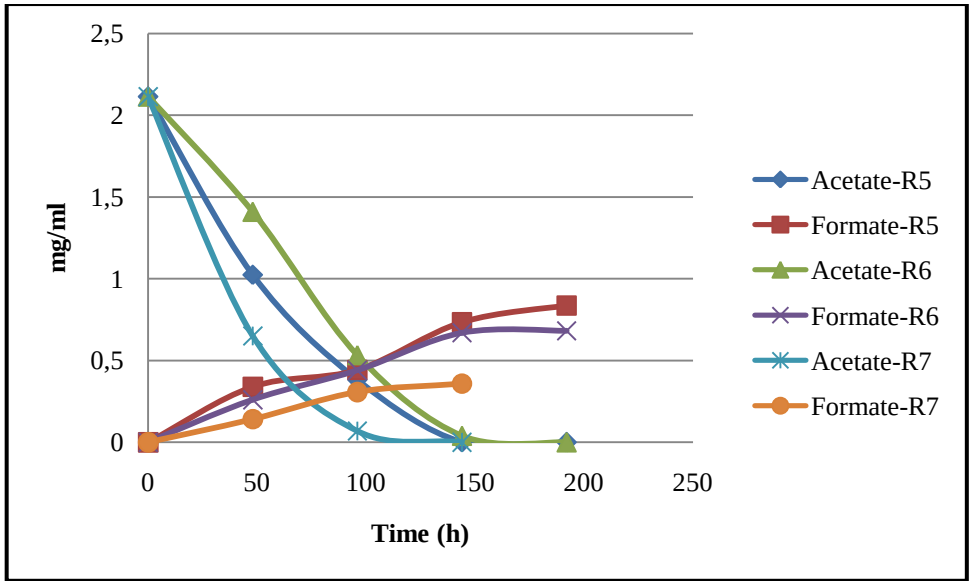

Figure B. 10 Acetate and formate concentrations during hydrogen production by *Rhodobacter capsulatus* DSM1710 immobilized in 5% agar

Table B. 11 Acetate and formate concentrations during hydrogen production by *Rhodobacter capsulatus* DSM1710 immobilized in 6% agar

(R1) Time (h)	Acetate mg/ml	Formate mg/ml	(R2) Time (h)	Acetate mg/ml	Formate mg/ml
0	2.1146	0	0	2.1146	0
44	0.8650	0.1815	44	1.1838	0.2370
96	0.4785	0.4024	96	0.7616	0.3645
120	0.1322	0.6098	120	0.6800	0.5458
170	0	0.6559	170	0.5165	0.703
(R3) Time (h)	Acetate mg/ml	Formate mg/ml	(R4) Time (h)	Acetate mg/ml	Formate mg/ml
0	2.1146	0	0	2.1146	0
48	1.5210	0.2909	44	1.4104	0.3342
96	1.1893	0.5227	96	0.4734	0.7764
144	0.7714	0.7943	144	0.3425	0.8396
192	0.3835	0.9352	192	0.3360	0.7665
			240	0.2944	0.3202

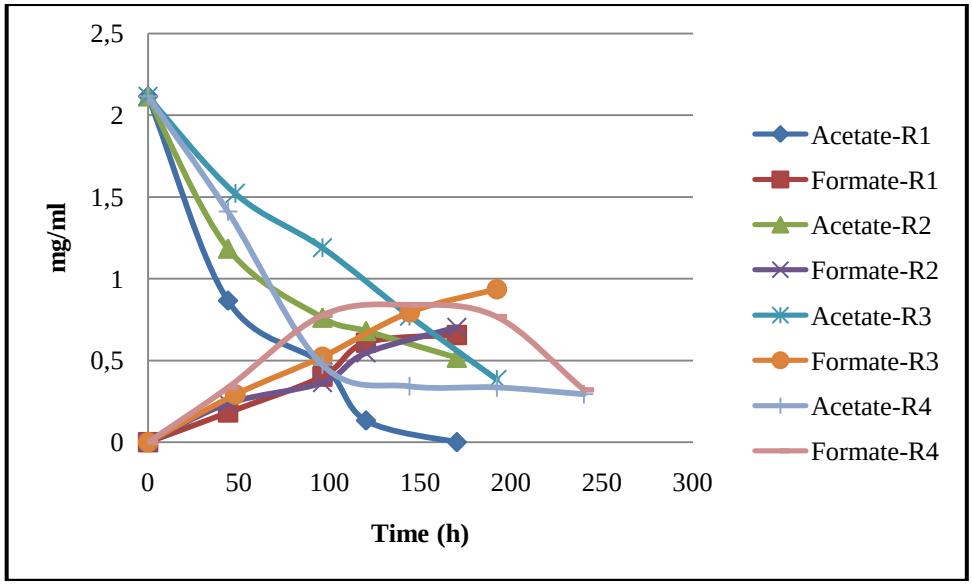

Figure B. 11 Acetate and formate concentrations during hydrogen production by *Rhodobacter capsulatus* DSM1710 immobilized in 6% agar

Table B. 12 Acetate and formate concentrations during hydrogen production by *Rhodobacter capsulatus* DSM1710 immobilized in 6% agar

(R5) Time	Acetate mg/ml	Formate mg/ml	(R6) Time	Acetate mg/ml	Formate mg/ml	(R7) Time	Acetate mg/ml	Formate mg/ml
0	2.1146	0	0	2.1146	0	0	2.1146	0
48	0.9889	0.3517	48	1.3179	0.2464	48	0.6723	0.2574
96	0.4069	0.4901	96	0.3728	0.5008	96	0.2474	0.4207
144	0.0742	0.8277	144	0.1787	0.5273	144	0.2293	0.5613
192	0.0644	0.8009	192	0.1277	0.6170			

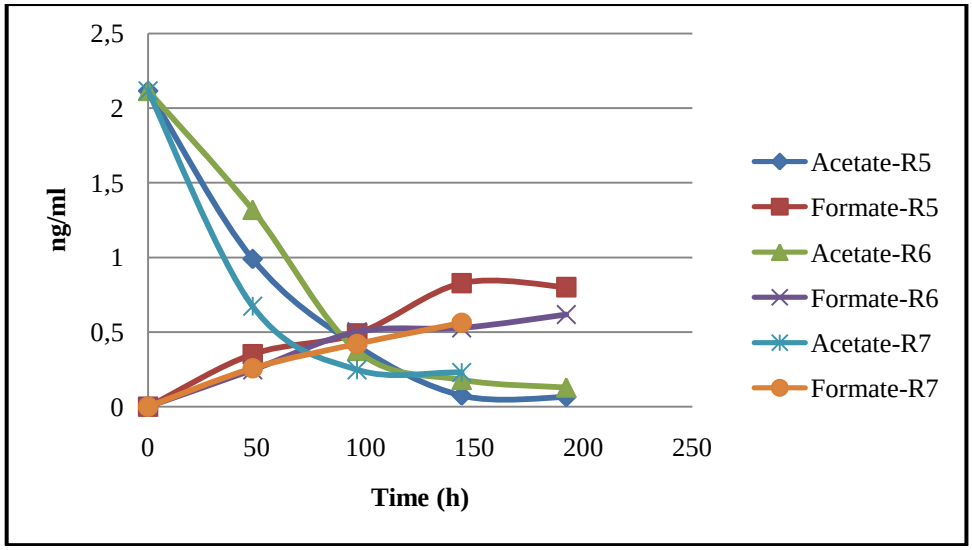

Figure B. 12 Acetate and formate concentrations during hydrogen production by *Rhodobacter capsulatus* DSM1710 immobilized in 6% agar

Table B. 13 Final aceate and formate concentrations duringhydrogen production by immobilized bacteria at 5 mg DCW/ml fed with different acetate concentrations

Rhodobacter capsulatus DSM 1710						
Round	Initial Acetate conc. 60 mM		Initial Acetate conc. 80 mM		Initial Acetate conc. 1000 mM	
	Ac. mg/ml	Form. mg/ml	Ac. mg/ml	Form. mg/ml	Ac. mg/ml	Form. mg/ml
R1-1	0.24	0.59	0.51	0.31	0.85	0.23
R2-1	0.11	0.36	0.72	0.07	2.15	0.09
R3-1	0.23	0.57	1.3	0.22	1.75	0.14
R1-2	0	1.01	0.14	0.86	1.48	0.43
R2-2	0	0.99	0.91	0.47	1.85	0.26
R3-2	0	0.89	1.59	0.29	2.2	0.23
Rhodobacter capsulatus YO3						
Round	Initial Acetate conc. 60 mM		Initial Acetate conc. 80 mM		Initial Acetate conc. 1000 mM	
	Ac. mg/ml	Form. mg/ml	Ac. mg/ml	Form. mg/ml	Ac. mg/ml	Form. mg/ml
R1-1	0.21	0.25	0.02	0.34	0	0.45
R2-1	0	0.31	0	0.87	0	1.4
R3-1	0.05	0.39	0.3	0.65	1.93	1.0
R1-2	0	0.27	0	0.48	0	0.63
R2-2	0	0.61	0	0.54	0	1.4
R3-2	0	0.64	0	0.82	0.2	1.5

Table B. 14 Final aceate and formate concentrations duringhydrogen production by immobilized bacteria at 2.5 mg DCW/ml fed with different acetate concentrations

Rhodobacter capsulatus DSM 1710						
Round	Initial Acetate conc. 60 mM		Initial Acetate conc. 80 mM		Initial Acetate conc. 1000 mM	
	Ac. mg/ml	Form. mg/ml	Ac. mg/ml	Form. mg/ml	Ac. mg/ml	Form. mg/ml
R1-1	0	0.22	0.02	0.13	0	0.43
R2-1	0	0.34	0.45	0.15	0.51	0.1
R3-1	0.03	0.34	0.06	0.21	0.4	0.23
R1-2	0.06	0.46	0.63	0.2	1.14	0.38
R2-2	0.97	0.19	1.27	0.22	1.45	0.13
R3-2	0.54	0.14	0.77	0.09	1.41	0.06
Rhodobacter capsulatus YO3						
Round	Initial Acetate conc. 60 mM		Initial Acetate conc. 80 mM		Initial Acetate conc. 1000 mM	
	Ac. mg/ml	Form. mg/ml	Ac. mg/ml	Form. mg/ml	Ac. mg/ml	Form. mg/ml
R1-1	0	0.26	0.16	0.32	0.49	0.34
R2-1	0.03	0.34	0.08	0.56	1.49	0.47
R3-1	0.07	0.45	0.09	0.45	0.32	0.56
R1-2	0.15	0.43	0	1.16	0.52	0.65
R2-2	0.55	0.08	0.70	0.36	1.0	0.48
R3-2	0.45	0.45	1.28	0.31	2.15	0.17

Table B. 15 Final aceate and formate concentrations duringhydrogen production by immobilized bacteria manipulated with 2.5 and 5% glycerol

Rhodobacter capsulatus DSM 1710			*Rhodobacter capsulatus* YO3		
Round	Ac. mg/ml	Form. mg/ml	Round	Ac. mg/ml	Form. mg/ml
2.5R1-1	0	1.1	2.5R1-1	0.31	0.46
2.5R2-1	0.07	0.96	2.5R2-1	0	0.80
2.5R2-1	0.06	0.68	2.5R2-1	0	0.72
2.5R2-2	0.64	0.57	2.5R2-2	0	0.55
2.5R3-1	0.35	0.40	2.5R3-1	0	1.04
2.5R3-2	0	0.83	2.5R3-2	0	0.71
5R1-1	0.69	0.47	5R1-1	0	0.76
5R2-1	0.89	0.78	5R2-1	0	0.86
5R3-1	0.20	0.98	5R3-1	0	1.oo

APPENDIX C

C. FORMULATIONS OF GROWTH MEDIA

Table C. 1 Growth Medium and Hydrogen Production Nutrient medium formulation

Minimal medium of Bieble and Pfennig (growth medium) 20/10		Nutrient medium for hydrogen production. 40/2	
Composition	g/L	Composition	g/L
KH_2PO_4	0.5	KH_2PO_4	0.5
$MgSO_4.7H_2O$	0.2	$MgSO_4.7H_2O$	0.2
Sodium glutamate (10 mM)	1.8	Sodium glutamate (2 mM)	0.36*
$CaCl_2.2H_2O$	0.05	$CaCl_2.2H_2O$	0.05
Acetic acid (20 mM)	1.15	Acetic acid (40 Mm)	2.29**
Vitamin solution	1 ml.	Vitamin solution	1 ml.
Trace element solution SL 7	1 ml.	Trace element solution SL 7	1 ml.
Fe-citrate solution	5 ml.	Fe-citrate solution	5 ml.

*For preparation of 4mM glutamate the amount of sodium glutamate described in the above table was doubled to 0.72 g/L.

** For preparation of 60 mM of acetate, 1.145 ml of acetate was used,for 80 mM acetate 4.58 ml acetate was used and for 100 mM acetate 5.725 ml of acetate was used

Table C. 2 Composition of trace elements solution Trace Element Solution

Composition	mg/L
HCl (25 % v/v)	1 ml.
$ZnCl_2$	70
$MnCl_2 . 4H_2O$	100
H_3BO_3	60
$COCl_2. 6H_2O$	200
$CuCl_2. 2H_2O$	20
$NiCl_2. 6H_2O$	20
NaMoO4 . 2H2O	40
Fe-citrate	1000

The ingredients of the solution was dissolved in 1000 ml. distilled water and sterilized by autoclaving.

Table C. 3 Vitamins solution composition

Composition	μg/L
Thiamine	500
Niacin	500
Biotin	15

Table C. 4 Growth medium of Halobacterium halobium

Composition	g/L
NaCl	250
MgSO4. 2H2O	20
trisodium citrate 5.5 H2O	3.64 g
KCl	2
Bacteriological peptone L37	10
pH 7	
Basal Medium no peptone in use	

Table C. 5 Chemicals and suppliers

KH_2PO_4, NaCl, $MgSO_4$.7H2O	MERCK	Sodium chloride	MERCK
NH_4Cl	MERCK	$MgSO_4.7H_2O$	MERCK
$CaCl_2.2H_2O$	MERCK	NaCl	MERK
Acetic acid	MERCK	Potassium Chloride	MERK
Yeast Extract	OXOID	Bacteriological Peptone	OXOID
Fe-citrate	MERCK	Bacteriological agar	OXIOD
HCl	MERCK	$ZnCl_2$	MERK
Thiamine, Niacin, Biotin	SIGMA	$MnCl_2$. $4H_2O$	MERK
NaOH	MERCK		
$ZnCl_2$, $MnCl_2.4H_2O$	MERCK		
H_3BO_3	MERCK		
$CoCl_2.6H_2O$	MERCK		
$CuCl_2.2H_2O$	MERCK		
Ni $Cl_2.6H_2O$	MERCK		
$NaMoO_4.2H_2O$	MERCK		

APPENDIX D

D. SAMPLE CALCULATIONS FOR HYDROGEN PRODUCTION

In this section, the calculation procedure for hydrogen productivity, substrate conversion efficiency (yield) and light conversion efficiency is shown. To illustrate, 60 mM/4 mM Ac/Glu feed will be used. The culture volume is 200 ml and 1045 m l of hydrogen gas was produced.

Hydrogen Productivity

This is the rate at which hydrogen gas is produced by the bacterial culture. It can be expressed in terms of the volume or moles of hydrogen gas produced.

Volumetric Productivity

The volumetric hydrogen productivity is the rate at which hydrogen is produced in terms of volume. It is expressed as ml H2/l/h.

Volumetric H_2 Productivity = Volumue of produced H_2(ml)/Volume of culture (ml).Time

Volume of hydrogen produced = $(V_{final} - V_{initial}) \times Y_{H2}$

where V is the volume (ml) of the total gas in the collection column and y is the mole fraction of hydrogen in the total gas produced.

A sample calculation for the volumetric productivity using effect of cell concentration on hydrogen production by YO3 strain fed with 60 mM/ 4 mM Ac/Glu which are shown during R1, Table A. 35 and introduced in section 4.3.2.

Volumetric Hydrogen Productivity = 1045×1000 ml.H_2/200 ml × 92 h = 56.8 ml.H_2/L/h

Molar Productivity

This is the rate at which hydrogen gas is produced in terms of moles. It is expressed as mmol H2/lc.h. It determining the molar productivity, several assumptions are made. They are:

1. The total gas collected obeys the ideal gas law.
2. There is no leakage from or into the gas collection column and the photobioreactor.
3. The atmospheric pressure is constant at 687 mmHg.

Using the fist assumption, the moles of hydrogen produced can be determined using the ideal gas equation.

PV = NRT

Equation can be rearranged as:

N = CV where C= P/RT is a conversion factor (concentration)

The conversion factor C was determined at the temperature range experienced in the outdoor conditions. The values obtained considering Ankara's atmospheric pressure 687 mmHg (Berktaş and Bircan, 2003) and at 760 mmHg are shown in Table I1.

Table D. 1 Molar productivities conversion factor

T (°C)	C(mmol/ml) 687 mmHg	C(mmol/ml) 760 mmHg
5	0.040	0.044
10	0.039	0.043
15	0.038	0.042
20	0.038	0.042
25	0.037	0.041
30	0.037	0.040
35	0.036	0.040
40	0.035	0.039
45	0.035	0.038

It is observed that the conversion factor remains more or less the same at 0.04 mmol/ml. The molar productivities were determined using the conversion factor (C= P/RT) 0.0445 mmol/ml.
Molar Productivity (N) = Conversion Factor ×Volumetric Productivity (I1.5)
A sample calculation for the molar productivity using data for the 60 mM/ 4 mM Ac/Glu fed immobilized bacteria in cell culture bottles is shown.

Molar Productivity = 0.0445 × mmol/ml × 56.8 ml/L.h = 2.53 mmol.H_2/L.h

Substrate Conversion Efficiency (Yield)

The substrate conversion efficiency (yield) determines how effectively the substrate (organic acid) fed to the bacterial culture is used in hydrogen production. It is the ratio of the amount hydrogen produced to the amount of theoretical hydrogen produced from the given carbon sources. Acetate and glutamate were the two carbon sources used. Shown in Equation (I2) is the substrate conversion (yield) determination.
Substrate conversion efficiency
= (Actual moles of H_2 produced/Theoretical moles of H_2 produced) × 100

Acetate Conversion

Acetate conversion efficiency
= $\left(\{\text{Acetate input (mM)} - \text{Acetate output (mM)}\}/\text{Acetate input}\right) \times 100$

Light Conversion Efficiency

where ρH2 is the density of the produced hydrogen gas in g/l, VH2 is the volume of produced hydrogen gas in l, I is the light intensity in W/m2, A is the irradiated area in m2 and t is the duration of hydrogen production in hours.

Light Conversion Efficiency (η) = $(33.61 \times \rho_{H2} \times V_{H2})/(1 \times A \times t)$

APPENDIX E

E. SAMPLE GC ANALYSIS OUTPUT

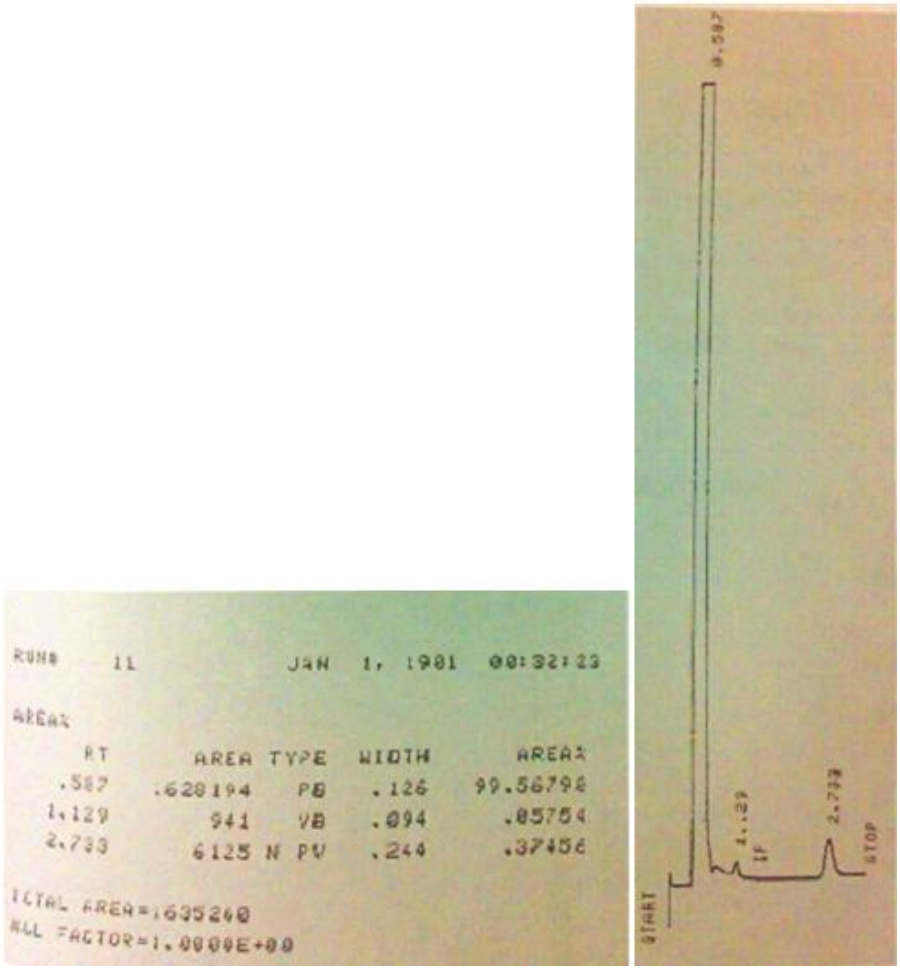

Figure E. 1 A sample GC output

CURRICULUM VITAE

PERSONAL INFORMATION

Surname, Name: Elkahlout E. M., Kamal

Nationality: Palestinian (Palestine)

Date and Place of Birth: 24 July 1967, Palestine-Jabalia

Marital Status: Married with seven children

Phone: 00905069852370

email: kelkahlout@gmail.com

EDUCATION

Degree	Instituation	Year of Graduation
Ph.D.	METU Biotechnology	2011
M.Sc.:	METU Biotechnology	2002
B.Sc.:	The Islamic University of Gaza (IUG), Palestine	1992
High School:	Al-Faloja High School, Jabalia - Palestine	1985

WORK EXPERIENCE

Year	Place	Enrolment
1994-1999	The Islamic University of Gaza (IUG), Department of Biology	Teaching Assistant
2002-2003	The Islamic University of Gaza (IUG), Department of Biolo	Acting Vice-Chairman
2002-2005	The Islamic University of Gaza (IUG), Department of Biology	Lecturer

LANGUAGES

Arabic (Native),

English (Fluent),

Hebrew (Fluent),

Turkish (Fair)

PUBLICATIONS

Zabut B., El-Kahlout K., Yücel M., Gündüz U., Türker L.,Eroğlu İ. 2006 "Hydrogen gas production by combined systems of *Rhodobacter sphaeroides* O.U.001 and *Halobacterium salinarum* in a photobioreactor" International Journal of Hydrogen Energy, 31, 1553-1562

Bashiti A. T., El-Kahlout K., Majed Sh. Hammad Sh. M., Zabut M. B., 2009 "Baker's Yeast Production from Cactus Opuntia Cladodes Extract: Optimization of pH and Temperature" The Islamic University Journal (Series of Natural Studies and Engineering), 17 (2) , 11-27

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Yücel M., El-Kahlout K.E., Zabut, B.M., Gündüz, U., Türker L., Eroglu, I., "Effect of Bacteriorhodopsin on Hydrogen Gas Production by Rhodobacter Sphaeroides O.U.001 in a Photobioreactor" "COST-841 Working Group-3 Workshop on Biosynthesis and Regulation of Hydrogenases", , (2002), p.67.

Zabut B., El-Kahlout K., Yücel M., Gündüz U., Türker L.,Eroğlu İ. 2005 " HYDROGEN GAS PRODUCTION BY COUPLED SYSTEMS OF RHODOBACTER SPHAEROIDES O.U.001 and HALOBACTERIUM SALINARUM IN A PHOTOBIOREACTOR" Proceedings

International Hydrogen Energy Congress and Exhibition IHEC 2005 Istanbul, Turkey, 13-15 July 2005.

Elkahlout K., Eroğlu I., Gündüz U., Yücel U., 2010 "HYDROGEN PRODUCTION BY PHOTOSYNTHETIC BACTERIA RHODOBACTER CAPSULATUS IMMOBILIZED IN AGAR GEL" 10th International Conference on Clean Energy (ICCE-2010)Famagusta, N. Cyprus, September 15-17, 2010

Elkahlout K., Eroğlu I., GÜNDÜZ T., Yücel M., 2011 "A novel Immobilized System for Hydrogen Production by *Rhodobacter capsulatus* in Panel Photobioreactor" accepted abstract for oral presentation, INTERNATIONAL CONFERENCE ON HYDROGEN PRODUCTION (ICH_2P), JUNE 19-22, 2011, Thessaloniki, GREECE

COURSES & WORKSHOPS

- Attended practical training course on public Health and Sea-shore quality (Aug. 1996) by the British Council – Gaza and the Islamic University of Gaza.
- Attended practical training course on Food Microbiology (July. 1996) by the Islamic University of Gaza -Gaza.
- Attended a training course in PCM, project management cycle and logframe approach (Jan, 2003) by the Islamic University of Gaza.
- Attended practical training course in webpage design (April, 2003) by the Islamic University of Gaza.
- Attended practical training course in webct program (October, 2003) by the Islamic University of Gaza.
- Attended "Workshop On HPLC, Separation Modes, Applications And The Further Systems" held on 25-26 June 2009, in METU Central Laboratory, Molecular Biotechnology R & D Center, Ankara, Turkey.

HOBBIES

Reading, light sports and cultural activities