PHOTOBIOLOGICAL HYDROGEN PRODUCTION FROM SUGAR BEET MOLASSES

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PHOTOBIOLOGICAL HYDROGEN PRODUCTION FROM SUGAR BEET MOLASSES

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

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ABSTRACT

PHOTOBIOLOGICAL HYDROGEN PRODUCTION FROM SUGAR BEET MOLASSES

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The main aim of this study was to investigate biological hydrogen production from sucrose and molasses by purple non-sulphur bacteria (PNS). The hydrogen production capacities of four different PNS bacteria (*Rhodobacter capsulatus* (DSM 1710), *Rhodobacter capsulatus* YO3 (Hup^-), *Rhodopseudomonas palustris* (DSM 127) and *Rhodobacter sphaeroides* O.U.001 (DSM 5864)) were tested on sucrose and molasses. The photobiological hydrogen production were performed in 50 ml and 150 ml small scale photobioreactors, in batch mode. The produced hydrogen

quantities, bacterial growth profiles and pH of the media were recorded through the photobiological hydrogen production processes. Organic acids and sucrose consumption rates were determined by HPLC during the experiments. The maximum hydrogen productivitiy of 0.78 (mmol/l_c.h) and 0.55 (mmol/l_c.h) was obtained by *R. palustris* (DSM 127) on sucrose and molasses, respectively. Secondly, co-cultivation of these bacterial strains was studied. The maximum hydrogen productivity by co-cultivation of *R. sphaeroides* O.U.001 (DSM 5864) and *R. palustris* (DSM 127) was found as 1.0 (mmol/l_c.h).

Keywords: Biological Hydrogen Production, Purple Non-Sulphur Bacteria, Sucrose, Molasses, Photobioreactor

ÖZ

ŞEKER PANCARI MELASINDAN FOTOBİYOLOJİK HİDROJEN ÜRETİMİ

Sağır, Emrah

Yüksek Lisans, Biyokimya Bölümü

Tez Yöneticisi : Prof. Dr. Meral Yücel

Ortak Tez Yöneticisi : Dr. Ebru Özgür

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Bu çalışmanın asıl amacı mor kükürtsüz bakteriler tarafından sükroz ve melastan biyolojik hidrojen üretimini araştırılmasıdır. Dört farklı mor kükürtsüz bakteri'nin (*Rhodobacter capsulatus* (DSM 1710), *Rhodobacter capsulatus* YO3 (Hup^-), *Rhodopseudomonas palustris* (DSM 127) ve *Rhodobacter sphaeroides* O.U.001 (DSM 5864)) sükroz ve melas üzerine hidrojen üretim kapasiteleri test edilmiştir. Fotobiyolojik hidrojen üretimleri 50 ve 150 ml'lik küçük ölçekli fotobiyoreaktörlerde gerçekleştirilmiştir. Fotobiyolojik hidrojen üretimi sürecinde üretilen hidrojen miktarı, büyüme profilleri ve ortamın pH'ı kaydedilmiştir. Deneyler süresince organik asitler ve sükroz tüketim oranları kromatografi (HPLC) ile belirlenmiştir. Maksimum hidrojen üretim hızı *R. palustris* (DSM 127) tarafından sükroz ve melastan sırası ile 0.78 ($mmol/l_c.h$) and 0.55 ($mmol/l_c.h$) olarak elde edilmiştir. İkinci olarak bu bakterilerin birlikte kültürleri çalışılmıştır. Maksimum hidrojen üretim hızı

R. sphaeroides O.U.001 (DSM 5864) and *R. palustris* (DSM 127)' in birlikte oluşturduğu kültür tarafından 1.0 (mmol/l_c.h) olarak bulunmuştur.

Anahtar Kelimeler: Biyolojik Hidrojen Üretimi, Mor Kükürtsüz Bakteriler, Sükroz, Melas, Fotobiyoreaktör

To Memory of My Mother,

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Acetyl-CoA	Acetyl Coenzyme A
ADP	Adenosine di-Phosphate
ATP	Adenosine tri-Phosphate
C	Carbon
C_0	Initial concentration
C_f	Final concentration
COD	Chemical Oxygen Demand
C. saccharolyticus	*Caldicellulosiruptor saccharolyticus*
Fd	Ferredoxin
GC	Gas Chromatography
gdcw	Gram dry cell weight of bacteria
H_2	Hydrogen
H	Hours
HPLC	High Pressure Liquid Chromatography
hup^-	Uptake Hydrogenase Deficient
l	liter
l_c	liter-culture
mM	Millimolar
mmol	Millimole
N	Nitrogen
NAD	Nicotineamid Adenine Dinucleotide
PBR	Photobioreactor
PHB	Poly-β-hydroxy butyrate
p_i	Inorganic Phosphate
PNS	Purple Non-Sulphur
PFL	Pyruvate formate lyase
PFOR	Pyruvate ferredoxin (flavodoxin) oxidoreductase
TCA	Tricarboxylic acid

R. capsulatus	*Rhodobacter capsulatus*
R. palustris	*Rhodopseudomonas palustris*
R. sphaeroides	*Rhodobacter sphaeroides*

CHAPTER 1

INTRODUCTION

Nowadays, the depletion of fossil fuels which have limited reserves is a huge problem of the world for energy requirement. As fossil fuels are coming to a shortage, alternative energy sources are searched for substitution of fossil fuels. Hydrogen is accepted as a new renewable energy carrier that can respond for our energy demand. Hydrogen is a clean and renewable fuel for the future energy production. It is the most abundant element in the earth, and has a high content of energy (142 kJ/g) when compared with other sources (Das and Veziroglu., 2001). Advances in hydrogen production avenue are expected by the aid of genetic studies, co-culture studies and reactor designs for immobilized cells to increase the hydrogen yield (McKinlay and Harwood., 2010).

A sustainable energy supply is important for the future populations of human in the world. Many attemps are being realized in the energy sector and studies in this area takes growing attentions in a rapid way. The priorities for alternative production methods are supposed to have a manner of economically low cost and sustainable in a long term hydrogen production. Biohydrogen production from renewable resources, like biomass is considered as one of the most promising systems for future energy requirement. General mechanisms of producing hydrogen and biological hydrogen production methods are given in chapter 1. In the same chapter, photofermentation is explained. The metabolism and characteristics of the photofermentative bacteria is given in detail. The procedures, experimental materials and methods part are examined in chapter 2.

Experimental studies are given in chapter 3 in detail. In the same chapter the results of the experiments and discussions are available. The experimental results, figures, tables and drawings are also present in chapter 3.

Concluding remarks are explained in conclusion part in chapter 5. Then references, appendices and experimental data are available in the last part of the thesis.

1.1 Hydrogen Production Systems

Hydrogen can be produced in two main broads. The method of application depends on the type of source. New green technologies have interest for a long term sustainable hydrogen production by increasing the efficiency of systems. There are essentially two kinds of hydrogen production systems. It may be obtained from conventional or thermochemical processes and non-thermal or renewable sources. These processes are summarized below; (Kothari et al, 2008).

1. Hydrogen from thermochemical production methods

Hydrogen is produced by thermochemical processing of hydrocarbons such as coal , natural gas, wastes, or biomass (Ogden, 1999).

a) Steam Reforming of Methane : The most common method for producing hydrogen is steam reforming of methane. In this process, hydrogen is obtained in three steps; steam reforming, water-gas shift reaction and purification of hydrogen. The steps of the reactions are shown in Equation (1.1) and (1.2).

$$CH_4 + H_2O \Leftrightarrow CO + 3H_2 \quad \text{(steam reforming)} \qquad (1.1)$$

$$CO + H_2O \longrightarrow CO_2 + H_2 \text{ (water-gas shift reaction)} \qquad (1.2)$$

This method is widespread and hydrogen is produced by the reaction of steam with methane. Steam reforming of methane (natural gas) results in the production of carbon monoxide, which is a greenhouse gas, causes global warming (Pilavachi et al, 2009).

b) Partial Oxidation of Hydrocarbons

In partial oxidation of hydrocarbons, there is an exothermic reaction of the hydrocarbons with oxygen and steam at mid-pressure. Equation (1.3) indicates hydrogen production in partial oxidation of hydrocarbons.

$$2C_nH_m + H_2O + 23/2O_2 \longrightarrow nCO_2 + nCO_2 + (m+1)H_2 \qquad (1.3)$$

c) Hydrogen from Water : Splitting of water into hydrogen and oxygen via electrolysis is another common method which hydrogen is produced. Renewable sources may be utilized in this process but it necessitates high amount of electricity.

These conventional methods are fossil fuel dependent and not environmentally friend. They are highly energy intensive and cause greenhouse effect by producing various chemicals in nature.

2. Non-thermal hydrogen production methods

Hydrogen can be produced by nothermal methods such as solar power, wind power and hydrogen power.

a) Solar power: When the water emits energy (285.57 kJ/mole) from UV radiation of the sun, molecular hydrogen is produced. Oxygen is also by-product of the reaction by the solar power.

b) Wind power: Wind power can be used for electricity source in electrolysis to produce hydrogen. As a source, wind turbine generated electricity is the most clean way of producing hydrogen.

c) Hydropower: Hydropower is considered as a source for producing hydrogen via electrolysis. Splitting of water into oxygen and hydrogen is performed by using electricity produced from hydropower.

d) Biological hydrogen production: Biological hydrogen production is an opportunity as an alternative, less energy intensive method since renewable sources can be widely used as raw materials. The concept of biological hydrogen production comprises a broad range of microorganisms such as algae, cyanobacteria, anaerobic and photosynthetic bacteria (Uyar, 2008).

1.2 Biological Hydrogen Processes

Biological hydrogen production are carried out by the utilization of fermentative bacteria, photosynthetic bacteria, algae and cyanobacteria under special conditions (Nandi and Sengupta, 1998). The biological hydrogen production can be categorized as below; (Das and Veziroğlu, 2001).

a. Biophotolysis of water using algae and cyanobacteria.

b. Fermentative hydrogen production from organic compounds.

c. Photodecomposition of organic compounds by photosynthetic bacteria.

d. Hybrid systems using photosynthetic and fermentative bacteria.

1.2.1 Biophotolysis

Biophotolysis is the photo-evolution of hydrogen and oxygen gases by the splitting of water in biological systems under light. Equation 1.4 shows the biophotolysis reaction. The biophotolysis is performed by algae and cyanobacteria at anaerobic conditions (Gaffron and Rubin, 1992).

$$H_2O + \text{light} \rightarrow H_2 + {}^1/_2\, O_2 \qquad (1.4)$$

Although biophotolysis is a direct and clean energy method for hydrogen production, it seems that this method has some barriers and drawbacks. Hydrogen production efficiency is low and the produced oxygen affects and inhibits nitrogenase and hydrogenase enzymes which are directly involved in biophotolysis reaction (Yu and Takahashi, 2007).

1.2.2 Dark Fermentation

Dark fermentative bacteria are able to oxidize organic substrates during the heterotrophic growth in the absence of light. Pyruvate, a product of the glycolysis pathway, is oxidized to acetyl-CoA. The formation of acetyl-CoA entails the reduction of ferredoxin (Fd). Molecular hydrogen is produced by the action of hydrogenase enzyme by the oxidation of the reduced ferrodoxin (Hallenbeck and Benemann, 2002). Various organic acids can be produced during dark fermentation processes by degradation of biomass or other organic wastes such as sugars like glucose and sucrose.

1) Pyruvate:*formate lyase* (*PFL*)

$$\text{Pyruvate} + \text{CoA} \xrightarrow[\text{PFL}]{} \text{acetyl}-\text{CoA} + \text{formate} \qquad (1.5)$$

2) *Pyruvate:ferredoxin* (*flavodoxin*) *oxidoreductase* (*PFOR*) (1.6)

$$\text{Pyruvate} + \text{CoA} + 2\text{Fd (ox)} \longrightarrow \text{acetyl}-\text{CoA} + CO_2 + 2\text{Fd(red)}$$

Pyruvate is broken down by one of the two enzymatic system which were shown in reactions in (1.5) and (1.6).

Major products of the dark fermentation are small molecular weight organic compounds such as acetic acid, butyric acid and propionic acid due to incomplete utilization of the substrates in the operation. One of the major drawback of this process is low hydrogen production rate. Sequential dark and photo-fermentation systems can be more efficient in biological hydrogen production than a sole dark fermentative process (Kapdan and Kargi, 2006).

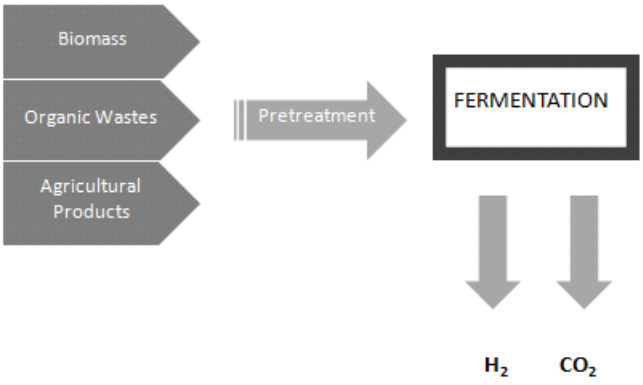


Figure 1.1 Dark fermentative hydrogen production adapted from Hallenbeck and Benemann, (2002).

1.2.3 Photofermentation

Photosynthetic Purple non-sulfur (PNS) bacteria presents a remarkable photoheterotrophic hydrogen production mechanism with their nitrogenase enzyme by reducing organic substances under light energy.

Photobiological hydrogen production by PNS bacteria offers some important advantages over other processes by the aid of photosynthetic bacteria in the processes. Photoheterotrophic bacteria are accepted as the most favorable microorganisms for the biological hydrogen production (Akkerman et al., 2002; Fascetti and Todini, 1995; Miyake and Kawamura, 1987). The advantageous features of photoheterotrophic bacteria for hydrogen production are listed below (Das and Veziroğlu, 2001).

a) They have high conversion yields for hydrogen production,

b) They benefit from the wide range of light spectrum,

c) High consumption ability on different organic substrates or wastes, and

d) Give opportunities in biological waste treatment or prevent environmental pollution.

The major drawback of photoheterotrophic hydrogen production by PNS bacteria is their low production rates compared to dark fermentative process. Besides, major improvements in the bioreactor design are still necessary for the cost-effective photobiological hydrogen production.

This bacteria were also studied for hydrogen production from organic wastes (Eroglu et al., 2004)

Uyar et al., (2009) studied photoproduction of hydrogen by *Rhodobacter capsulatus* and reported that this PNS bacteria is a good candidate for photofermentation and also for a combined dark and photofermentative process.

1.2.4 Integrated Systems

New hydrogen gas production systems may be developed to improve hydrogen yield. Sequential and combined dark and photo-fermentation processes are considered as a promising approach in terms of enhancing the hydrogen production. The effluent which comes from the dark fermentative operation, is aimed for utilization by photofermentative bacteria to produce more hydrogen (Kapdan and Kargi, 2006).

A current example to the integrated hydrogen production system is "HYVOLUTION", which is a EU 6^{th} Framework project, coordinated by Institute of Food and Biobased Research (DLO-FBR), The Netherlands. Hyvolution project intends to generate hydrogen from biomass by first dark fermentation (thermophilic) and then photofermentation processes. In thermophilic fermentation, biomass is utilized by thermophilic microorganisms. Hydrogen, carbondioxide and organic intermediates are produced in thermophilic process and these intermediate products are consumed in the second step by photofermentative bacteria and more hydrogen is produced by the integrated system. About nine moles of hydrogen per mole of hexose is aimed considering, 75% efficiency (Claassen and Vrije, 2006).

Two step processes were also studied using sucrose obtained from different wastes. Tao et al. (2007) reported a two-step dark and photo-fermentation process. Hydrogen rate was raised from 3.67 mol H_2/mol sucrose in dark fermentation to 6.63 mol H_2/mol sucrose by *R. sphaeroides* with a two-step fermentation of sucrose. All of the organic acids produced in dark fermentation is consumed in the photofermentation part of two-step process. Özgür et al. (2010) studied on hydrogen production by sequential dark and photo-fermentation with sucrose from sugar beet molasses as

carbon source. The maximum hydrogen is produced by *C. saccharolyticus* and *R. capsulatus* hup$^-$ mutant strain in dark and photo-fermentation, respectively. They improved the yield of hydrogen from 4.2 mol H_2/mol sucrose in dark fermentation to 13.7 mol H_2/mol sucrose by the sequential process.

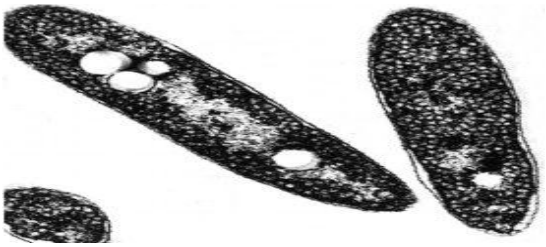
1.3 General Characteristics of Purple Non-sulphur Bacteria

Purple non-sulphur bacteria (PNS) are photosynthetic microorganisms which can grow at near pH 6-9 and a temperature range of 25-35 ^oC (Sasikala et al., 1993). They have ability to grow in various modes such as aerobically chemoautotrophic and chemoheterotrophic or anaerobically phototrophic and photoheterotropic (Koku., 2002).

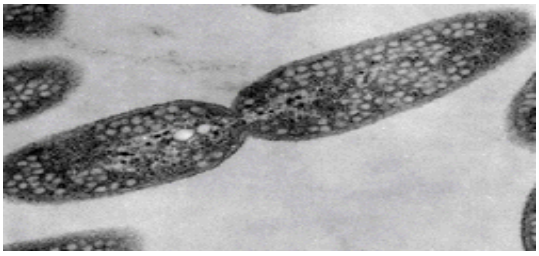
Rhodobacter capsulatus is a gram-negative purple non-sulphur bacterium. It is a rod-shaped photosynthetic microorganism with a cell diameter of 0.5-1.2 μm. This bacteria has a photosynthetic membrane and cell division occurs by binary fission and (Imhoff et al., 1995). Shown in Figure 1.2 is a microscopic image of *Rhodobacter capsulatus.*

Rhodobacter sphaeroides is also a PNS bacterium and can be in various shapes but generally has an ovoid cell shape with a photosynthetic membrane and bacteriochlorophyll *a*. Their cells have a dimension of 0.5 μm wide and 2-2.5 μm long (Pellerin and Gest, 1983).

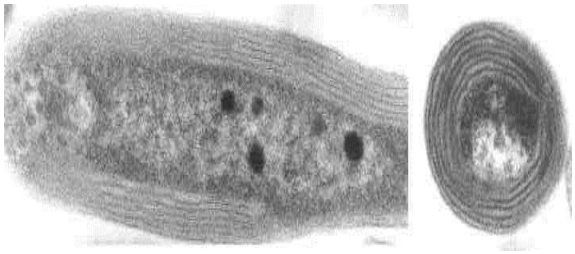
Rhopseudomonas palustris is known as one of the most metabolically suitable organism for biological processes. They are gram-negative bacteria which belong to Bradyrhizobiaceae family and Rhodopseudomonas genus. *R. palustris* is a rod-shaped phototropic bacterium which could reproduce by budding (Whittenbury and McLee., 1967). The photosynthetic reaction center of the Rhodopseudomonas bacteria has bacteriochlorophyll *b* and have a range of different modes (Lang and Oesterhelt, 1989).



(a)



(b)



(c)

Figure 1.2 The microscopic images of PNS bacteria. *R. capsulatus* (a) (from Institut für Molekulare Enzymetechnologie), *R. sphaeroides* (b) (from Institute of Doe Joint Genome), and *R. palustris* (c) (from Dr. Gert-Wieland Kohring, University of Saarland, Germany.

Table 1.1 Classification of hydrogen-generative bacteria (Basak and Das, 2007)

Available energy form	Enzyme for H2	Class of bacteria	Sub-class of bacteria	Genus of bacteria	Electron donor
Photosynthesis	Hydrogenase	Green algae	-	*Chlamydomonas*	Water
				Chlorella	Water
		Cyano-bacteria	Heterocyst	*Anabeana*	Water
			Non-heterocyst	*Oscillataria*	Water
	Nitrogenase	Photo-synthetic	Purple-non-sulfur	*Rhodobacter*	Organic acids
		bacteria	(PNS) bacteria	*Rhodopseudomonas*	Organic acids
			(Facultative anaerobe)	*Rhodospirillum*	Organic acids
			Purple sulfur bacteria	*Chromatium*	Sulfates
				Thiocapsa	Sulfates
Non-photosynthesis	Hydrogenase	Obligate anaerobe	-	*Clostridium*	Sugars
		Facultative anaerobe	-	*Methanobacterium*	Sugars
			-	*Escherichia*	Sugars
			-	*Enterobacter*	Sugars
	Nitrogenase	Nitrogen fixing	Facultative aerobes	*Azotobacter*	Sugars
		bacteria		*Clostridium*	Sugars
			Facultative anaerobes	*Klebsiella*	Sugars

Table 1.2 The classification of the PNS bacteria used in this study (Tabanoğlu, 2002; Uyar, 2008).

Domain	Bacteria	Bacteria	Bacteria
Phylum	Proteobacteria	Proteobacteria	Proteobacteria
Class	Alphaproteobacteria	Alphaproteobacteria	Alphaproteobacteria
Order	Rhodobacterales	Rhodobacteraceae	Rhizobiales
Family	Rhodobacteraceae	Rhodobacteraceae	Bradyrhizobiaceae
Genus	Rhodobacter	Rhodobacter	Rhodopseudomonas
Species	*capsulatus*	*sphaeroides*	*palustris*

R. palustris is commonly found in various places in nature such as water ponds and marine coasts. This bacteria are accepted as one of the most metabolically suitable

microorganism. For gaining energy, they can utilize light, different organic substances or inorganics. Many alternative growth modes are available in *R. palustris.* They are able to grow in the absence or presence of oxygen and have degradative effect on many organic compounds (Larimer et al., 2003).

1.4 Hydrogen Production Metabolism of PNS Bacteria

Hydrogen evolution is carried out by utilization of organic substrates under an illumination in an anaerobic medium. The hydrogen production metabolism of PNS bacteria consists of several main components; the enzyme systems, the carbon flow (TCA cycle) and the photosynthetic membrane apparatus. These components are found in close interaction via exchange of protons, electrons and ATP during metabolic processes in the cell (Koku et al., 2002). Figure 1.3 shows the carbon metabolism of PNS bacteria.

The oxidation of organic subtrates in TCA cycle gives off electrons, protons and carbondioxide. The liberated protons and electrons are then carried to nitrogenase. This transfer is performed by the carrier molecules; nicotinamide adenine dinucleoide (NAD) and Ferredoxin (Vignais et al., 1985). The remaining protons are provided to nitrogenase as the result of ATP synthase activity. Finally, molecular hydrogen is evolved by reduction of protons by nitrogenase (Koku, 2002). Figure 1.4 depicts a part of hydrogen metabolism that occurs on the photosynthetic membrane of PNS bacteria. The overall hydrogen production metabolism is illustrated in Figure 1.5.

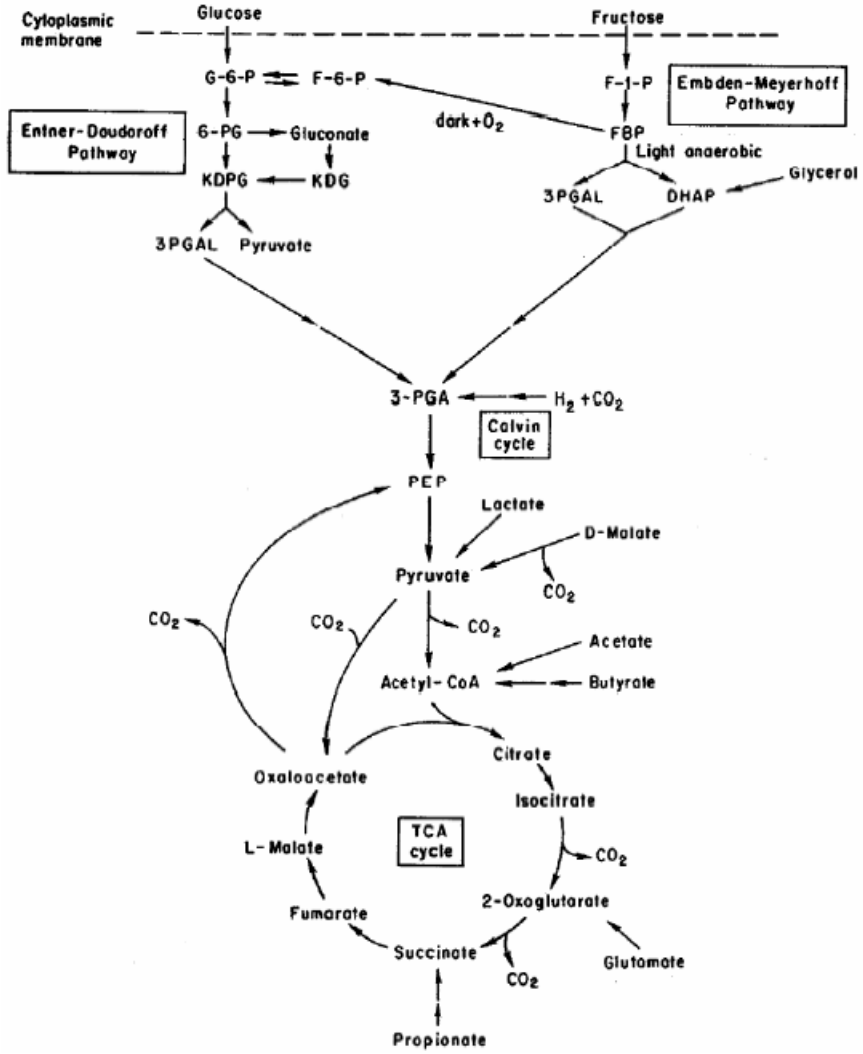


Figure 1.3 A sketch of the carbon metabolism in PNS bacteria (Koku, 2001).

1.4.1 Nitrogenase

Purple non-sulphur bacteria produce hydrogen via their nitrogenase enzymes. Hydrogen production is mostly obtained by nitrogenase activity. There are three different kinds of nitrogenase which have been identified depending on their active center elements. They are molybdenum centered (Mo – nitrogenase), vanadium centered (V – nitrogenase) and iron centered (Fe – nitrogenase). Four ATP is consumed for production of one mole molecular hydrogen in the absence of nitrogen

(Basak and Das, 2007). Equation 1.1 shows the hydrogen production in the absence of nitrogen by PNS bacteria.

When molecular nitrogen is present in environment, nitrogenase reduces di-nitrogen into ammonia as a part of their nitrogen fixation pathway. Equation 1.2 shows nitrogen fixation reaction by nitrogenase activity. Many PNS bacteria have Mo – nitrogenase for hydrogen production (Koku et al., 2002).

$$2H^+ + 2e^- + 4ATP \rightarrow H_2 + 4ADP + 4P_i \quad (1.1)$$

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H2 + 16ADP + 16P_i \quad (1.2)$$

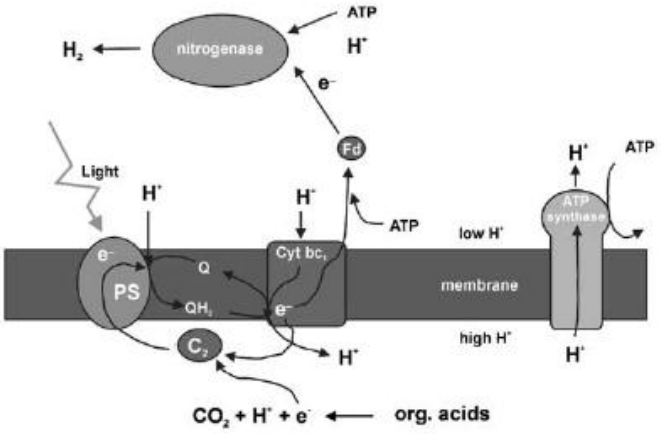


Figure 1.4 Photofermentation by PNS bacteria Akkerman et al. (2002).

1.4.2 Hydrogenase

Hydrogenase is membrane-bound critical enzyme of PNS bacteria. Hydrogenase may both catalyse the production of hydrogen at small amounts and consumption of hydrogen which is produced by their metabolism (Koku et al., 2002). Hydrogenase catalyzes the reversible reaction (1.7) below.

$$H_2 \longleftrightarrow 2H^+ + 2e^- \qquad (1.7)$$

Oxygen and carbon monoxide have inhibitory force on hydrogenase. Although hydrogenases of PNS bacteria generate hydrogen, most of the hydrogen in the cell is produced by the action of nitrogenase. Consumption of hydrogen by the uptake hydrogenase activity could be prevented by mutations on hydrogenase for evolution of more hydrogen from the PNS bacteria. Öztürk et al. (2006) developed a mutant strain, *R. capsulatus* YO3, which was derived from *R. capsulatus* MT 1131 by deleting uptake hydrogenase via interposon mutation and reported 70% hydrogen production efficiency. Using this strain Androga et al (2010) and Avcioglu et al (2011) reported improved hydrogen productions in outdoor panel photobioreactors using defined media and dark fermenter effluent of molasses, respectively.

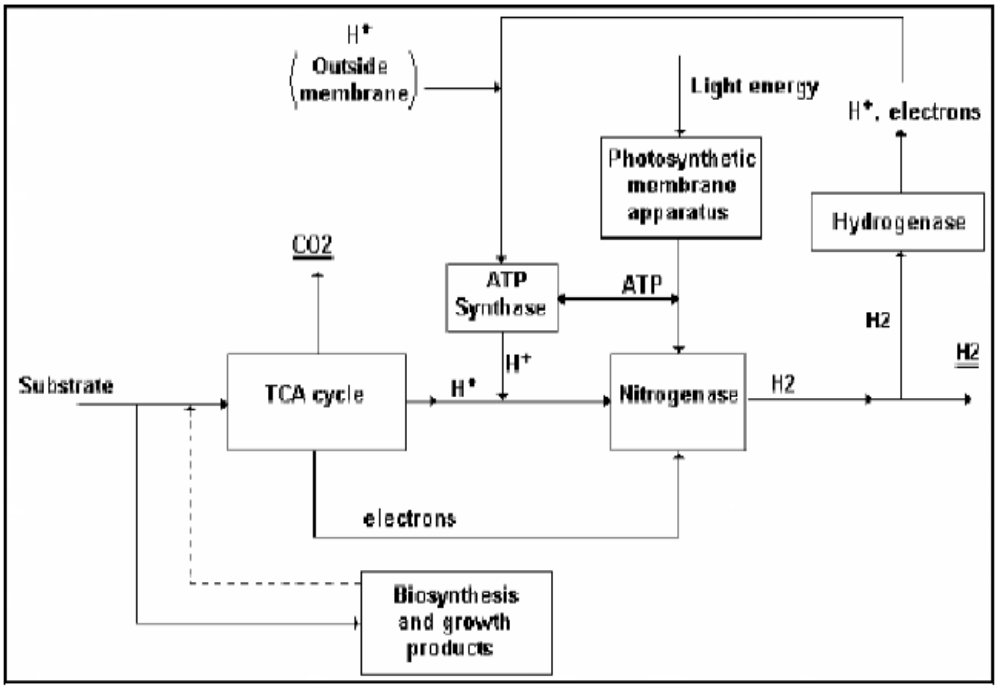


Figure 1.5 Hydrogen production metabolism of PNS bacteria (Koku et al., 2002).

1.4.3 By-Products of Biological Hydrogen Production

PHB is a valuable polymer of the photofermentation by-product. The accumulation of this product in the cell occurs when superfluous carbon and energy present and insufficient nitrogen for growth (Hustede et al., 1993). Poly-β-hydroxybutyrate (PHB) is synthesized for storage of carbon and energy by the bacteria. The accumulation of PHB is affected and determined by the type of substrate and pH of the hydrogen production medium. PHB is considered as an economical by-product since it is a biodegradable polymer. Therefore, this product may be used in different industrial applications (Koku, 2002).

Caratenoid pigments (carotene and xanthophyll) of PNS bacteria is also accepted as a by-product of the hydrogen production process. They protect the bacteria from photoinhibitory effect of the sun and enhance light energy absorption of bacteriochlorophyll which is photosynthetic pigment of photosynthetic bacteria. They have potential usages in medical and food industry (Androga, 2009).

1.5 Molasses as a Feedstock for Hydrogen Production

The biological hydrogen are generated especially by anaerobic dark fermentation, photofermentation and combined systems with microorganisms. Different sources of materials were investigated and used for hydrogen production in those systems. Organic biomass and wastes comprise a major part of the sources since they contain organic substrates. Biomass is a widespread renewable resource for energy production. The biomass is produced by either agricultural or industrial processes. Energy crops such as sugar cane, sugar beet and corn are common plants used in energy production. The conversion of energy from biomass occurs in two ways; thermal or biological methods (Claassen et al., 1999).

Sugar beet molasses is a source of sucrose which can be used as substrate in anaerobic dark and photofermentative hydrogen production processes. Molasses is obtained by repeated crystallization of raw sugar in refineries during the sugar production. They are the final effluents before the refined sugar production. Molasses, the end product in sugar factories, contain of 12-17 % water and 49 % sucrose. Sucrose is predominantly present in molasses rather than other carbohydrates such as glucose, fructose and raffinose (Olbrich, 1963).

Hydrogen production on sucrose was studied abundantly in dark fermentative processes (Chen et al., 2001). The produced compounds in the effluent was used as substrate for the photofermentative process. (Tao et al., 2007; Özgür et al., 2010).

Therefore, sequential production systems could be applied for the maximum utilization of sucrose or other organic compunds.

Bolliger et al. (1985) studied with several photosynthetic non-sulphur bacteria for photoproduction of hydrogen by using a sugar refinery wastewater. They used sugar containing wastewater as carbon source for the bacteria and the yield of produced hydrogen was 0.01 l H_2/h/gdcw with 5% wastewater.

Sunita and Mitra (1993) reported hydrogen evolution by photosynthetic non-sulphur bacteria isolated from a wastewater pond by using different organic compunds as carbon sources. *Rhodopseudomonas sp*. (SM1NSOU) was chosed for hydrogen production since it was the fast-growing bacterial culture. Total hydrogen for 1 lt substrate was, 214 ml for waste water and 290 ml for sewage.

Tanisho and Ishiwata (1994) obtained the maximum hydrogen production rate as 36 mmol-H_2/(l-culture h) by *Enterobacter aerogenes* in a 300 ml fermenter containing 250 ml diluted molasses of which 2% were sugars.

Uchiyama et al. (1996) studied with *Rhodopseudomonas* sp. using effluents from an anaerobic reactor fed with sugar wastewater effluents as carbon sources and obtained the maximum hydrogen rate as 0.126 l H_2/h/gdcw.

Türkarslan et al. (1998) reported photobiological hydrogen production by the wastewater of milk industry with *R. sphaeroides* O.U.001. This bacteria utilized 3-fold diluted wastewater as carbon source and produced 85 ml hydrogen through 90 hours process in a 150 ml cylindirical glass column bioreactor.

Yiğit et al. (1999) studied hydrogen production from a sugar factory waste water using *R. sphaeroides* O.U.001. Hydrogen medium was formed by 30% waste water in 50 ml flasks. The amount of produced hydrogen was 35 ml in 108 hours at 36 ^{o}C and under 200 W m^{-2} illumination.

Yetis et al. (2000) reported photoproduction of hydrogen from a pretreated sugar refinery waste water by *R. sphaeroides* O.U.001 in a column reactor. They obtained maximum hydrogen production as 2.67 l at a dilution rate of 0.0013 h^{-1}.

Sung et al. (2002) studied hydrogen production by anaerobic microbial culture using pure sucrose as substrate. The hydrogen yield was 1.5291 mole H_2/mole sucrose. *Clostridium and Bacillus* species was dominant in the microbial community. They found that pH 5.5 was optimum for the operation in continuous-flow bioreactors.

Hussy et al (2005) studied continuous fermentative hydrogen production from sucrose and sugar beet using anaerobically digested sewage sludge. For refined sucrose, 1.7 ± 0.2 mol/mol hexose were obtained with a nitrogen sparging 2.3 l working volume of CSTR reactor.

Zhang et al. (2005) showed the effect of iron concentrations by mixed bacteria on hydrogen production with sucrose. They obtained the maximum yield as 2.73 mol/mol sucrose at 1600 mg $FeSO_4$ l^{-1}.

Ren et al. (2006) reported the maximum hydrogen production rate of 5.57 m^3H_2/m^3 reactor/d in a continuous flow anaerobic reactor with a volume of 1.48 m^3 for 200 days operation.

Xu et al. (2007) investigated the effects of pre-treatment methods of molasses in anaerobic systems. They reported that yeast extract increased fermentability and hydrogen yield by *Ethanoligenens sp.* B49. The hydrogen yield was 78.97 mmol/l_c. They also showed that molasses of 4 g/l was found as optimum concentration.

Li et al. (2007) studied hydrogen production from diluted molasses by anaerobic bacteria in a 27.48 l effective volume of anaerobic baffled reactor (ABR) for 26 days. The initial pH was adjusted as 6.6 and molasses were diluted to 5000 mg/l COD level. The hydrogen yield was obtained as 32.51 l/day.

Xu et al. (2007) studied hydrogen production from molasses with a fermentative strain, *Ethanoligenens sp.* B49. They reported that feasible COD of molasses was

20.6 g/l and also showed that addition of nitrogen increased hydrogen production. The yield of hydrogen was raised up from 44.82 mmol/l to 78.97 mmol/l by applying optimal concentrations of molasses.

Wang et al. (2009) reported maximum hydrogen production yield of 1.85 mol H_2/mol hexose by *Clostridium butyricum* W5 using 100 g/l molasses at 39 ^oC and pH of 6.5 as initial level in a laboratory scale batch bioreactor with a working volume of 1.5 l.

Özgür et al. (2010) studied on hydrogen production by sequential dark and photo-fermentation with sucrose from sugar beet molasses as carbon source. The yield of hydrogen was increased from 4.2 mol H_2/mol sucrose in dark fermentation by 13.7 mol H_2/mol sucrose by the sequential process.

Li et al. (2011) reported the highest hydrogen production of 11.39 l/d using mixed culture fermentation with molasses wastewater in a CSTR at 35 ^oC.

Özkan et al. (2011) studied photofermentative hydrogen production by dark fermenter effluent of sugar beet thick juice in solar 4 l fed-batch panel photobioreactors using *R. capsulatus* YO3 (Hup^-). The productivity of hydrogen was 1.12 mmol/L_c/h through 15 days of process.

Avcioglu et al. (2011) reported biological hydrogen production using dark fermenter effluent of molasses by PNS bacteria. They achieved maximum hydrogen productivity as 0.50 mmol H_2/L_c.h and 0.67 mmol H_2/L_c.h by *R. capsulatus* DSM 1710 and *R. Capsulatus* YO3 (Hup^-) respectively.

Chu et al. (2011) reported maximum biohydrogen production rate with suspended sludge bioreactor 1.2 l (SSB) and immobilized cell bioreactor (ICB) systems with condensed molasses solubles. The hydrogen rate was 14.04 ± 2.08 for SSB and 7.60 ± 1.05 l/d on 40 g COD/l.

Qu et al. (2012) reported the increase of hydrogen yield from 1.76 l/d to 6.45 l/d in a continuous two-stage (dark-dark) fermentation system from molasses wastewater with two 9.2 l CSTRs.

1.6 Co-cultivation of PNS Bacteria

One of the options for the enhancement of hydrogen productivity is co-cultivation of the microorganisms in hydrogen production medium. Improvement of hydrogen production by the syntrophic interactions between different bacteria are considered as an interesting research area in recent years (Fang et al., 2006). The interaction of the bacteria in the same media has a great importance for the fate of hydrogen production, growth and pH change through the operation time. Co-cultivation was applied for both dark and photofermentative bacteria on glucose as the hydrogen production substrate (Zhu et al., 2001; Miyake et al., 1984).

Co-cultivation of *Rhodobacter sphaeroides* and *Lactobacillus delbrueckii* was applied as co-immobilized cultures and 7.1 mol H_2 was produced per mole of glucose (Asada et al., 2006).

Fang et al. (2006) studied co-culture of *Clostridium butyricum* and *Rhodobacter sphaeroides* on glucose by photofermentation. The FISH method was also shown as an effective method in the quantification of bacteria in co-cultivation studies.

1.7 Scope of The Thesis

The objective of this study was to produce photobiological hydrogen by utilization of sugar beet molasses via direct photofermentation process. Molasses contain high

amount of sucrose, which can be utilized by PNS bacteria for growth and hydrogen production. The raw molasses obtained from Etimesgut Sugar Factory (Ankara, Turkey) was used as substrate after 40, 60 and 80 fold dilutions.

Four different photosynthetic PNS bacteria (*Rhodobacter capsulatus* (DSM 1710), *Rhodobacter capsulatus* YO3 (Hup^-), *Rhodopseudomonas palustris* (DSM 127) and *Rhodobacter sphaeroides* O.U.001 (DSM 5864)) were used for biological hydrogen production.

Studies were carried out starting with adaptation of the PNS bacteria to sucrose. After the adaptation studies, growth and hydrogen production on defined sucrose media were tested. Then, molasses were diluted to certain sucrose concentrations and they were also used for growth and hydrogen production of PNS bacteria.

In addition, hydrogen production was performed by co-cultivation of these bacteria on sucrose from molasses. Modes of pH, growth and hydrogen production of the bacteria in bioreactors were examined and compared in.

The process provided not only a clean fuel, hydrogen, but also contributed cleaning of environment by performing waste treatment. Such a bioremediative aid by preventing pollution and providing recyclability, was considered as remarkable for a green earth.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacterial Strains

Four different bacterial strains were used in this study. *Rhodobacter capsulatus* (DSM 1710), *Rhodopseudomonas palustris* (DSM 127) and *Rhodobacter sphaeroides* O.U.001 (DSM 5864) strains were taken from DSMZ (Deusche Sammlung von Mikroorganismen und Zellkulturen GmbH – German Collection of Mikroorganisms and Cell Cultures, Germany). *Rhodobacter capsulatus* YO3 (Hup^-), a mutant strain lacking the uptake hydrogenase, was genetically modified by Dr. Yavuz Öztürk (GMBE, TÜBİTAK-MAM, Gebze) from *Rhodobacter capsulatus* MT 1131 (Öztürk *et al*., 2006).

2.1.2 Chemicals

Potassium dihydrogen phosphate, magnesium sulfate heptahydrate, Calcium chloride dihydrate, Monosodium glutamate, Agar, Sucrose, Iron-citrate, Vitamin, Trace elements were the chemicals used in the experiments and obtained from MERCK Chemicals. The list of the chemicals are given in Appendix A.

Raw molasses were obtained from sugar factory (Ankara Şeker Fabrikası) in Ankara, Turkey. The composition of molasses is given in Appendix F.

2.1.2.1 Solid Media

Solid media was used for activation and contamination detection of bacteria in stocks. Solid media is formed by addition of agar (1% w/v) to the growth media. The solid media was prepared by dissolving agar in the growth medium before autoclaving the media. After sterilization process, the media was poured into agar plates. Bacterial inoculation was performed when solidification of agar is completed.

2.2 Methods

2.2.1 Growth Media

Biebl and Pfennig (1981) medium with acetate (20 mM) and glutamate (10 mM) as carbon and nitrogen sources respectively, was used for the bacterial growth. The ingredients of the medium were dissolved in distilled water and initial pH was adjusted to 6.4-6.5 by titrating with NaOH. The medium was sterilized by autoclaving for 20 minutes at 121 ^{0}C. Vitamins, trace elements and iron-citrate were other components which were added after cooling the solution. The ingredients of growth medium is given in Appendix A.

2.2.2 Sucrose-Adaptation Media

The PNS bacteria were adapted to sucrose using two extra steps. After growing of the bacteria in acetate-glutamate (20/10 mM) medium, they were transferred into a second growth medium with acetate-sucrose-glutamate (20/10/10 mM) and then the bacteria were finally inoculated into sucrose-glutamate (10/10 mM) medium. When the optical density was nearly 2.0 at 660 nm, the culture was used for hydrogen production. Glutamate (10 mM) was used as nitrogen source for all the growth media (Table 2.1).

Table 2.1 The growth media of PNS bacteria for activation (1), and adaptation of bacteria to sucrose, (2) and (3).

Growth media	Acetate (mM)	Sucrose (mM)	Glutamate (mM)
1	20	-	10
2	10	20	10
3	-	10	10

2.2.3 Hydrogen Production Media

Hydrogen production media were based on sucrose as the substrate. Different sucrose concentrations (5-7.5-10 mM) were used as carbon source in the experiments. Firstly, pure sucrose was used for defined hydrogen media. Glutamate (2 mM) was the nitrogen source of the bacteria. Secondly, molasses was diluted to obtain sucrose concentrations of 5 mM, 7.5 Mm and 10 mM (Table 2.2) and used for hydrogen production.

Table 2.2 Dilutions carried out on molasses feedstock to obtain desired sucrose concentrations.

Concentration of Sucrose	Dilution Factors of Molasses
5 mM	80X
7,5 mM	60X
10 mM	40X

2.3 Experimental Setup

Experiments for hydrogen production were performed in 55 ml glass bottles. The glass bottles were used as photobioreactors which contained of 45 ml of hydrogen production media and 5 ml of bacterial culture. The transfer of the medium and culture into the glass reactors were done in a sterile cabinet. Sterilization of the glass bottles and brown caps were achieved by autoclaving. Argon gas was flushed through the top of brown caps by a needle to make the environment fully anaerobic. The photobioreactors (PBRs) were kept at 30 ^{0}C in an incubator (Nüve, ES250). The illumination of the system was ensured by 60-100 W tungsten lamps. Light intensity of the reactors were adjusted by a luxmeter (Lutron LX-105). The produced gas was collected in volumetric cylindirical glass tubes and measured through of water. Experimental setup is shown in Figure 2.1.

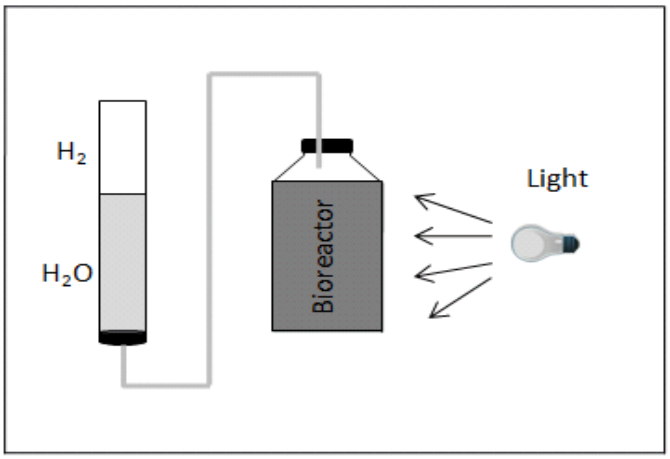


Figure 2.1 Schematic illustration of experimental setup.

2.4 Hydrogen Production Procedure

The bacterial stock culture which were stored in deep freezer at -80 were activated by streak plate method. Firstly, the original stock bacteria were transferred to plates containing solid growth medium that are composed of agar solution with 20 mM acetate and 10 mM glutamate in a laminar chamber. Then, the agar plates were incubated for growth at a temperature of about 30 ^{0}C and an illumination of 2000-2200 lux by using tungsten lamps. After 5-7 days, a grown bacterial colony was taken and then inoculated into 2 mL eppendorfs which contain agar free liquid growth medium. The inoculation was done at mid logarithmic phase of the growth which corresponds to around 1.0-1.5 optical density at 660 nm. 10% (v/v) inoculum was injected into the liquid growth medium by means of a sterile syringes.

The bioreactors were filled with 5 ml of bacterial culture and 45 of ml hydrogen production medium. Then, they were placed into cooling incubators and operated for 7-10 days. All the steps were performed in sterile cabinets.

Figure 2.2 The picture of experimental setup for hydrogen production.

2.5 Analyses

2.5.1 pH Analysis

A pH-meter (Mettler Toledo 3311) was used to measure pH of the liquid samples taken from the PBRs. Standard buffer solutions at pH of 4.0, 7.0 and 9.2 were used for calibration of the pH-meter before measuring each samples.

2.5.2 Cell Concentration

Growth of the bacteria was monitored by a spectrophotometer (Shimadzu UV-1201) at 660 nm wavelength. Optical densities of the samples were measured using a fresh medium as the blank solution. Cell concentrations were determined by conversion of absorbance values to dry cell weights (Uyar, 2008). For calculation of dry cell weights, the calibration curve was used (Appendix B).

2.5.3 Analysis of Sugar and Organic Acids

Sugar analysis was performed by using High Performance Liquid Chromatography (HPLC, Shimadzu 10A) in the Chemical Engineering Department at the Middle East Technical University, Ankara, Turkey. Transgenomic, CARBOSep CHO-682 Lead column was used with a refractive index detector. Filtered samples (5 μL) were injected with an autosampler (Shimadzu SIL-10AD) In the analysis, ultra pure water was used as the mobile phase with a flow rate of 0.25 mL/min. The oven temperature was maintained at 80 ^{0}C during the analysis. Alternatively, HPLC analysis for sugar was carried out using Inertsil NH_2 (250mm x 4.6mm) column with mobile phase of acetonitrile:water (75:25). The oven temperature was set to 40 ^{0}C and 1.1 ml/min mobile phase flow was provided through the analysis. Appendix C is given as a sample chromatogram and a calibration curve for sucrose.

Organic acid contents in the samples were analyzed by HPLC (Shimadzu 20A). Alltech I0A-1000 (300 mm x 7.8 mm) ion-exchange column was used to detect organic acids in the liquid samples. An autosampler (Shimadzu SIL-10AD) was provided for the injection of 20 μL samples and the detection of organic acids was determined by a UV detector (Shimadzu FCV-10AT) at 210 nm. The oven temperature was maintained at 66 ^{0}C and 0.085 M H_2SO_4 was used as mobile phase in the system. Flow rate of the mobile phase was set to 0.4 ml/min. Calibration curves were constructed for standard organic acids at different concentrations. A sample chromatogram and a calibration curve is shown in Appendix D. Organic acids remaining or produced during the process were acetic acid, lactic acid, butyric acid, propionic acid, formic acid and malic acid.

2.5.4 Analysis of Gas Composition

The composition of the total gas produced by bioreactors was analyzed by gas chromatography (GC, Agilent Technologies 6890N). The gas chromatography device was equipped with a thermal conductivity detector and a column (Supelco Carboxen 1010). Gas composition analysis was performed with a flow rate of 25 mL/min by using argon gas as the carrier gas in the system.

A micro gas syringe (Hamilton, 22 GA 500 μL) was used for injection of the gas sample to the GC system. The gas sample (100 μL) taken from gas collector. The oven, injector and detector temperatures were 140 ^{0}C, 160 ^{0}C and 170 ^{0}C, respectively. A representative gas analysis chromatogram is given in Appendix E.

2.5.5 Molasses Composition and Pretreatment of Molassses

Molasses was taken from a sugar factory in Ankara, Turkey (Ankara Sugar Factory). Their components were analyzed in Düzen Norwest Laboratory, Ankara, Turkey. Total aminoacids, alcohol (Ethyl alcohol), phenol and minerals (Fe, Mo, S, K) were the parameters analyzed. Sugar composition and organic acid analysis were carried out by HPLC in the METU Molecular Biology & Biotechnology Research and Development Center. The results of the analyses is given in Table 2.3. A detailed table of undiluted molasses composition analysis is given in Appendix F.

Table 2.3 The composition of undiluted molasses used in the experiments.

Component	**Units**	**Concentration**
Sucrose	M	1.16
Alcohol (ethyl alcohol)	% (v/v)	0.16
Phenol	mg/L	25.4
Lactic acid	mg/mL	7.58
Iron (Fe)	μg/L	516.9
Molybdenum (Mo)	μg/L	99.7
Sulphur (S)	mg/L	187.1
Potassium (K)	mg/L	5365

2.6 Data Analysis and Calculations

In the evaluation of the hydrogen production analysis, there are some important parameters such as substrate conversion efficiency, hydrogen productivity and light conversion efficiency.

Substrate conversion efficiency was calculated by the formula below;

The substrate was sucrose either as pure or on molassses with different concentrations in this study. Equation 2.1, is the efficiency of the substrate conversion was determined by the ratio of mole of theoretical hydrogen production over consumed sucrose to experimental hydrogen production amounts.

Substrate conversion efficiency (%) = (Moles of theoretically produced H_2 over consumed substrate) / (Experimental moles of H_2) x 100 (2.1)

Hydrogen productivity is the rate of hydrogen production through the duration of H_2 production. It was calculated and the results were compared to determine the productivities of hydrogen by PNS bacteria on sucrose or molasses media.

Hydrogen Productivity = Cumulative millimoles of hydrogen produced / volume of culture (l) x t (hour) (2.2)

Light Conversion efficiency is defined as the ratio of the total produced energy of hydrogen to the total energy input to the photobioreactor by light radiation. Light conversion efficiency is calculated by the formula below (2.3);

$$\eta(\%) = \frac{33.6 \times \rho_{H_2} \times V_{H_2}}{I \times A \times t} \times 100$$

Hydrogen density is 0.089 g/l. V_{H2} is the volume of produced hydrogen in L. The constant number 33.6 is the energy density of hydrogen in Watt.h/g. I, is the value of conversion of lux to Watt/m^2. A, is the irradiated area of photobioreactors. It is 0.002 m^2 and 0.011 m^2 for 50 ml and 150 ml bioreactors, respectively. Hydrogen production time, t is in hour.

Appendix I is given for sample calculations of substrate conversion efficiency, hydrogen productivity and light conversion efficiency.

CHAPTER 3

RESULTS AND DISCUSSION

Photofermentative hydrogen production using sucrose and molasses feedstock as sole carbon source was studied. Hydrogen production by photofermentative PNS bacteria on various organic acids is well characterized. Although they can also consume sugars, hydrogen production on sugars has not been studied in detail. The strains used in this study were adapted to grow and produce hydrogen on organic acids, as well. For this reason, adaptation of the bacteria to sucrose was necessary and the present study started with the optimization of growth and hydrogen production conditions of purple non-sulphur (PNS) bacteria on sucrose as sole carbon source. The results of adaptation studies of PNS bacteria on sucrose was given in section 3.1. Growth and hydrogen production of different PNS bacteria on sucrose and molasses were discussed and compared in sections 3.2, 3.3 and 3.4. Finally, the results of co-cultivation experiments were given in section 3.5.

3.1 Optimization of Purple Non-sulphur (PNS) Bacterial Growth on Sucrose

In this part, results of the adaptation studies of PNS bacteria to sucrose were given. Various sucrose, acetate and mixture of sucrose-acetate concentrations were tested for *R. capsulatus* (DSM1710) and *R. capsulatus* YO3 (Hup^-). The biomass growth, hydrogen production capacities and pH modes of these strains were determined and

compared. Ideal sucrose and buffer concentrations were found for optimum growth and hydrogen production.

3.1.1 Adaptation of PNS Bacteria to Sucrose as Carbon Source

The PNS bacteria, which were previously grown on acetate containing growth medium, were adapted to sucrose through several inoculation steps, where acetate concentration was decreased while sucrose concentration was increased in each inoculation step. After growing of the bacteria in acetate-glutamate (20/10 mM) medium, they were transferred into a second growth medium containing acetate-sucrose-glutamate (20/10/10 mM). Then the bacteria were inoculated into sucrose-glutamate (10/10 mM) medium. When the optical density was nearly 2.0 at 660 nm, the culture was transferred to hydrogen production medium. All the experiments were carried out in anaerobic conditions under illumination.

3.1.2 The Effect of Sucrose on Growth

in 50 ml glass photobioreactors.. Glutamate (2 mM) was used for the nitrogen source for the bacteria. The maximum biomass for *R.capsulatus* (DSM1710) and *R.capsulatus* YO3 (Hup^-) were 0.74 g/l_c and 0.96 g/l_c respectively. The growth on sucrose (20 mM) was more than acetate (30 mM) medium for both of the strains. Figure 3.1 and 3.2 show the growth of *R.capsulatus* (DSM1710) and *R.capsulatus* YO3 (Hup^-) of the cultures, respectively, during 230 hours of operation time.

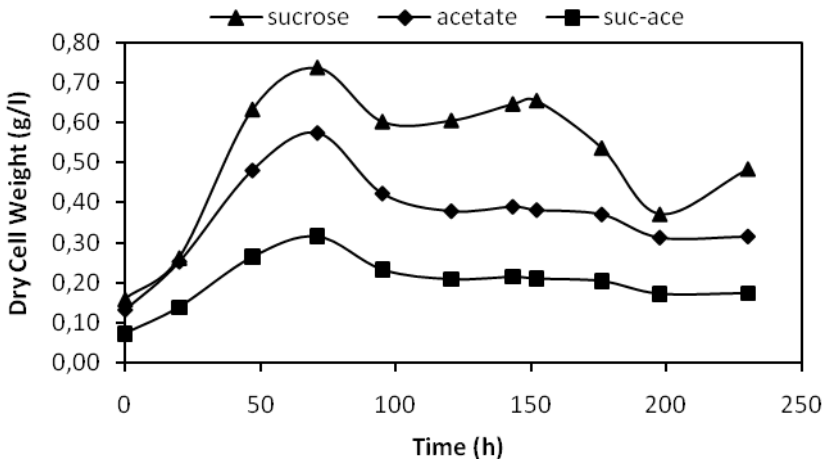


Figure 3.1 The growth of *R. capsulatus* (DSM1710) on sucrose (20 mM), acetate (30 mM) and a mixture of sucrose-acetate (20 mM/10 mM) defined media.

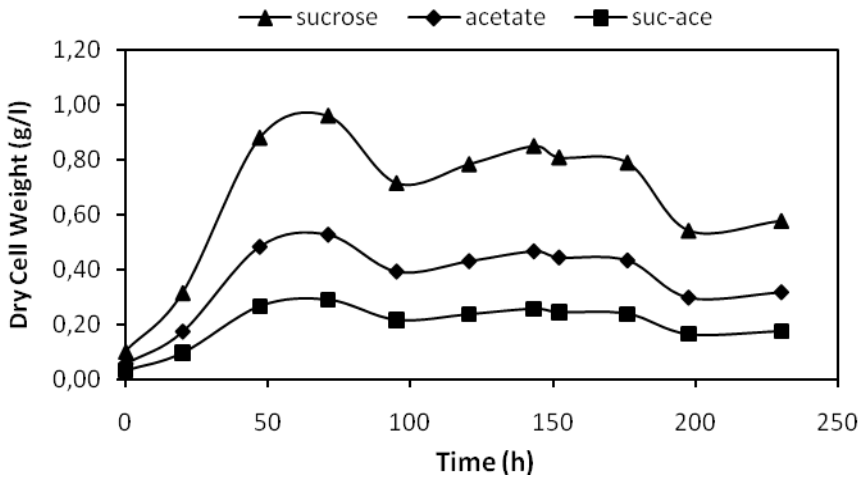


Figure 3.2 The growth of *R.capsulatus* YO3 (Hup-) on sucrose (20 mM), acetate (30 mM) and a mixture of sucrose-acetate (20 mM/10 mM) defined media. Glutamate (2 mM) was the nitrogen source for all the bacteria.

The hydrogen production levels on sucrose were lower than acetate in both strains. *R.capsulatus* YO3 (Hup^-) produced two times more hydrogen on sucrose (20 mM) than *R.capsulatus* (DSM 1710). Shown in Figure 3.3 and 3.4 are cumulative hydrogen production on different concentrations of acetate and sucrose media. Acetate showed better profile than sucrose as a substrate in hydrogen production.

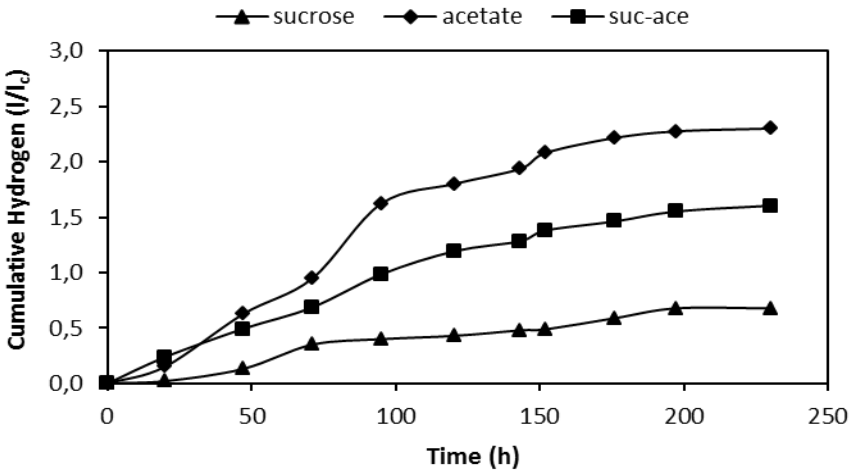


Figure 3.3 The cumulative hydrogen production of *R. capsulatus* (DSM 1710) on sucrose (20 mM), acetate (30 mM) and a mixture of sucrose-acetate (20 mM/10 mM) defined media. Glutamate (2 mM) was the nitrogen source for all the bacteria.

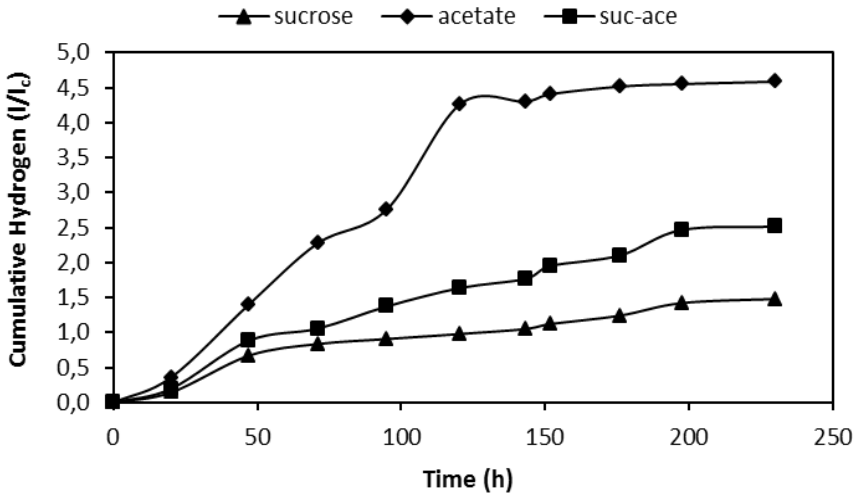


Figure 3.4 The cumulative hydrogen production of *R.capsulatus* YO3 (Hup-) on sucrose (20 mM), acetate (30 mM) and a mixture of sucrose-acetate (20 mM/10 mM) defined media. Glutamate (2 mM) was the nitrogen source for all the bacteria.

The variations in pH during the hydrogen production process on sucrose by *R.capsulatus* (DSM1710) and *R.capsulatus* YO3 (Hup$^-$) are given in Figure 3.5. The change of pH was in the range of 5.0-6.5. Produced organic acids may cause drops in pH during the process. Acetic acid and formic acid were produced abundantly as intermediates (Appendix H).

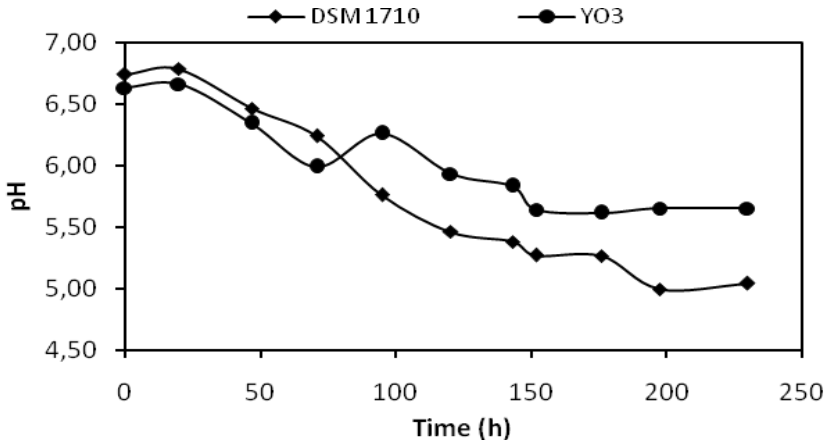


Figure 3.5 The changes of pH on 20 mM sucrose media by *R.capsulatus* DSM 1710 and *R.capsulatus* YO3 (Hup-).

This study also showed that *R. capsulatus* YO3 (Hup$^-$) is more promising in hydrogen production than wild type. The cumulative hydrogen production was 0.36 g/l_c for *R. capsulatus* (DSM1710) and 0.7 g/l_c for *R. capsulatus* YO3 (Hup$^-$). Hydrogen was produced intensively through the first 48 hours at which exponential cell growth occurs.

3.1.2 The Effect of Buffer Concentrations

Previous study in section 3.1 showed that pH decreased during the hydrogen production process. The rapid decline and unstable pH mode which was observed with 20 mM KH_2PO_4 buffer on 20 mM sucrose media. Therefore, different KH_2PO_4 buffer concentrations (30-40-50 mM) were used to control pH during the growth and hydrogen production on sucrose.

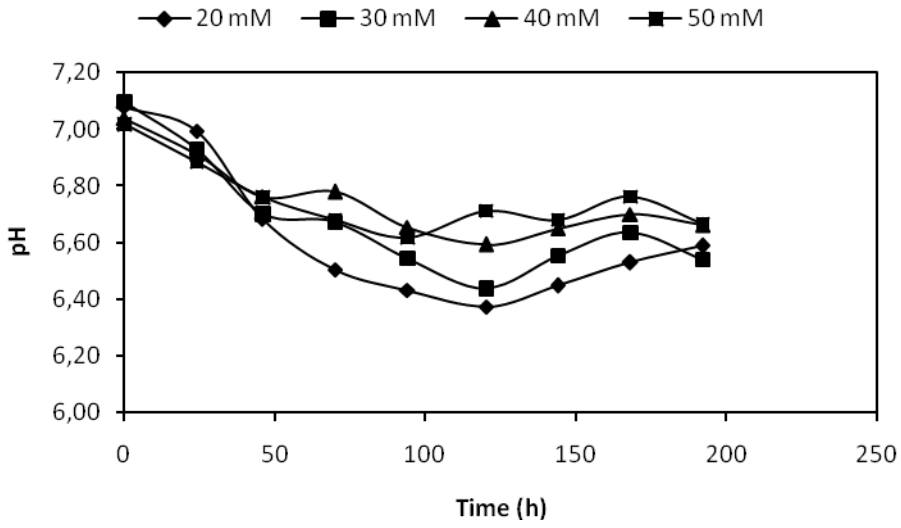


Figure 3.6 pH curves of *R.capsulatus* YO3 (Hup-) in 5 mM sucrose defined medium (DF) which is prepared by different concentrations of KH_2PO_4 buffers (initial pH was 7.0)

Hydrogen production media were prepared with 5 mM sucrose (DF) and various buffer concentrations by adjusting the initial pH as 7.0. The variation in pH during hydrogen production by *R. capsulatus* YO3 and *R. capsulatus* DSM 1710 are illustrated in Figure 3.6 and 3.7 respectively.

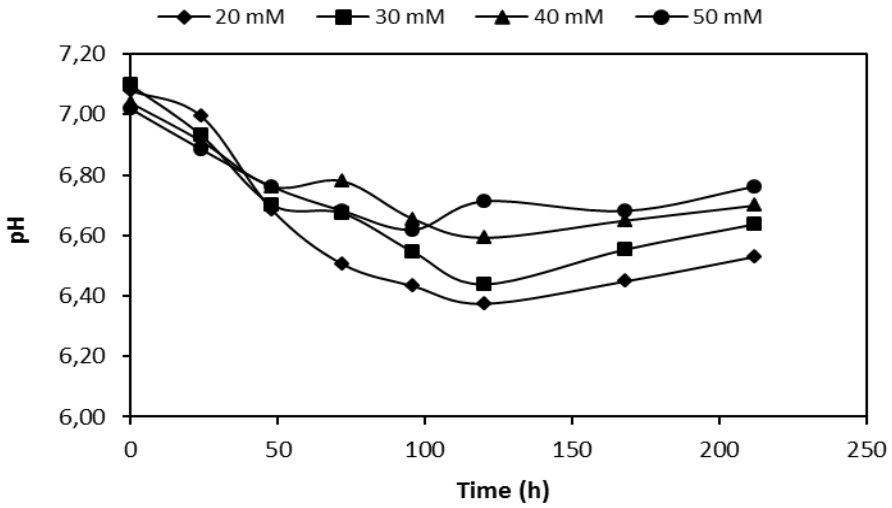


Figure 3.7 pH curves of *R.capsulatus* YO3 (Hup-) in 5 mM sucrose from molasses which is prepared by different concentrations of KH_2PO_4 buffers (initial pH was 7.0)

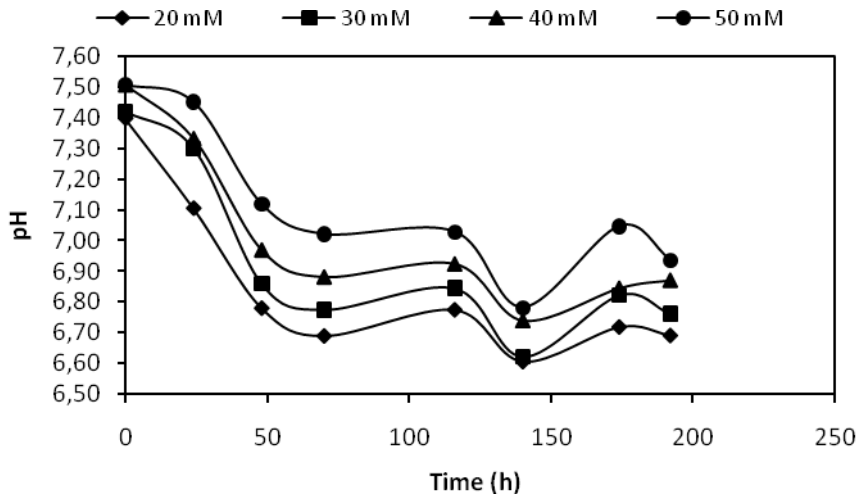


Figure 3.8 pH curves of *R.capsulatus* YO3 (Hup-) in 5 mM sucrose defined medium which is prepared by different concentrations of KH_2PO_4 buffers (initial pH was 7.5)

The effect of buffer concentrations were also examined on molasses. Molasses was diluted to have sucrose concentration of 5 mM and various buffer concentrations. The initial pH was adjusting to 7.5. The variation in pH during hydrogen production by *R. capsulatus* YO3 and *R. capsulatus* DSM 1710 are illustrated in Figure 3.8 and 3.9, respectively.

More rapid decrease in pH on molasses medium than pure sucrose medium was observed. Initial pH of 7.5 were more effective than pH of 7.0 for providing a stable pH mode in both hydrogen production media. Nath and Das (2009) reported that initial pH raised up hydrogen production and biomass growth during photofermentation by *R. sphaeroides* and they also stated that optimum initial pH was 7.0. Increasing buffer concentrations ensured a stable pH mode at range of below 5.5 to 6.5.

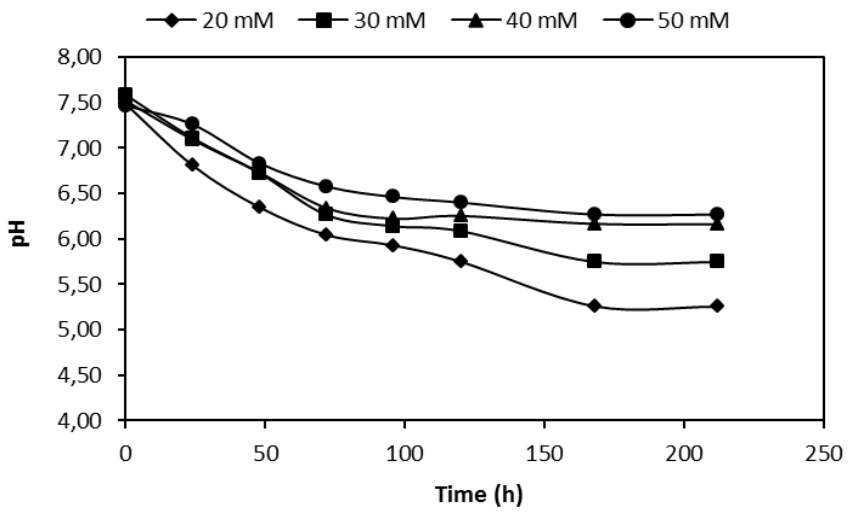


Figure 3.9 pH curves of *R.capsulatus* YO3 (Hup-) in 5 mM sucrose from molasses which was prepared by different concentrations of KH_2PO_4 buffers (initial pH was 7.5)

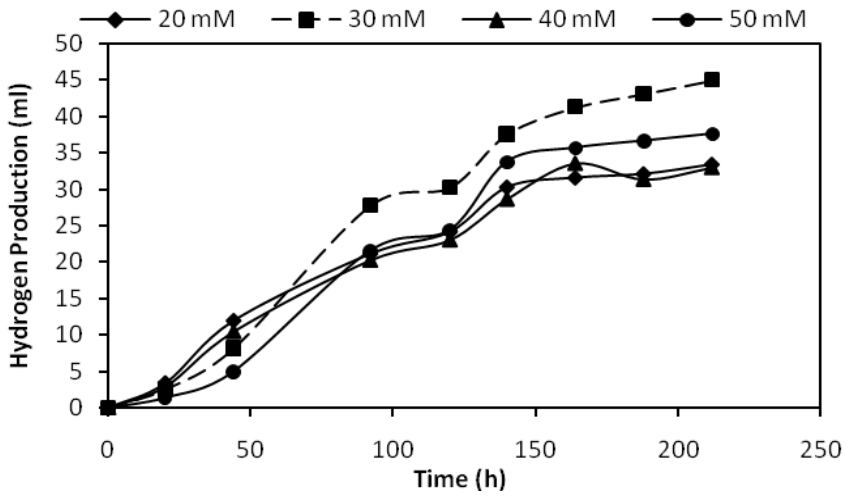


Figure 3.10 The cumulative hydrogen production (ml) at different concentrations of KH_2PO_4 buffers on 5 mM sucrose from molasses in 50 ml PBRs.

Hydrogen production ability was more using 30 mM KH_2PO_4 buffer than the other concentrations. Figure 3.10 shows the cumulative hydrogen production at different KH_2PO_4 buffer concentrations on 5 mM sucrose from molasses.

3.2 Growth and Hydrogen Production on Sucrose by Different Strains of Purple Non-sulfur (PNS) Bacteria

Growth and hydrogen production by four different strains of bacteria, *R. capsulatus* YO3 (hup-), *R. capsulatus* DSM 1710, *R. sphaeroides* O.U.001 and *R. palustris*, were studied on three different (5, 7.5 and 10) sucrose concentrations in 50 ml photobioreactors under 2000-2200 lux illumination at 30-32 ^{o}C. Initial pH was set to 7.2-7.4 and 30 mM KH_2PO_4 was used as buffer in all hydrogen production media.

3.2.1 Experiments on *R. capsulatus* (DSM 1710)

The number of bacterial cells showed a dramatic increase at exponential phase after a short lag phase. Most of the hydrogen was generated at time duration of 0-48 hours. The maximum biomass was approximately 0,6 gdcw/l_c and same for all PBRs. Therefore, sucrose may be considered as a good carbohydrate source for growth. Figure 3.11 is the depiction of biomass growth of *R. capsulatus* (DSM 1710) on different sucrose concentrations.

The products of the hydrogen metabolism of PNS bacteria on sucrose utilization are organic acids such as acetic acid, lactic acid, propionic acid and butyric acid. During the growth and metabolism of sucrose results the production of those intermediates. Due to acidic nature, they are able to lower the pH. The organic acids first produced and then consumed during the hydrogen production process. Shown in Table 3.1 is the initial and final organic acid concentrations during hydrogen production by *R. capsulatus* (DSM 1710) on sucrose.

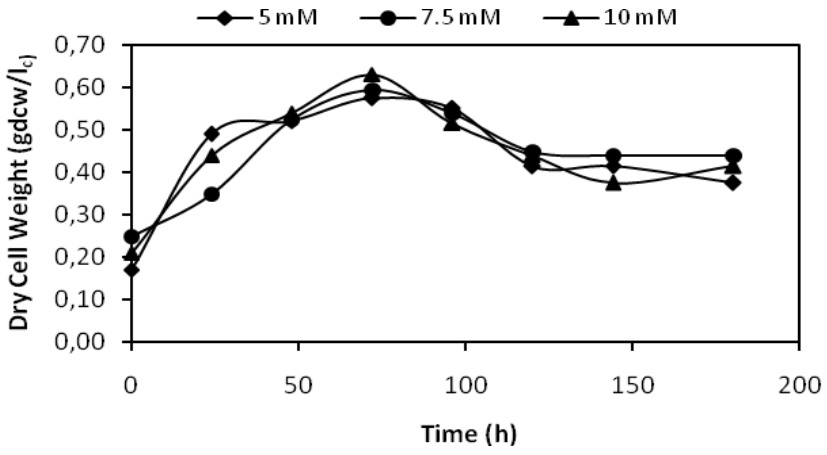


Figure 3.11 The growth of *R. capsulatus* DSM 1710 on 5 mM, 7.5 mM and 10 mM sucrose hydrogen production media.

The pH, which was initially set as 7.2-7.3, showed a great decline and it was 6.0-6.5 at the end of 192 hours process. The difference between initial and final pH of the PBRs was more in increasing start up sucrose concentrations, most probably due to production of more organic acids. This event explains the one of the reasons in pH drops through the hydrogen production. The change in pH was given in Figure 3.12.

A proportional link can be inferred between biomass and hydrogen production of bacteria. Both parameters show increments till about 48 hours. However, after 48 hours of the operation, both hydrogen production and growth slowed down.

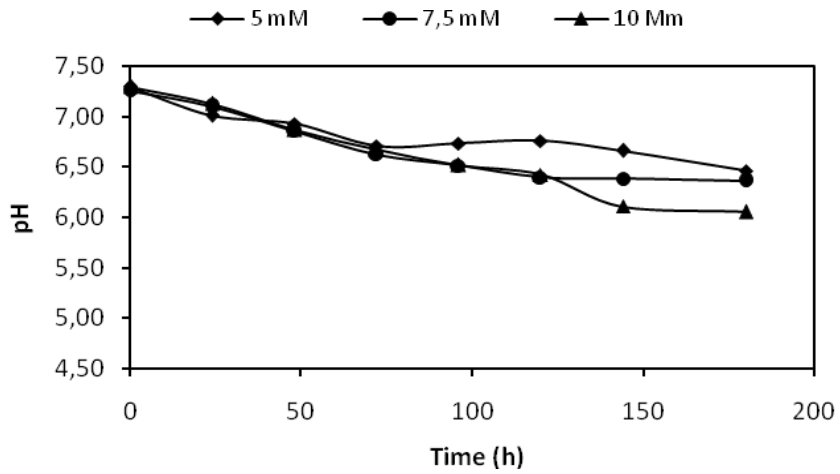


Figure 3.12 The pH changes of *R. capsulatus* DSM 1710 on 5 mM, 7.5 mM and 10 mM sucrose hydrogen production media

The total hydrogen production on 5, 7.5 and 10 mM sucrose media were 0.2, 0.86 and 0.6 l/l_c, respectively. Figure 3.13 shows the cumulative hydrogen production of *R.capsulatus* DSM 1710 on 5 mM, 7.5 mM and 10 mM sucrose hydrogen production media.

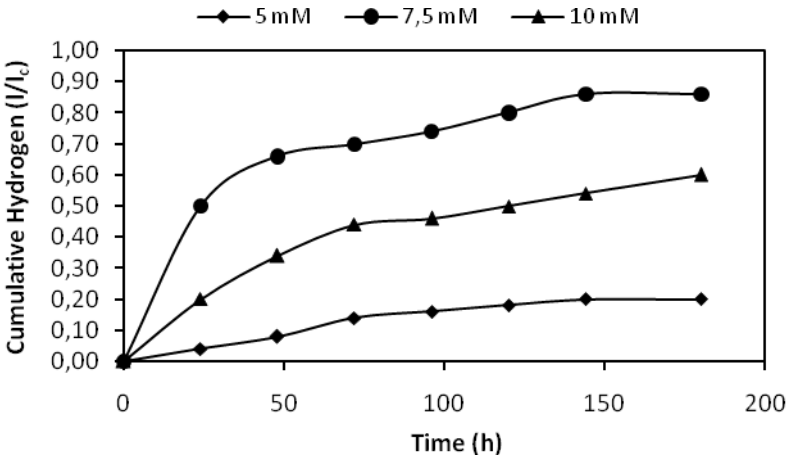


Figure 3.13 The cumulative hydrogen production of *R. capsulatus* DSM 1710 on 5 mM, 7.5 mM and 10 mM sucrose hydrogen production media.

Table 3.1 Concentrations of organic acids in photofermentation of *R.capsulatus* DSM 1710 on 5 mM, 7.5 mM and 10 mM sucrose hydrogen production media.

Hydrogen production media	5 mM Sucrose		7.5 mM Sucrose		10 mM Sucrose	
Organic acids	C_0 (mM)	C_f (mM)	C_0 (mM)	C_f (mM)	C_0 (mM)	C_f (mM)
Acetic acid	0.117	3.320	0.041	1.177	0.021	4.172
Formic acid	0.643	16.403	0.392	2.569	0.265	6.113
Lactic acid	0.43	0.08	0.23	0.99	0.04	0.00

C_0 * The initial concentration (mM)

C_f * The final concentration (mM)

Lactic acid is produced and consumed in all the hydrogen production media, whereas acetic acid and formic acid was generally directed in just production rather than consumption after producing them. Figure 3.15 shows the formic acid production on different sucrose media. The production and consumption of organic acids are given

in Table 3.1. Figure 3.14 indicates organic acid production by *R.capsulatus* (DSM 1710) on 5 mM sucrose defined medium. Sucrose was consumed by *R.capsulatus* DSM 1710 as %100, %71 and %50 in 5 mM, 7.5 mM and 10 mM sucrose containing PBRs, respectively. Given in Table 3.2 and Figure 3.16 is the consumption of sucrose by *R.capsulatus* (DSM 1710) on different sucrose media.

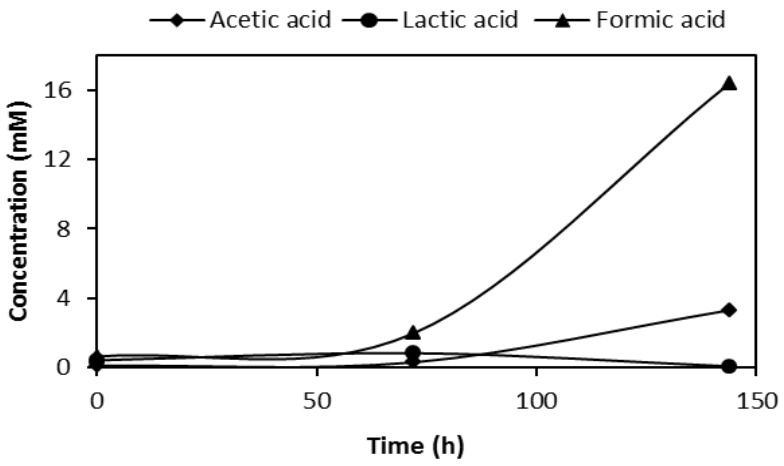


Figure 3.14 Organic acid production by *R. capsulatus* (DSM 1710) on 5 mM sucrose defined medium.

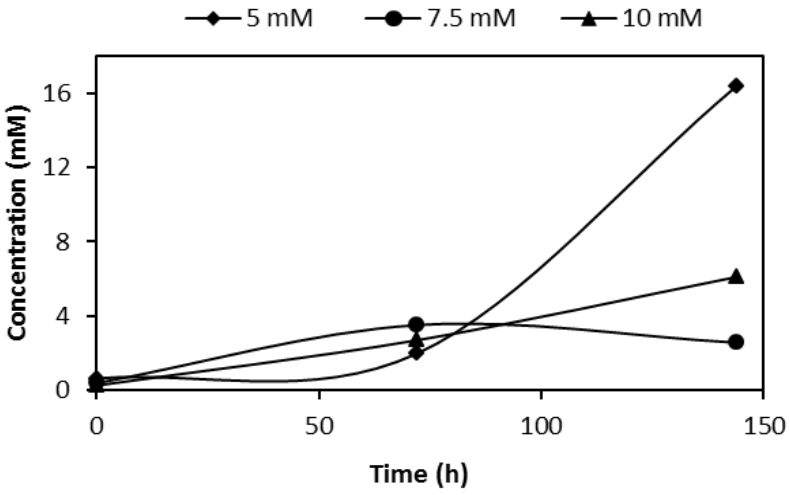


Figure 3.15 Formic acid production by *R. capsulatus* DSM 1710 on different sucrose concentrations.

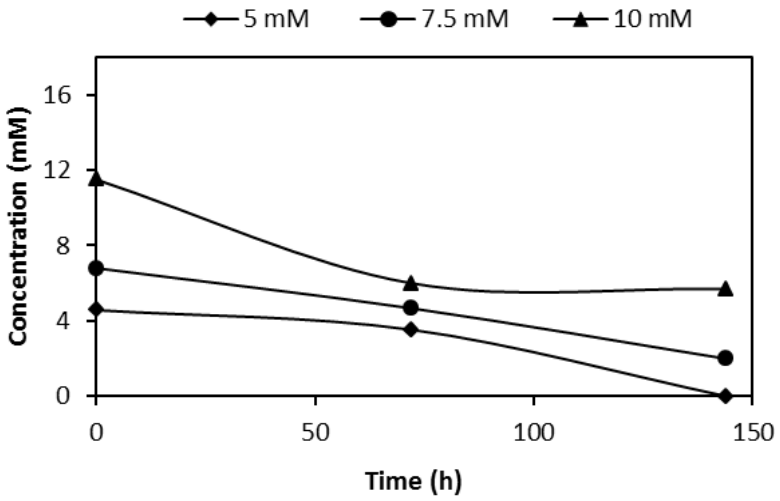


Figure 3.16 Sucrose consumption by *R. capsulatus* DSM 1710 on 5 mM, 7.5 mM and 10 mM sucrose hydrogen production media.

Table 3.2 The utilization of sucrose by *R.capsulatus* (DSM 1710) on 5 mM, 7.5 mM and 10 mM sucrose hydrogen production media.

Sucrose concentrations (PBRs)	C_0 (mM)	C_f (mM)	Sucrose consumption (%)
5 mM	4.57	0	100
7.5 mM	6.80	1.99	71
10 mM	11.5	5.7	50.4

3.2.2 Experiments on *R. capsulatus* YO3 (Hup$^-$)

The growth curves of *R. capsulatus* YO3 (Hup$^-$) showed that bacteria increase cell populations from 0.15 gdcw/l_c to 0.4 gdcw/l_c between 0-48 time period. After that, steady state was observed. The cells in media of 10 mM sucrose grow faster and had maximum biomass of 0.62 gdcw/l_c.

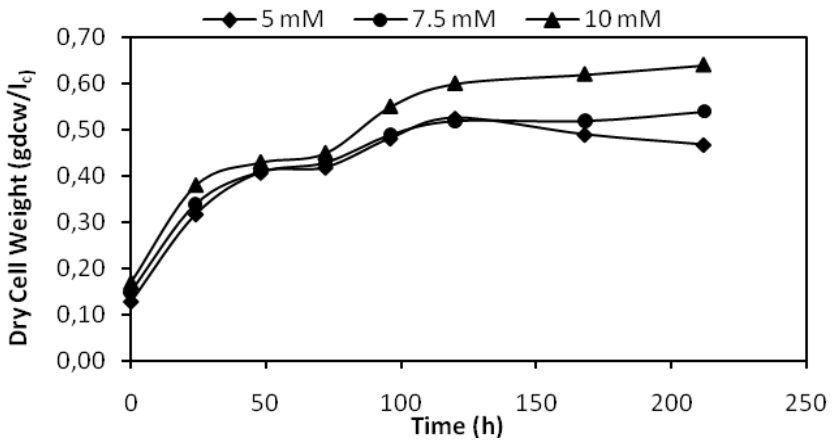


Figure 3.17 The growth of *R. capsulatus* YO3 (Hup-) on 5 mM, 7.5 mM and 10 mM sucrose hydrogen production media.

The pH dramatically declined from initial value of 7.2-7.4 to about 5.8-6.0 levels in all the three PBRs. Figure shows the organic acids production in the PBRs. The growth and pH changes of *R.capsulatus* YO3 (Hup$^-$) were given in Figure 3.17 and 3.18 respectively.

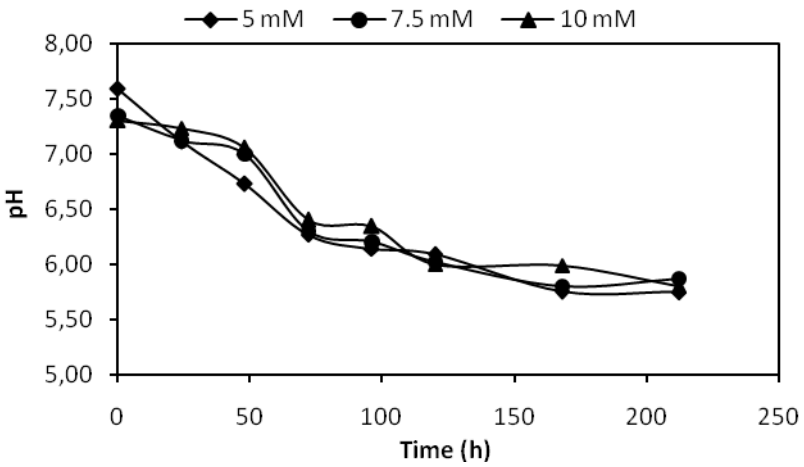


Figure 3.18 The mode of pH of *R. capsulatus* YO3 (Hup-) on 5 mM, 7.5 mM and 10 mM sucrose hydrogen production media.

The cumulative hydrogen production was higher on 5 mM sucrose PBR than the others. On the contrary, the hydrogen production showed a fluctuated profile during 230 hours for the same PBR and the maximum hydrogen production was 0.8 l/l_c. It was observed near two times more hydrogen was generated in 5 mM sucrose than 7.5 and 10 mM sucrose containing PBRs. The cumulative hydrogen production was given in Figure 3.19.

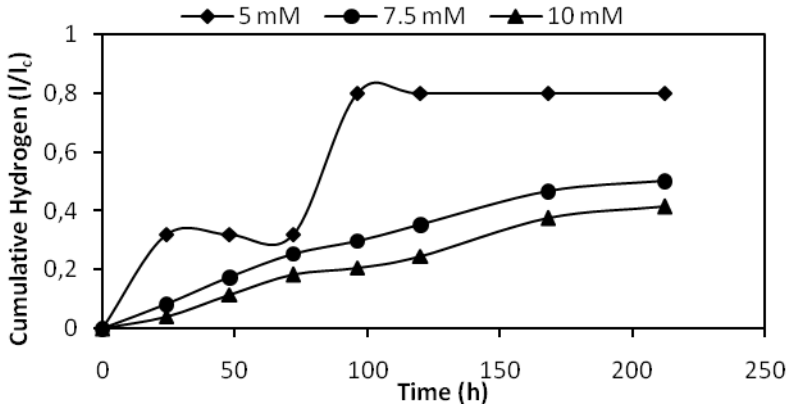


Figure 3.19 The cumulative hydrogen production (l/lc) of *R. capsulatus* YO3 (Hup-) on 5 mM, 7.5 mM and 10 mM sucrose hydrogen production media.

Acetic acid and formic acid were produced and not consumed during 216 hours operation time. Lactic acid was produced and used by the bacteria. The organic acid products were formed by the action of the bacteria on sucrose through hydrogen production. Figure 3.20 shows organic acid production on 5 mM sucrose.

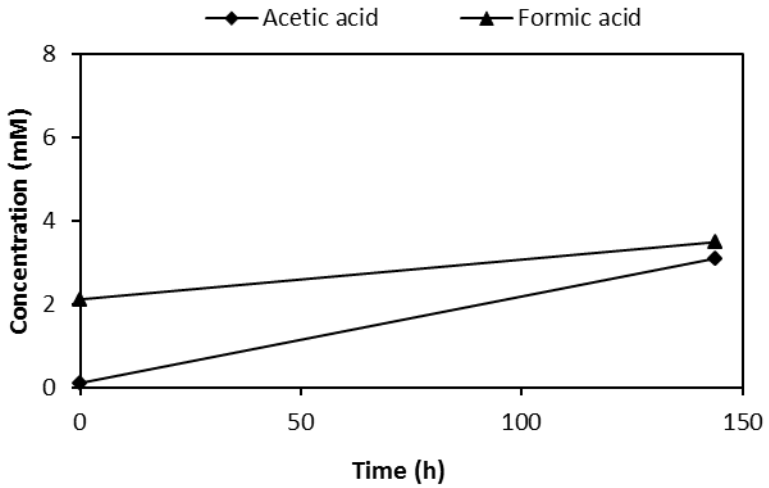


Figure 3.20 Acetic acid and formic acid production on 5 mM sucrose by *R. capsulatus* YO3 (Hup$^-$).

Main organic acids was acetic acid and formic acid in all of the hydrogen media. These compounds were directed into just production rather than consumption. Therefore, acidity of the media were increased and hydrogen production was adversely affected.

Table 3.3 Concentrations of organic acids in photofermentation of *R.capsulatus* YO3 (Hup$^-$) on 5 mM, 7.5 mM and 10 mM sucrose hydrogen production media. Glutamate (2 mM) was used as the nitrogen source.

Hydrogen production media	5 mM Sucrose		7.5 mM Sucrose		10 mM Sucrose	
Organic acids	C_0 (mM)	C_f (mM)	C_0 (mM)	C_f (mM)	C_0 (mM)	C_f (mM)
Acetic acid	0.112	3.098	0.32	4.54	0.23	5.65
Formic acid	2.117	3.488	3.12	4.32	3.45	5.32
Lactic acid	0	0	0.2	0	0.12	0.08

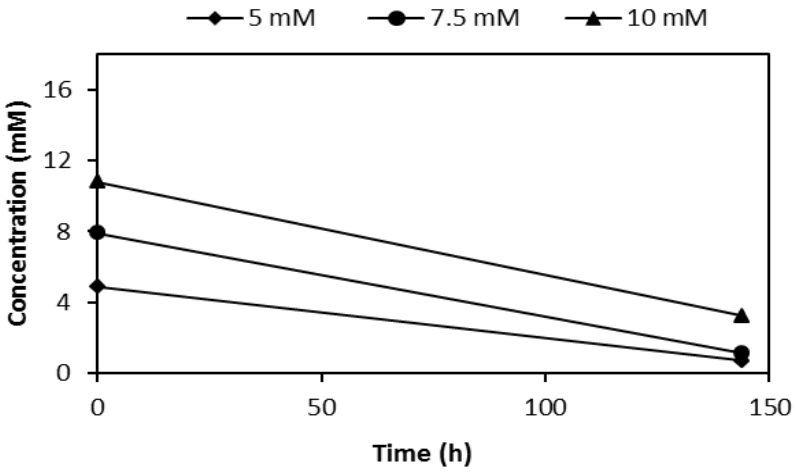


Figure 3.21 The consumption curves of *R. capsulatus* YO3 (Hup-) on different sucrose concentrations.

The consumption (%) of sucrose was 85, 85.5 and 70 in 5, 7.5 and 10 mM sucrose containing PBRs, respectively. Figure 3.21 and Table 3.4 indicate utilization of different sucrose concentrations by *R.capsulatus* YO3 (Hup$^-$).

Table 3.4 The utilization of sucrose by *R.capsulatus* YO3 (Hup^-) on 5 mM, 7.5 mM and 10 mM sucrose hydrogen production media.

Sucrose concentrations (PBRs)	C_0 (mM)	C_f (mM)	Sucrose consumption (%)
5 mM	4.88	0.73	85
7.5 mM	7.89	1.14	85.5
10 mM	10.78	3.25	70.0

3.2.3 Experiments on *R. palustris* (DSM 127)

R R. palustris (DSM 127) was used for hydrogen production on different sucrose defined media at 5 mM, 7.5 and 10 mM concentration in 50 ml bioreactors. The pH changes, growth and hydrogen production were followed during 200 hours batch process. The pH was stabilized at 6.7 level after a rapid decrease from the initial pH of 7.4 in 5 mM sucrose containing PBR. On the contrary, pH was declined and reached to about 6.5 level in 7.5 and 10 mM sucrose PBRs (Figure 3.16).

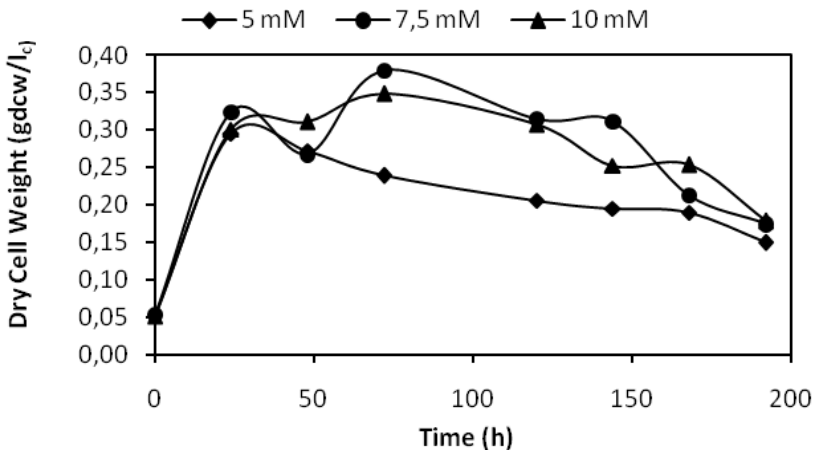


Figure 3.15 The growth of *R. palustris* (DSM 127) on 5 mM, 7.5 mM and 10 mM sucrose hydrogen production media.

Figure 3.15 illustrates the growth profile. A rapid growth was observed during the frst 24h of operation. The cell growth was more on 7.5 mM and 10mM sucrose concentrations, while at 5 mM a decline in biomass concentration was observed after 24h.

Hydrogen production was higher with 5 mM sucrose and 7.5 mM as 1.4 l/l_c and 1.5 l/l_c during 200 hours operation. The maximum biomass and hydrogen production was observed on 7.5 mM sucrose containing PBR. Figure 3.17 shows the cumulative hydrogen production of *R. palustris* (DSM 127).

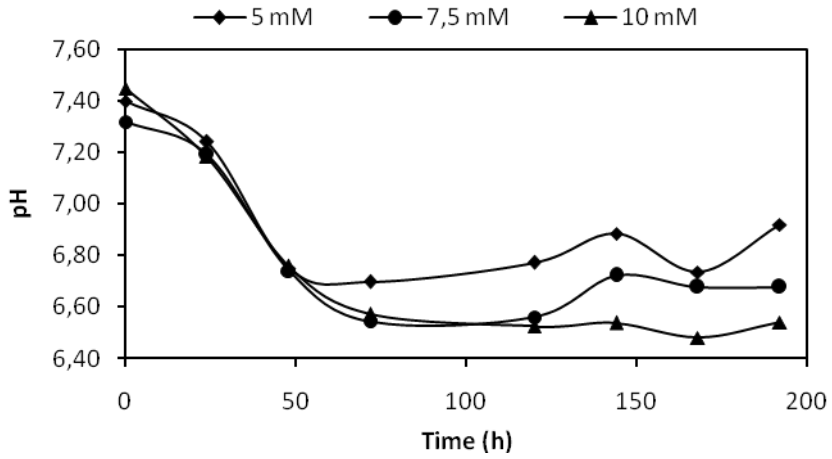


Figure 3.16 The pH changes of *R. palustris* (DSM127) on 5 mM, 7.5 mM and 10 mM sucrose hydrogen production media.

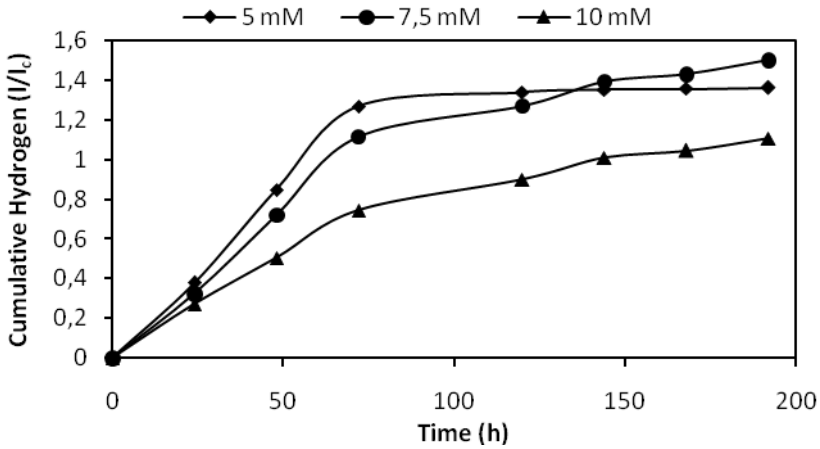


Figure 3.17 The cumulative hydrogen production of *R. palustris* (DSM127) on 5 mM, 7.5 mM and 10 mM sucrose hydrogen production media.

A rapid increase in production of acetic and formic acids was observed at 72th hours in both 7.5 mM and 10 mM sucrose PBRs. On the contrary, a decline in production of acetic, lactic and formic acids were recorded in 5 mM PBR. Moreover, the acetic and lactic acids were produced and then consumed in 5 mM PBR. The pHs of hydrogen media were also at lower levels due to high organic acid amounts in the PBRs. Table 3.5 summarizes initial and final concentrations of the organic acid products.

Table 3.5 Concentrations of organic acids in photofermentation of *R. palustris* (DSM127) on 5 mM, 7.5 mM and 10 mM sucrose hydrogen production media.

Hydrogen production media	5 mM Sucrose		7.5 mM Sucrose		10 mM Sucrose	
Organic acids	C_0 (mM)	C_f (mM)	C_0 (mM)	C_f (mM)	C_0 (mM)	C_f (mM)
Acetic acid	0.074	0	0.088	0	0.213	19.15
Formic acid	0.420	3.056	0.429	10.46	0.579	5.76
Lactic acid	0.15	0	0.14	0	0.19	0

Sucrose utilization by *R. palustris* (DSM127) on 5 mM, 7.5 mM and 10 mM sucrose hydrogen production media was quite high, compared to other PNS bacterial strains, tested. The sucrose consumption (%) in PBRs is given in Table 3.6.

Table 3.6 The utilization of sucrose by *R. palustris* (DSM127) on 5 mM, 7.5 mM and 10 mM sucrose hydrogen production media.

Sucrose concentrations (PBRs)	C_0 (mM)	C_f (mM)	Sucrose consumption (%)
5 mM	4.94	0.19	96.1
7.5 mM	7.99	1.51	81.1
10 mM	9.54	1.06	88.9

3.2.4 Experiments on *R. sphaeroides* O.U.001 (DSM 5864)

In this part, *R. sphaeroides* O.U.001 (DSM 5864) was investigated for biohydrogen production by sucrose. A 180 hours operation was done using 50 ml bioreactors. The biomass growth indicated that *R. sphaeroides* O.U.001 (DSM 5864) grows well on sucrose. Figure 3.18 shows the growth of the bacteria.

pH variation during hydrogen production were the same range in all the PBRs. All of the final pHs were in the range 6.5-7.0 and began to drop after 48^{th} hours by the effect of side products in the media especially acidic compounds. Figure 3.19 is given as the changes of pHs in the PBRs.

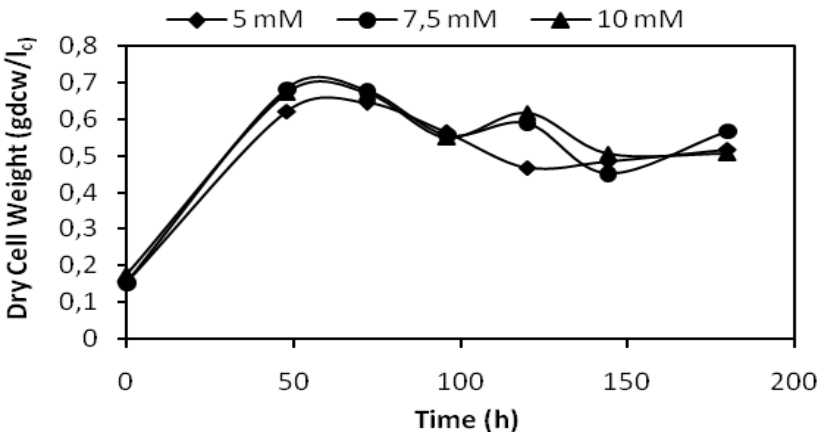


Figure 3.18 The growth of *R. sphaeroides* O.U.001 (DSM 5864) on 5 mM, 7.5 mM and 10 mM sucrose containing hydrogen production media.

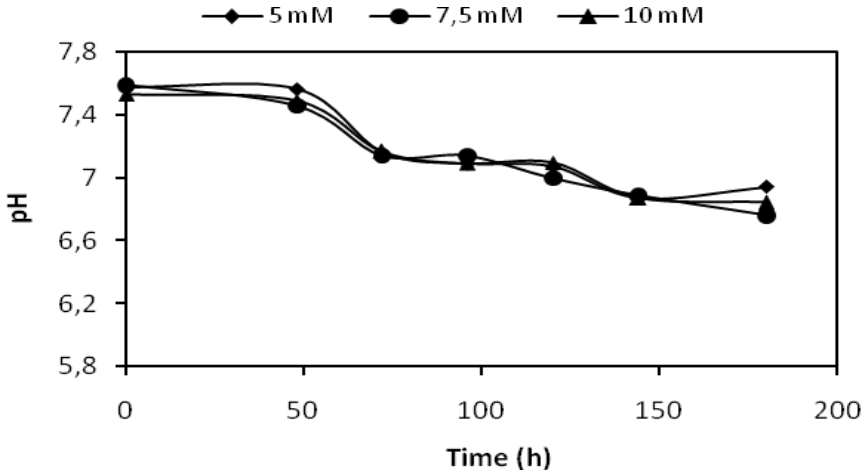


Figure 3.19 The pH changes of *R. sphaeroides* O.U.001 (DSM 5864) on 5 mM, 7.5 mM and 10 mM containing sucrose hydrogen production media.

The maximum hydrogen was produced by 5 mM sucrose medium as 0.86 l/l$_c$. The other two PBRs (7.5 mM and 10 mM) produced 0.60 l/l$_c$ and 0.74 l/l$_c$. Hydrogen production was continued exponentially till 96th hours. Figure 3.20 is given as the cumulative hydrogen production by *R. sphaeroides* O.U.001 (DSM 5864) on 5 mM, 7.5 mM and 10 mM sucrose hydrogen production media.

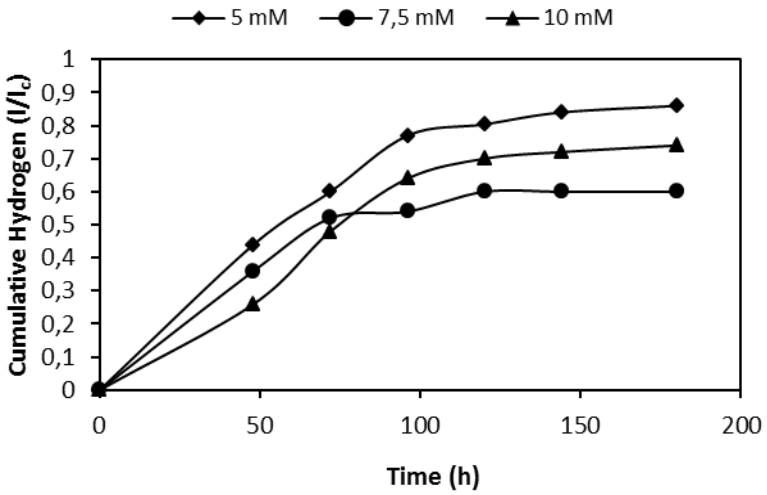


Figure 3.20 The cumulative hydrogen production of *R. sphaeroides* O.U.001 (DSM 5864) on 5 mM, 7.5 mM and 10 mM sucrose containing hydrogen production media.

Formic acid and lactic acid were detected at small amounts whereas acetic acid was not found in the fermentation media of the all PBRs. Acetic acid may be produced and utilized in a short time or its production did not occur. The produced organic acids were shown in Table 3.7. Sucrose conversion of 5 mM PBR was the highest (78.6) and when compared with the other two PBRs. Table 3.8 shows the sucrose conversion by *R. sphaeroides* O.U.001 (DSM 5864) in three different sucrose media.

Table 3.7 Concentrations of organic acids in photofermentation of *R. sphaeroides* O.U.001 (DSM 5864) on 5 mM, 7.5 mM and 10 mM sucrose hydrogen production media.

Hydrogen production media	5 mM Sucrose		7.5 mM Sucrose		10 mM Sucrose	
Organic acids	C_0 (mM)	C_f (mM)	C_0 (mM)	C_f (mM)	C_0 (mM)	C_f (mM)
Acetic acid	0	0	0	0	0	0
Formic acid	0.101	0.683	0	1.07	0.016	1.299
Lactic acid	0.04	0.1	0	0.06	0.05	0.07

Table 3.8 The utilization of sucrose by *R. sphaeroides* O.U.001 (DSM 5864) on 5 mM, 7.5 mM and 10 mM sucrose hydrogen production media.

Sucrose concentrations (PBRs)	C_0 (mM)	C_f (mM)	Sucrose consumption (%)
5 mM	4.88	1.04	78.6
7.5 mM	7.45	4.50	40.0
10 mM	9.89	4.59	53.5

3.3 Growth and Hydrogen Production on Molasses by Different Strains of Purple Non-sulfur (PNS) Bacteria

The objective of this part of the experiments is to investigate the utilization of sucrose from molasses. This part of the study also intends to see the growth and hydrogen production on different sucrose concentrations which was prepared by the dilutions of molasses. Dilution factors were illustrated in section 2 and in Table 2.1. Four different PNS bacteria were used for hydrogen production on three different (5, 7.5 and 10) sucrose concentrations in 50 ml photobioreactors under 2000-2200 lux illumination. Initial pH was adjusted to 7.2-7.4 and 30 mM KH_2PO_4 buffer was used to keep the pH stable in all hydrogen production media. Vitamins, trace elements and iron-citrate were added to the media.

3.3.1 Experiments on *R. capsulatus* (DSM 1710)

There was found an inverse proportion between the growth and concentration of sucrose. Bacterial growth was greater in 5 mM sucrose medium than the others and reached the maximum biomass in 96 hours. Then a steady growth phase has started. The maximum biomass was recorded as 0.6 gdcw/l_c, 0.3 gdcw/l_c and 0.2 gdcw/l_c for

in order of 5 mM, 7.5 mM and 10 mM sucrose from molasses. The growth of *R. capsulatus* (DSM 1710) was given in Figure 3.21.

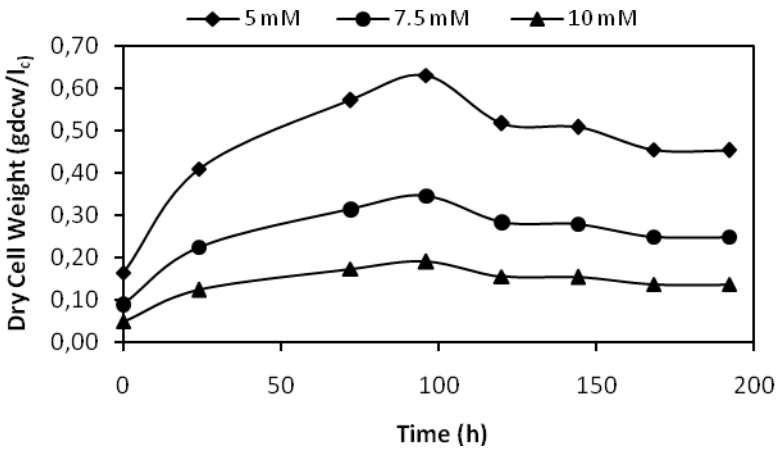


Figure 3.21 The growth of *R. capsulatus* (DSM 1710) in different sucrose concentrations (5, 7.5 and 10 mM) by dilutions of molasses.

A continuous decline in pH was observed during 0-48 hours time interval. The final pHs of the bioreactors were in the range of 5.5-5.8. The pH curves indicated nearly the same manner in all PBRs of *R. capsulatus* (DSM 1710). The pH variations were given in Figure 3.22. Although the biomass growth was not higher than the other PBRs, hydrogen production was the highest. The cumulative hydrogen production of 5 mM, 7.5 mM and 10 mM PBRs were found as 0.26 l/l_c, 0.20 l/l_c and 0.5 l/l_c respectively. Figure 3.23 is hydrogen production curves by *R. capsulatus* (DSM 1710) on molasses media with different sucrose concentrations.

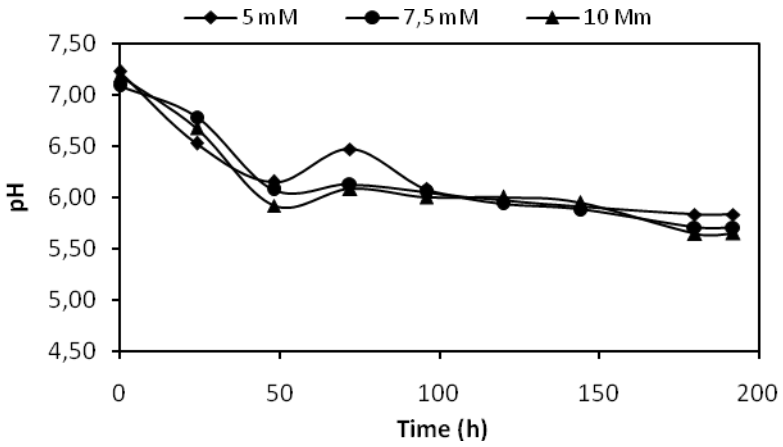


Figure 3.22 pH variation during hydrogen production by *R. capsulatus* (DSM 1710) on different molasses media with different sucrose concentrations (5, 7.5 and 10 mM).

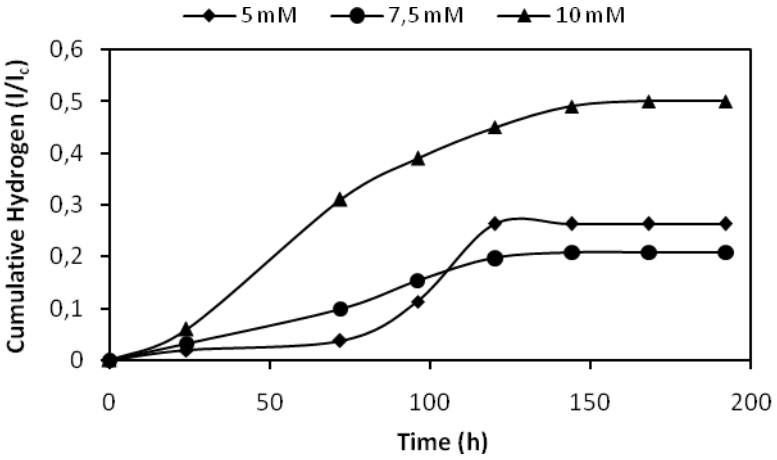


Figure 3.23 The cumulative hydrogen production by *R. capsulatus* (DSM 1710) on molasses media with different sucrose concentrations (5, 7.5 and 10 mM).

R. capsulatus (DSM 1710) started intensive hydrogen production after 18 hours and continued until 192 hours on molasses medium with 10 mM sucrose. Hydrogen was produced at a slow rate after 96 hours in the process. However at 5 mM and 7.5 mM sucrose containing molasses media, hydrogen production was lower.

Acetic, lactic, formic, propionic and butyric acids were detected in the effluents of PBRs. The concentration of formic acid was high at high sucrose concentrations. However, acetic acid production was higher at low sucrose concentrations. Formic acid was produced through the first 48 hours, then it was consumed by *R. capsulatus* (DSM 1710). Butyric acid production was also at great amounts. Lactic acid and propionic acid were the other organic acids. Shown in Table 3.9 is the concentrations of organic acids by *R. capsulatus* (DSM 1710).

Nearly half of the sucrose in molasses was utilized for growth and hydrogen production. The initial and final sucrose concentrations in PBRs were given in Table 3.10. Figure 3.24 is given for organic acid concentrations by *R. capsulatus* (DSM 1710) on molasses medium containing 5 mM sucrose.

Table 3.9 Concentrations of organic acids in photofermentation of *R. capsulatus* (DSM 1710) on 5 mM, 7.5 mM and 10 mM sucrose (molasses) hydrogen production media.

Hydrogen production media	5 mM Sucrose		7.5 mM Sucrose		10 mM Sucrose	
Organic acids	C_0 (mM)	C_f (mM)	C_0 (mM)	C_f (mM)	C_0 (mM)	C_f (mM)
Acetic acid	0.1	7.80	0	8.47	0.03	4.76
Formic acid	0	0.72	0	0.69	0	0.56
Lactic acid	0.02	2.81	0.05	1.77	0	0.68
Propionic acid	0	2.093	0.02	0	0	0
Butyric acid	0	1.30	0	2.48	0	3.31

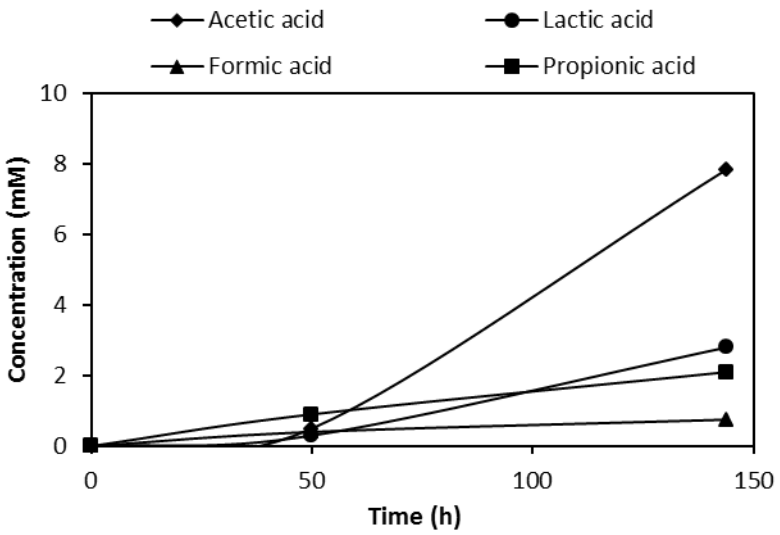


Figure 3.24 Organic acid concentrations of *R. capsulatus* (DSM 1710) on molasses medium containing 5 mM sucrose.

Table 3.10 The utilization of sucrose by *R. capsulatus* (DSM 1710) on 5 mM, 7.5 mM and 10 mM sucrose (molasses) hydrogen production media.

Sucrose concentrations (PBRs)	C_0 (mM)	C_f (mM)	Sucrose consumption (%)
5 mM	4.90	2.32	52.6
7.5 mM	7.47	3.36	55.0
10 mM	9.86	5.80	41.1

3.3.2 Experiments on *R. capsulatus* YO3 (Hup$^-$)

In this part uptake hydrogenase deleted mutant strain of *R. capsulatus* was investigated for growth and hydrogen production. The biomass growth was shown in Figure 3.25. Exponential growth phase was pursued till 120 hours except 5 mM sucrose containing PBR. In 5 mM sucrose PBR, after 48 hours a steady growth phase was observed continued towards 120 hours. Then, cell concentrations began to increase again through 150 hours at which the growth restart to slow down. This situation might be caused by the production of intermediates which could inhibit growth metabolism of the bacteria. The maximum cell growth were calculated as 1.2 gdcw/l_c, 0.96 gdcw/l_c and 1.18 gdcw/l_c respectively.

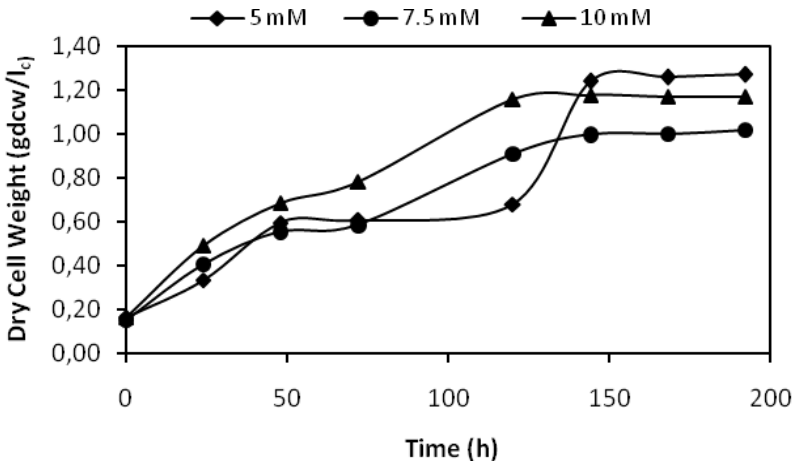


Figure 3.25 The growth of *R. capsulatus* YO3 (Hup-) in different sucrose concentrations (5, 7.5 and 10 mM) by dilutions of molasses.

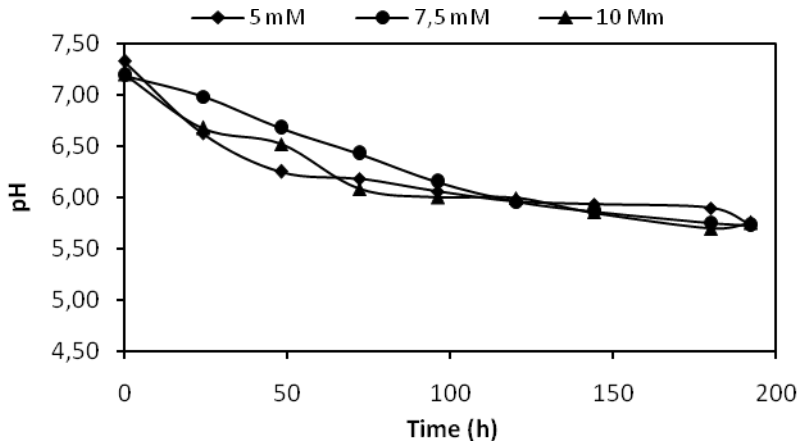


Figure 3.26 The pH variation during hydrogen production by *R. capsulatus* YO3 (Hup^-) on molasses with different sucrose concentrations (5, 7.5 and 10 mM).

The pH started to drop in each PBRs. The final pHs in all PBRs were around 5.5-5.8. Figure 3.26 shows the changes of pH in the PBRs. The complicated ingredients of molasses may bring about the fall in pH levels. Another strong reason might be the production of organic acids or other pH lowering compounds in photofermentation effluent.

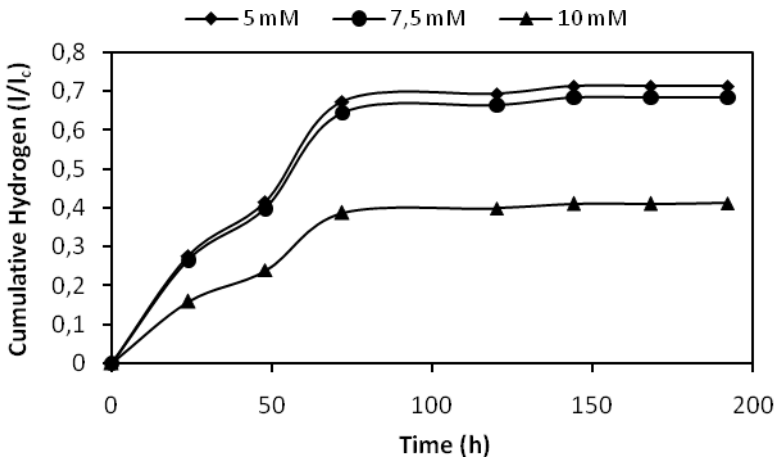


Figure 3.27 Figure 3.27 The cumulative hydrogen production by *R. capsulatus* YO3 (Hup-) on molasses with different sucrose concentrations (5, 7.5 and 10 mM).

Figure 3.27 shows the cumulative hydrogen production of *R. capsulatus* YO3 (Hup$^-$). More hydrogen was produced in 5 mM and 7.5 mM PBRs. The rate of hydrogen production was similar in both PBRs with a cumulative production of 0.6-0.7 l/l_c. The produced hydrogen was about two times higher than that of 10 mM PBR. It can be inferred that the hydrogen production metabolism was predominant over growth metabolism at sucrose concentrations lower than 10 mM.

Acetic acid produced and consumed simultaneously in all the PBRs. Formic acid first increase until 72th hours then drops. The concentration of propionic acid increase after 48th hours. More propionic acid was produced by *R.capsulatus* YO3 (Hup$^-$) rather than other bacterial strains. Lactic acid was produced and then used by the bacteria. Table 3.11 shows the initial and final concentrations of organic acids.

The sucrose was consumed more in 5 mM PBR than the others. The consumption of sucrose by *R.capsulatus* YO3 (Hup$^-$) was shown in Table 3.12.

Table 3.11 Concentrations of organic acids in photofermentation of *R.capsulatus* YO3 (Hup$^-$) on 5 mM, 7.5 mM and 10 mM sucrose (molasses) hydrogen production media.

Hydrogen production media	5 mM Sucrose		7.5 mM Sucrose		10 mM Sucrose	
Organic products	C_0 (mM)	C_f (mM)	C_0 (mM)	C_f (mM)	C_0 (mM)	C_f (mM)
Acetic acid	0	1.93	0.02	0.17	0	0.07
Formic acid	0	0.15	0	3.87	0	3.49
Lactic acid	0	3.69	0	0.09	0	0.05

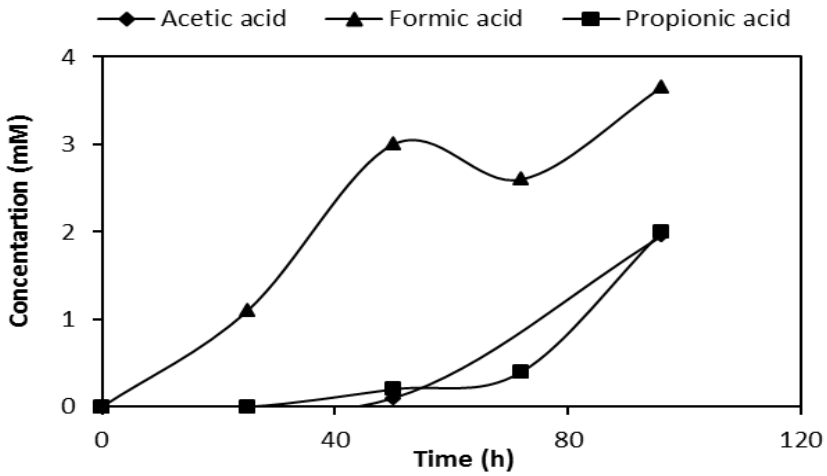


Figure 3.28 Acetic, lactic and propionic acid concentraitons during hydrogen production by *R. capsulatus* YO3 (Hup-) on molasses medium with 5 mM sucrose.

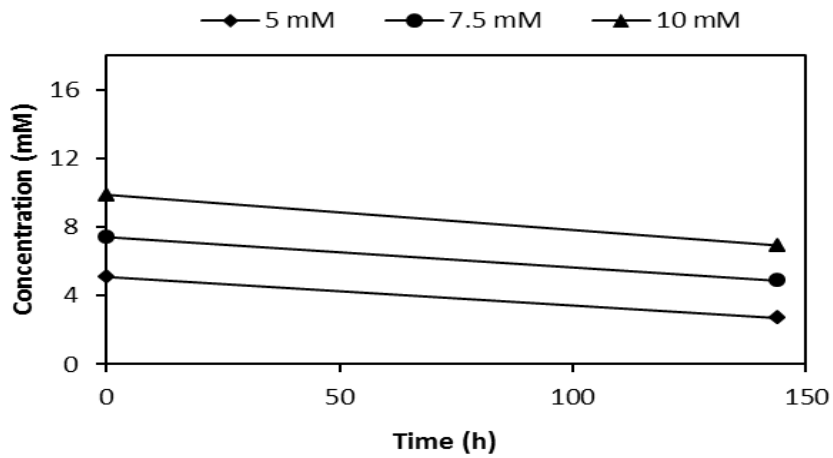


Figure 3.29 The consumption of sucrose by *R. capsulatus* YO3 (Hup^-).

Table 3.12 The utilization of sucrose by *R.capsulatus* YO3 (Hup^-) on 5 mM, 7.5 mM and 10 mM sucrose (molasses) hydrogen production media.

Sucrose concentrations (PBRs)	C_0 (mM)	C_f (mM)	Sucrose consumption (%)
5 mM	5.10	2.70	47.0
7.5 mM	7.42	4.87	34.3
10 mM	9.88	6.93	29.8

3.3.3 Experiments on *R. palustris* (DSM 127)

The growth, pH and hydrogen production by *R. palustris* (DSM 127) on molasses media containing different concentrations of sucrose (5 mM, 7.5 mM and 10 mM) were investigated for 192 hours. Shown in Figure 3.30 is the growth of *R. palustris* (DSM 127) on hydrogen production media.The biomass growth of 10 mM sucrose was greater than the other two PBRs. The maximum biomass was observed at 144 hours as 0.4 gdcw/l_c.

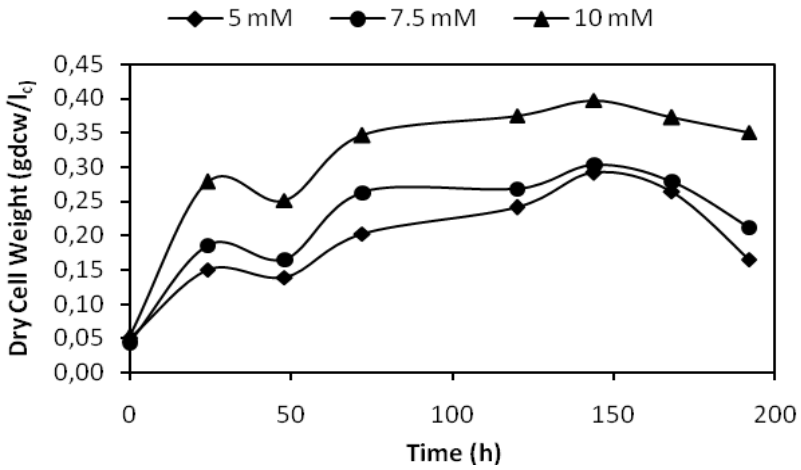


Figure 3.30 The growth of *R. palustris* (DSM 127) on molasses media with different sucrose concentrations (5, 7.5 and 10 mM).

The change of pH in PBRs was illustrated in Figure 3.31 The pH decreased from 7.3-7.4 to 6.5 in 5 mM and 7.5 mM sucrose, whereas the final pH was 5.8 in 10 mM sucrose. It was stabilized after 72 hours by all the three PBRs. This may be resulted due the consumption of organic acids by the carbon metabolism of PNS bacteria. Given in Figure 3.32 is the cumulative hydrogen production of *R. palustris* (DSM 127). The cumulative hydrogen production of 10 mM sucrose PBR was 1.4 l/l_c and it was higher than the other PBRs. 1.2 l/l_c and 1.0 l/l_c hydrogen was produced by 7.5 Mm and 5 mM sucrose from molasses media, respectively. Hydrogen production was produced mostly during 0-48 hours time interval.

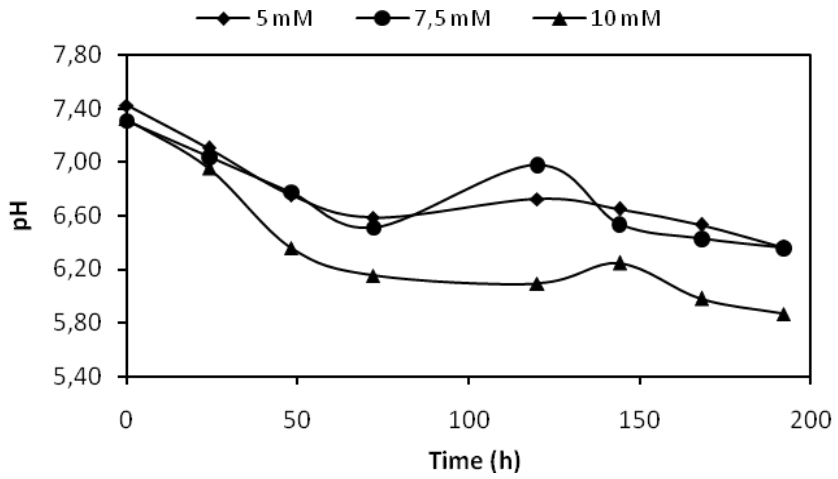


Figure 3.31 The variation in pH during hydrogen production by *R. palustris* (DSM 127) on molasses media with different sucrose concentrations (5, 7.5 and 10 mM).

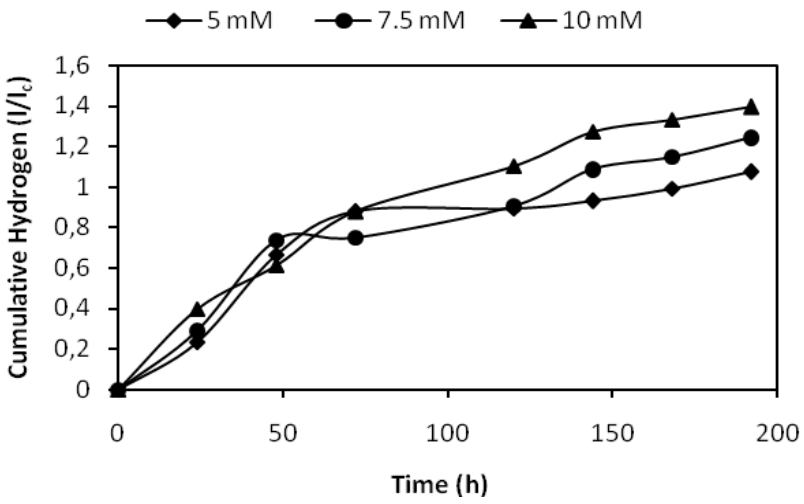


Figure 3.32 The cumulative hydrogen production of *R. palustris* (DSM 127) on molasses media with different sucrose concentrations (5, 7.5 and 10 mM).

Table 3.13 shows the organic acid production and consumption in PBRs by *R. palustris* (DSM 127). Acetate was mostly produced in PBR with 10 mM sucrose, while formic acid was predominant product in 5 mM and 7.5 mM sucrose containing PBRs through the process. Lactic acid and and acetic acid were highly produced in 10 mM sucrose containing PBR whereas formic acid concentration nearly constant. Trace amount of propionic was also present in the same medium.

The sucrose consumption of the *R. palustris* (DSM 127) was given in Table 3.14. The percentages of sucrose consumption by 5 mM PBR, 7.5 mM and 10 mM photobioreactors were measured as 51%, 59% and 28%, respectively.

Table 3.13 Concentrations of organic acids in photofermentation of *R. palustris* (DSM 127) on 5 mM, 7.5 mM and 10 mM sucrose (molasses) hydrogen production media.

Hydrogen production media	5 mM Sucrose		7.5 mM Sucrose		10 mM Sucrose	
Organic acids	C_0 (mM)	C_f (mM)	C_0 (mM)	C_f (mM)	C_0 (mM)	C_f (mM)
Acetic acid	0.967	1.624	1.435	1.246	0.692	6.539
Formic acid	3.148	6.878	4.228	9.371	3.726	3.738
Lactic acid	1.04	0.92	1.34	0.81	1.15	5.37

Table 3.14 The utilization of sucrose by *R. palustris* (DSM 127) on 5 mM, 7.5 mM and 10 mM sucrose (molasses) hydrogen production media.

Sucrose concentrations (PBRs)	C_0 (mM)	C_f (mM)	Sucrose consumption (%)
5 mM	4.98	2.43	51.2
7.5 mM	7.41	3.02	59.2
10 mM	9.58	6.9	28.0

3.3.4 Experiments on *R. sphaeroides* O.U.001 (DSM 5864)

The biomass growth of *R. sphaeroides* O.U.001 (DSM 5864) in 10 mM sucrose PBR was the highest as 0.8 gdcw/l_c during the operation. Shown in Figure 3.33 is the biomass growth of *R. sphaeroides* O.U.001 (DSM 5864).

The growth of 5 mM sucrose PBR showed a fluctuated growth curve, but the maximum hydrogen was produced in this PBR. The maximum hydrogen production

was 0.66 l/l_c, 0.4 l/l_c and 0.60 l/l_c in order of increasing sucrose concentrations (5-7.5-10 mM) and given in Figure 3.35.

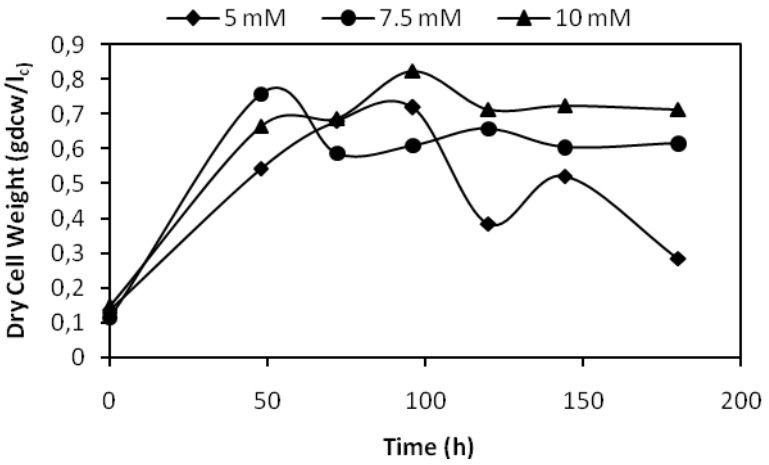


Figure 3.33 The growth of *R. sphaeroides* O.U.001 (DSM 5864) on molasses media with different sucrose concentrations (5, 7.5 and 10 mM).

R. sphaeroides O.U.001 (DSM 5864) had a more stable pH through the 180 hours photofermentation especially in 7.5 mM and 10 mM sucrose PBRs. The range of measured pH values were 6.2-6.8 during the operation time and shown in Figure 3.34. *R. sphaeroides* O.U.001 are not able to grow under pH of 6.0 (Sasikala et al., 1995).

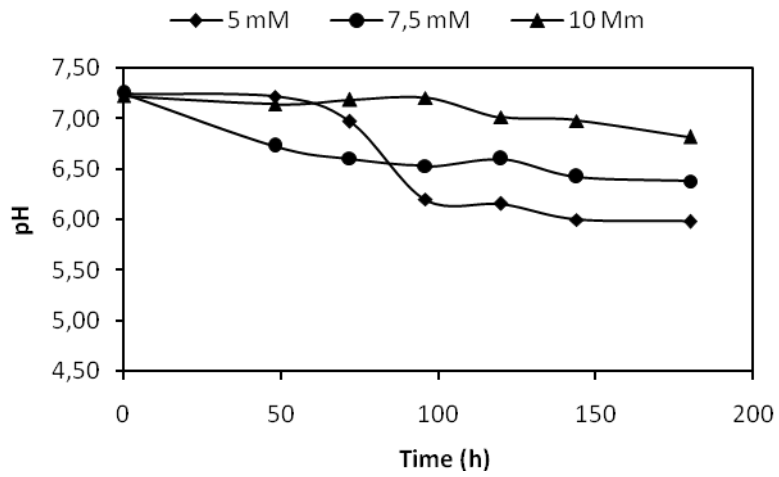


Figure 3.34 The variation in pH during hydrogen production by *R. sphaeroides* O.U.001 (DSM 5864) on molasses media with different sucrose concentrations (5, 7.5 and 10 mM).

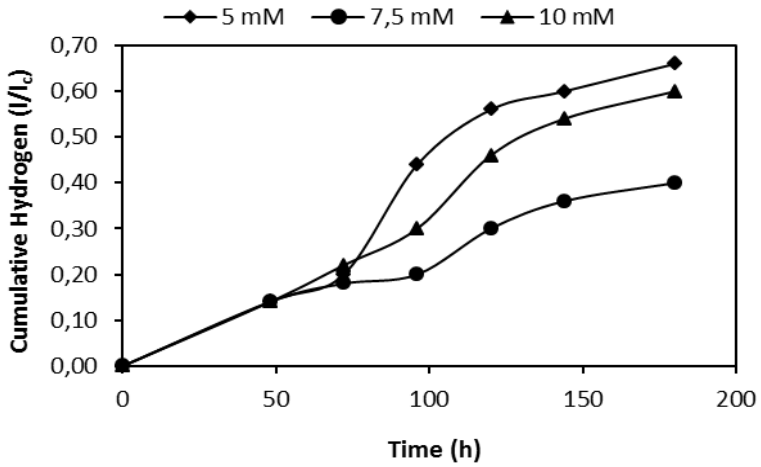


Figure 3.35 The cumulative hydrogen production by *R. sphaeroides* O.U.001 (DSM 5864) on molasses media with different sucrose concentrations (5, 7.5 and 10 mM).

Lactic acid was first consumed and then produced again. It was probably the first preferred substrate rather than others. Sevinç (2010) also reported that lactic acid was used primarily by the *R. capsulatus* DSM 1710 in a acetate-lactate hydrogen

medium. Acetic acid seems to be the secondary utilized organic acid in the hydrogen production medium. In 5 mM and 10 mm PBRs, formic acid was consumed through first 72 hours. After that, the concentration of formic acid increased dramatically. Table 3.15 shows the concentrations of organic acids during photofermentation of *R. Sphaeroides* O.U.001. Formic acid production with *R. sphaeroides* O.U.001 was reported due to low light intensity (Eroğlu, 2008). All of the organic acids were produced after 72th hours in 7.5 mM sucrose medium. Sucrose consumption was approximately 50% in all of the PBRs, and shown in Table 3.16.

Table 3.15 Concentrations of organic acids in photofermentation of *R. sphaeroides* O.U.001 (DSM 5864) on 5 mM, 7.5 mM and 10 mM sucrose (molasses) hydrogen production media.

Hydrogen production media	5 mM Sucrose		7.5 mM Sucrose		10 mM Sucrose	
Organic acids	C_0 (mM)	C_f (mM)	C_0 (mM)	C_f (mM)	C_0 (mM)	C_f (mM)
Acetic acid	0.241	3.298	0.365	0.784	1.129	0.313
Formic acid	0.958	5.373	1.509	11.405	3.417	4.944
Lactic acid	0.41	2.37	0.59	1.07	1.22	0.03

Table 3.16 The utilization of sucrose by *R. palustris* (DSM 127) on 5 mM, 7.5 mM and 10 mM sucrose (molasses) hydrogen production media.

Sucrose concentrations (PBRs)	C_0 (mM)	C_f (mM)	Sucrose consumption (%)
5 mM	5.12	2.51	50.9
7.5 mM	7.56	3.54	53.1
10 mM	9.53	4.47	53.0

3.4 Comparison of the Experiments

R. palustris (DSM 127) had the highest biomass growth (1.5 gdcw/l_c) on 5 mM defined sucrose media. On the other hand, *R. capsulatus* YO3 (Hup^-) have grown well on 5 mM sucrose from molasses medium with a maximum biomass of 1.25 gdcw/l_c. *R. sphaeroides* O.U.001 showed good growth profiles in both defined and molasses media. A more stable pH change could be the reason of having such growth curves. Total hydrogen production was greater with *R. palustris* (DSM 127) than the other bacteria in both defined sucrose and molasses hydrogen media. In 50 ml PBRs, 3.34 and 3.10 mmol hydrogen were produced during 192 hours by *R. palustris* (DSM 127) on defined sucrose and molasses media respectively. Figure 3.17 and Figure 3.19 is given for total hydrogen production of PNS bacteria through certain operation periods.

Hydrogen productivities were generally in higher values on defined media than molasses media. On the contrary, substrate conversion efficiencies were greater on molasses media than defined media. This is actually a pleasurable situation in terms of utilization of molasses. Shown in Figure 3.32 and Figure 3.33 are the comparison of hydrogen productivities. The productivities of *R. capsulatus* YO3 (Hup^-) on molasses was much higher than that of *R. capsulatus* (DSM 1710). Özgür et al., (2009) reported that *R. capsulatus* YO3 (Hup^-) had better productivity than *R. capsulatus* (DSM 1710) on 30 mM acetate/7.5 mM Lactate.

Table 3.17 Duration of the processes and total hydrogen production of different PNS bacteria on defined sucrose media in 50 ml bioreactors.

Microorganism	**Sucrose concentration (mM)**	**Duration of process (h)**	**Total Hydrogen (mmol H_2)**
R. capsulatus (DSM 1710)	5	180	0.45
	7.5	180	1.91
	10	180	1.34
R. capsulatus YO3 (Hup^-)	5	216	1.78
	7.5	216	1.11
	10	216	0.67
R. palustris (DSM 127)	5	192	3.03
	7.5	192	3.34
	10	192	2.46
R. sphaeroides O.U.001	5	180	1,91
	7.5	180	1,34
	10	180	1,65

It was observed that molasses media had generally more substrate conversion efficiency than defined sucrose media. 96.6% efficiency was obtained by *R. palustris* (DSM 127) on 10 mM sucrose from molasses. Substrate conversion efficiencies of *R. palustris* (DSM 127) and *R. sphaeroides* O.U.001 were 53.3% and 41.7% on 5 mM defined sucrose media.

Figure 3.32 is given for substrate conversion efficiencies of PNS bacteria. The experimental results on sucrose indicated that substrate conversion efficiencies varied from 7.41% to 96.6% by PNS bacteria.

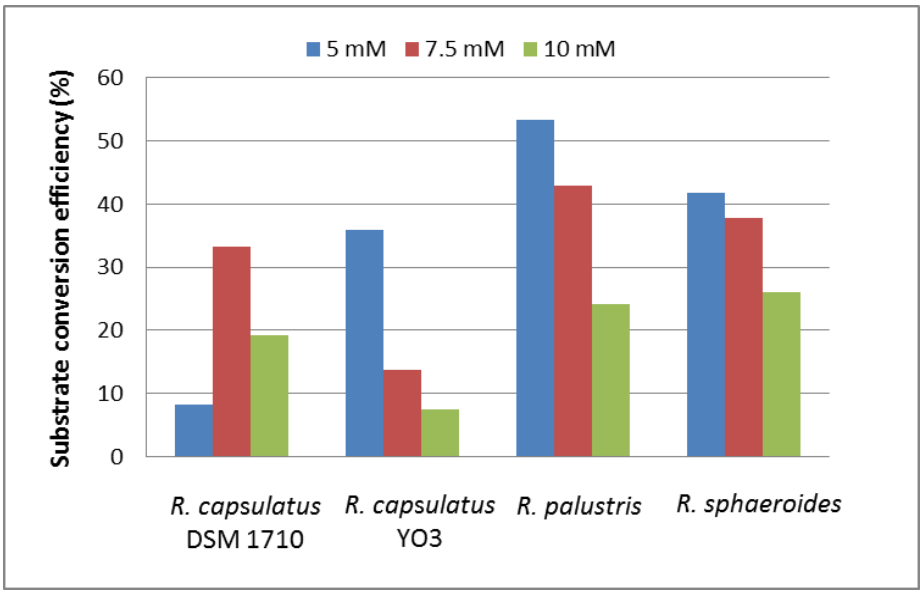


Figure 3.36 The substrate conversion efficiencies of different PNS bacteria on various sucrose concentrations in 50 ml bioreactors.

The concentration of sucrose also affects the substrate conversion efficiencies and hydrogen productivities (Figure 3.36). These parameters were generally lower when sucrose concentration is increased.

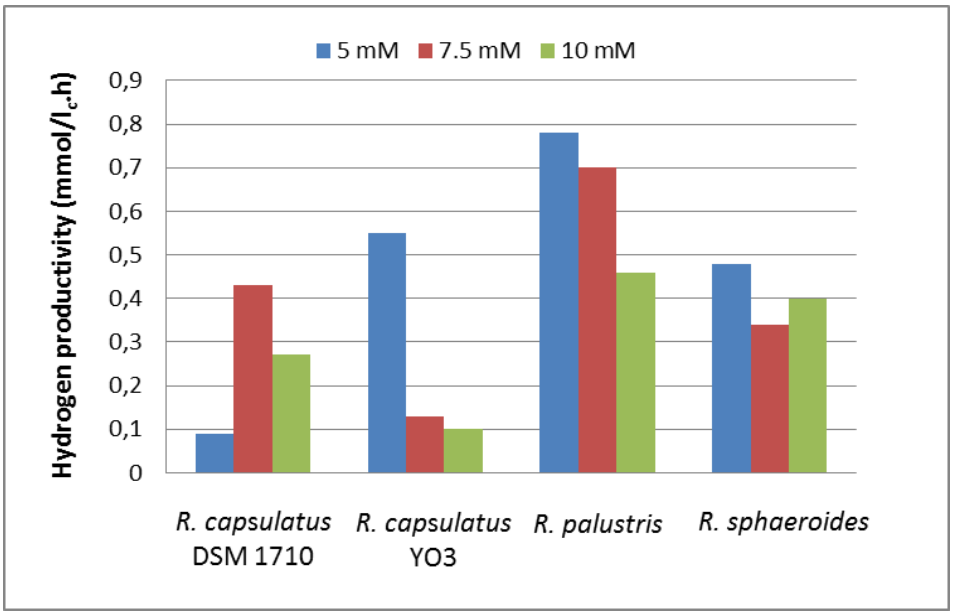


Figure 3.37 The hydrogen productivities of different PNS bacteria on various sucrose concentrations in 50 ml bioreactors.

Table 3.18 Summary of the results with different PNS bacteria on defined sucrose media in 50 ml bioreactors.

Microorganism	**Sucrose concentration (mM)**	**Biomass ($gdcw/l_c$)**	**Hydrogen productivity ($mmol/l_c.h$)**	**Substrate conversion efficiency (%)**	**Sucrose consumption (%)**	**Light conversion efficiency (%)**
R. capsulatus DSM 1710	5	0.60	0.09	8.14	100	0.07
	7.5	0.58	0.43	33.2	71.0	0.31
	10	0.57	0.27	19.3	50.04	0.21
R. capsulatus YO3 (Hup^-)	5	0.62	0.55	35.9	85.0	0.24
	7.5	0.55	0.13	13.8	85.5	0.15
	10	0.51	0.10	7.41	70.0	0.09
R. palustris DSM 127	5	1.40	0.78	53.3	96.1	0.46
	7.5	1.50	0.70	43.0	81.1	0.51
	10	1.10	0.46	24.2	88.9	0.37
R. sphaeroides O.U.001	5	0.60	0.48	41.7	78.6	0.30
	7.5	0.68	0.34	37.8	40.0	0.22
	10	0.68	0.40	26.0	53.5	0.27

Table 3.19 Duration of the processes and total hydrogen production of different PNS bacteria on molasses in 50 ml bioreactors.

Microorganism	**Sucrose concentration (mM)**	**Duration of process (h)**	**Total Hydrogen (mmol H_2)**
R. capsulatus (DSM 1710)	5	192	0.59
	7.5	192	0.47
	10	192	1.11
R. capsulatus YO3 (Hup^-)	5	192	1.59
	7.5	192	1.20
	10	192	0.69
R. palustris (DSM 127)	5	192	2.39
	7.5	192	2.76
	10	192	3.10
R. sphaeroides O.U.001	5	180	1.47
	7.5	180	0.89
	10	180	1.34

Shown in Figure 3.34 and Figure 3.35 are substrate conversion efficiencies and hydrogen productivities of PNS bacteria on molasses with different sucrose concentrations (5-7.5-10 mM).

Table 3.20 Summary of the results with different PNS bacteria on molasses in 50 ml bioreactors.

Microorganism	Sucrose concentration (mM)	Biomass ($gdcw/l_c$)	Hydrogen productivity ($mmol/l_c.h$)	Substrate conversion efficiency (%)	Sucrose consumption (%)	Light conversion efficiency (%)
R. capsulatus DSM 1710	5	0.6	0.20	18.9	52.6	0.09
	7.5	0.3	0.07	9.45	55.0	0.07
	10	0.2	0.18	22.9	41.1	0.16
R. capsulatus YO3 (Hup^-)	5	1.25	0.41	55.2	47.0	0.243
	7.5	0.98	0.40	39.2	34.3	0.18
	10	1.21	0.24	19.6	29.8	0.10
R. palustris DSM 127	5	0.4	0.55	78.3	51.2	0.36
	7.5	0.3	0.47	52.7	59.2	0.42
	10	0.28	0.54	96.6	28.0	0.47
R. sphaeroides O.U.001	5	0.7	0.27	47.1	50.9	0.23
	7.5	0.75	0.12	18.5	53.1	0.15
	10	0.8	0.18	22.0	53.0	0.21

The substrate conversion efficiencies and hydrogen productivities on molasses are shown in Figure 3.38 and 3.39, respectively.

Sucrose consumption (%) was calculated by measuring the final and initial sucrose concentrations in the media via HPLC analysis. Table 3.18 shows the consumption of sucrose by PNS bacteria. *R. palustris* (DSM 127) and *R. capsulatus* (DSM 1710) consumed more sucrose than the other strains on molasses. The consumption of sucrose were of 59.2% and 55.0% by those bacteria. The range of sucrose consumption varied between 41.1-59.2% on molasses media.

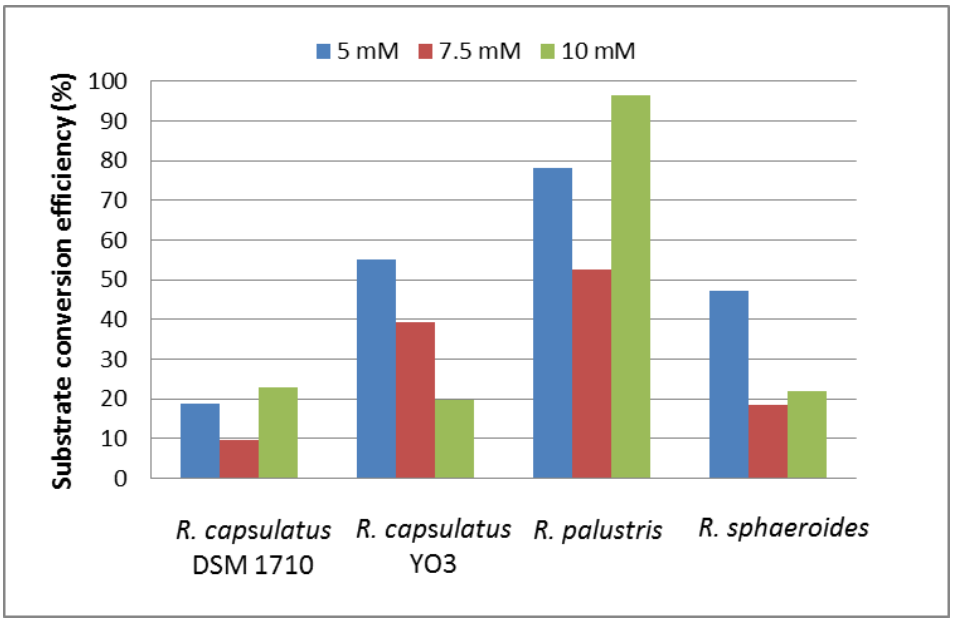


Figure 3.38 The substrate conversion efficiencies of different PNS bacteria on molasses with different sucrose concentrations (5-7.5-10 mM).

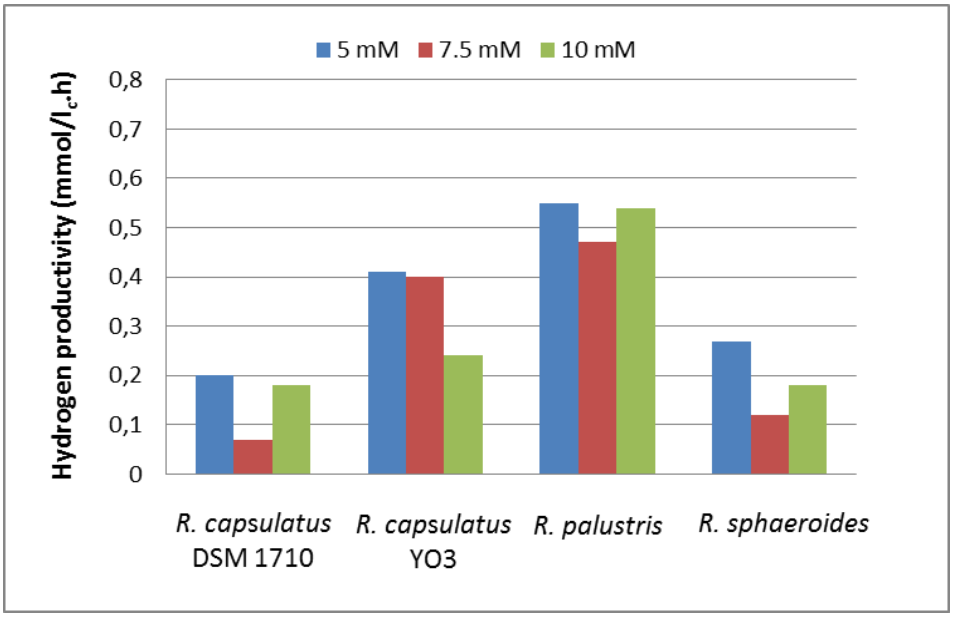


Figure 3.39 The hydrogen productivities of different PNS bacteria on molasses with different sucrose concentrations (5-7.5-10 mM).

3.5 Co-cultivation of PNS Bacteria

The objectives of the co-cultivation studies were improving hydrogen production, bacterial growth and pH stabilization by PNS bacterial co-cultures in photobioreactors.

In this part of the study different strains of PNS bacteria such as *R. capsulatus* YO3 (Hup^-), *R. palustris* (DSM 127) and *R. sphaeroides* O.U.001 (DSM 5864) were co-cultivated for hydrogen production on sucrose (5 mM) for all the photobioreactors. The growth ad hydrogen production parameters were studied with 150 ml photobioreactors with an illumination of 2000-2200 lux, and a temperature of 30-32 ^oC at cooling incubators.

Table 3.19 Co-cultured bacterial strains.

Co-cultured Bacteria
R. capsulatus YO3 (Hup^-) and *R. palustris* (DSM 127)
R. sphaeroides O.U.001 (DSM 5864) and *R. palustris* (DSM 127)
R. capsulatus YO3 (Hup^-) and *R. sphaeroides* O.U.001 (DSM 5864)
R. capsulatus YO3 (Hup^-), *R. sphaeroides* O.U.001 (DSM 5864) and *R. palustris* (DSM 127)

3.6.2 Co-cultivation of *R. capsulatus* YO3 (Hup$^-$) and *R. palustris* (DSM 127)

The bacterial inoculations 5% from each strain, was done with freshly grown cultures of *R. capsulatus* YO3 (Hup$^-$) and *R. palustris* (DSM 127) when their optical density (OD660) reached 2.0. Photobiological hydrogen production was followed in 150 ml PBRs on molasses medium with 5 mM of sucrose.

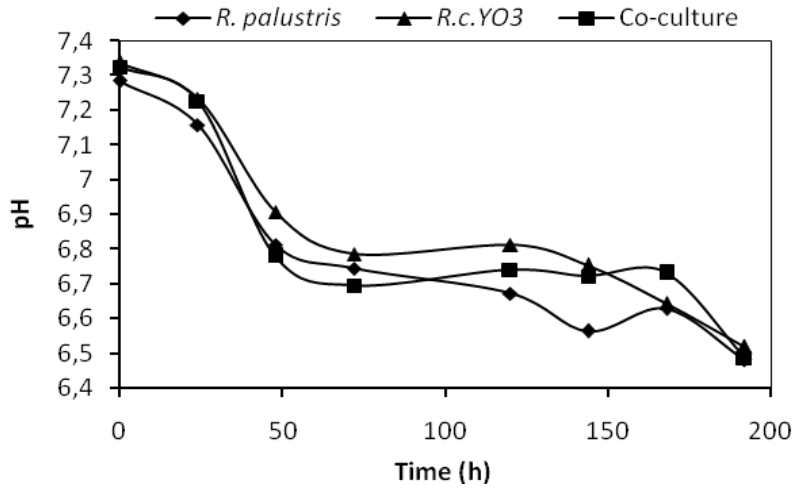


Figure 3.40 The pH variation in single cell and co-cultures of *R. capsulatus* YO3 (Hup-) and *R. palustris* (DSM 127), during hydrogen production on molasses medium with 5 mM sucrose.

The pH range was between 6.5-7.3 in both single cell cultures and co-culture of *R. capsulatus* YO3 (Hup$^-$) and *R. palustris* (DSM 127). The decline in pH was observed between 0-48 hours in all the PBRs. The biomass growth of the co-culture showed a more stable profile rather than single cell cultures. The maximum biomass of the single cell cultures and the co-culture were 0.68 gdcw/l_c, 0.94 gdcw/l_c, and 0.95 gdcw/l_c respectively. The growth showed more fluctuations in the single cell cultures. The pHs and biomass growth (OD) of both single cell cultures and co-culture of *R. capsulatus* YO3 (Hup$^-$) and *R. palustris* (DSM 127) were shown in Figure 3.40 and Figure 3.41 respectively.

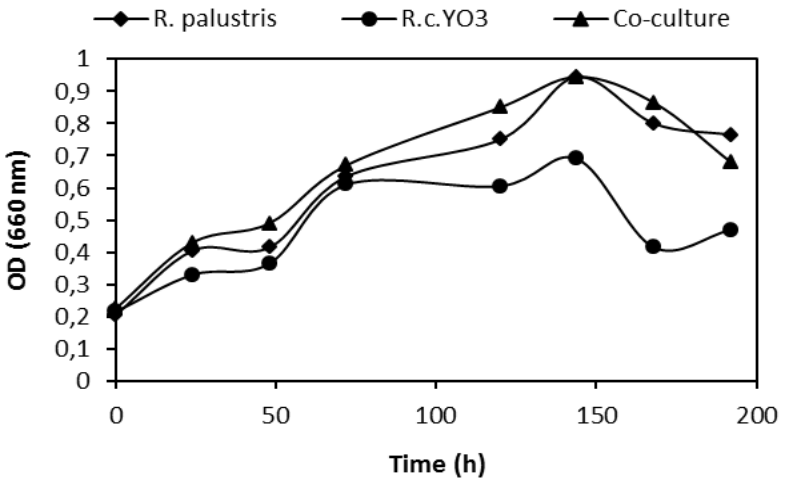


Figure 3.41 The growth of single cell and co-cultures of *R. capsulatus* YO3 (Hup-) and *R. palustris* (DSM 127) on molasses medium with 5 mM sucrose.

The maximum hydrogen production of the co-culture was 0,918 l/l_c. The cumulative hydrogen production was the greatest (1.73 l/l_c) with the single cell culture of *R. palustris* (DSM 127). The maximum hydrogen was 0,54 in *R. capsulatus* YO3 (Hup^-). Given in Figure 3.42 is the cumulative hydrogen production curves in single cell and co-cultures of *R. capsulatus* YO3 (Hup^-) and *R. palustris* (DSM 127).

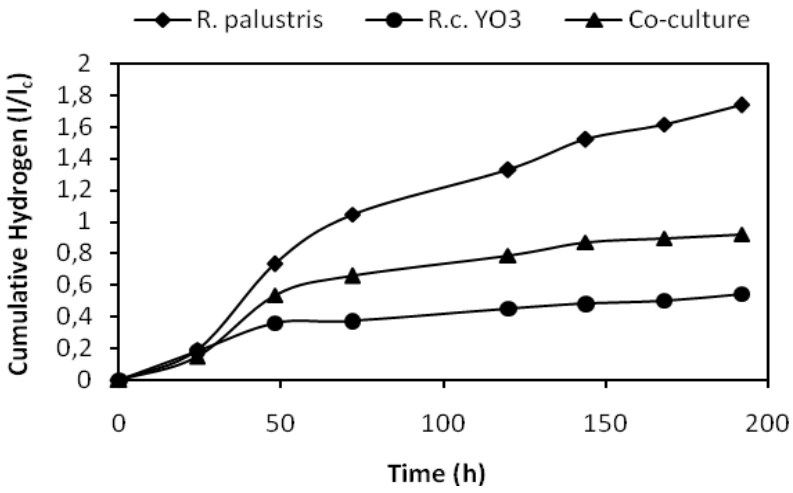


Figure 3.42 The cumulative hydrogen production of single cell and co-cultures of *R. capsulatus* YO3 (Hup$^-$) and *R. palustris* (DSM 127) on molasses medium with 5 mM sucrose.

Formic acid, lactic acid and acetic acid were the products of the photofermentation during 192 hours. In the single cell cultures, all of these organic acids were just produced through the operation time. There could be small negligible reductions in their amounts. On the other hand, the concentrations of formic acid, lactic acid and acetic acid raised up for the first 72 hours. After that, the concentrations of the organic acids began to decrease and finish at the end of the process. Shown in Table 3.18 is the concentrations of the organic acids in co-cultivation of *R. capsulatus* YO3 (Hup$^-$) and *R. palustris* (DSM 127).

The sucrose consumption was achieved as 97.6% by the co-culture of *R. capsulatus* YO3 (Hup$^-$) and *R. palustris* (DSM 127). The single cell cultures consumed sucrose as 53.3% and 51.2% of the concentrations. Table 3.21 indicates the sucrose consumption of the co-cultivation of *R. capsulatus* YO3 (Hup$^-$) and *R. palustris* (DSM 127).

Table 3.20 The concentrations of the organic acids in co-cultivation of *R. capsulatus* YO3 (Hup$^-$) and *R. palustris* (DSM 127).

Hydrogen production media	*R. palustris*		*R. capsulatus* YO3		Co-culture (*R. palustris*-*R. capsulatus* YO3)	
Organic acids	C_0 (mM)	C_f (mM)	C_0 (mM)	C_f (mM)	C_0 (mM)	C_f (mM)
Acetic acid	0.583	3.068	0.531	3.466	0.913	0.481
Formic acid	1.810	10.66	1.079	6.365	2.781	0.168
Lactic acid	0.68	2.27	0.54	1.78	0.90	0.04

C_0 * The initial concentration (mM)

C_f * The final concentration (mM)

Table 3.21 The sucrose consumption (%) during growth and hydrogen production by single cell and co-cultures of *R. capsulatus* YO3 (Hup-) and *R. palustris* (DSM 127) on molasses medium with 5 mM sucrose.

(PBRs) Sucrose (5 mM)	C_0 (mM)	C_f (mM)	Sucrose consumption (%)
R. palustris	5.012	2.34	53.3
R. capsulatus YO3	4.98	2.43	51.2
Co-culture (*R. palustris*-*R. capsulatus* YO3)	5.02	0.12	97.6

3.6.3 Co-cultivation of *R. palustris* (DSM 127) and *R. sphaeroides* O.U.001 (DSM 5864)

The bacterial inoculation (5% from each strain) was done with freshly grown cultures of *R. palustris* (DSM 127) and *R. sphaeroides* O.U.001 (DSM 5864) when their optical density (OD660) reached 2.0. Photobiological hydrogen production was followed in 150 ml PBRs on molasses medium with 5 mM of sucrose. The process was continued for 240 hours. The pHs of the PBRs declined through the time. The range of pH was 7.4-5.5 in all the media (Figure 3.43). The pH stayed at between 6.0-7.4 in the co-culture whereas drops in pH was observed mostly in single cell *R. palustris* (DSM 127).

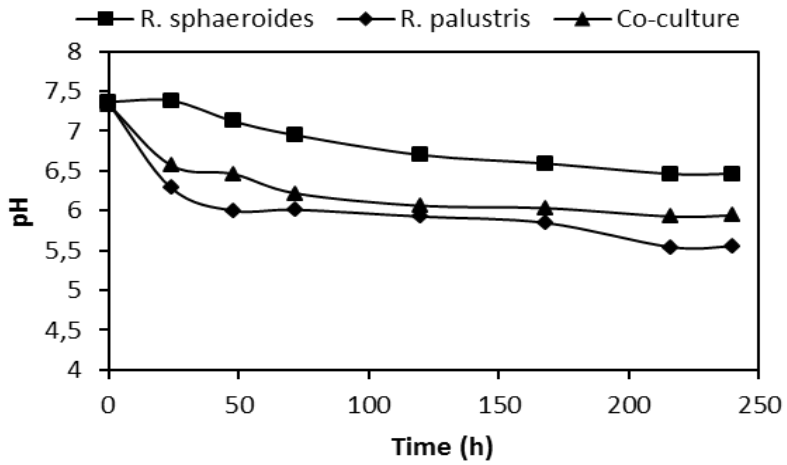


Figure 3.43 The pH variation in single cell and co-cultures of *R. sphaeroides* O.U.001 (DSM 5864) and *R. palustris* (DSM 127), during hydrogen production on molasses medium with 5 mM sucrose.

The cell growth was greater in *R. sphaeroides* O.U.001 (DSM 5864) and co-culture. The maximum biomass was 1.10 gdcw/l_c and 1.15 gdcw/l_c respectively. Given in Figure 3.44 is the biomass growth (OD) of single cell and co-cultures of *R. palustris* (DSM 127) and *R. sphaeroides* O.U.001 (DSM 5864).

The maximum hydrogen was about the same as 1.19 l/l_c in both co-cultivation and single cell *R. palustris* (DSM 127).

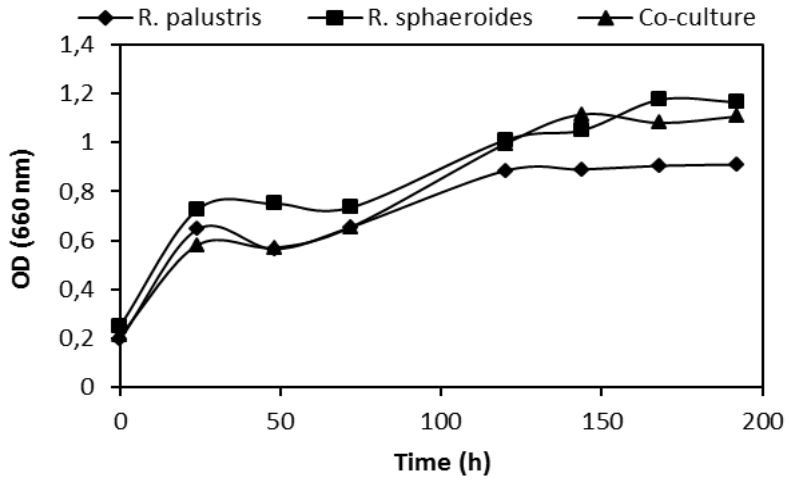


Figure 3.44 The growth of single cell and co-cultures of *R. sphaeroides* O.U.001 (DSM 5864) and *R. palustris* (DSM 127) on molasses medium with 5 mM sucrose.

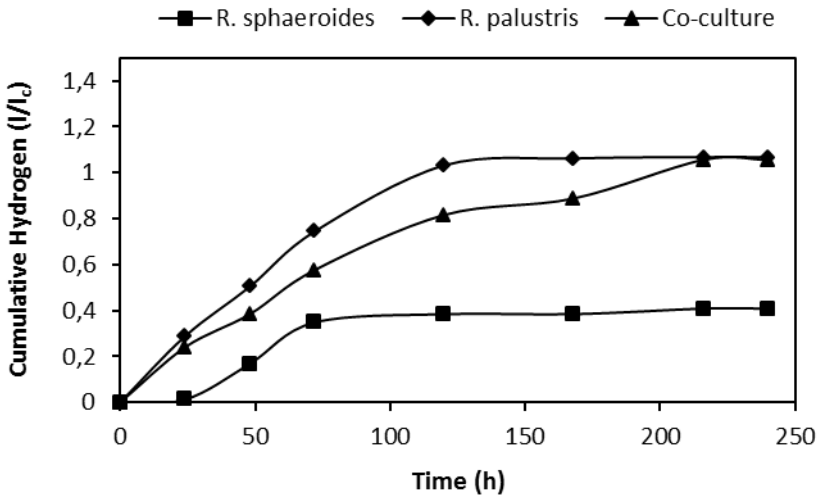


Figure 3.45 The cumulative hydrogen production of single cell and co-cultures of *R. sphaeroides* O.U.001 (DSM 5864) and *R. palustris* (DSM 127) on molasses medium with 5 mM sucrose.

The production of the formic acid, acetic acid and lactic acid increased through the operation time. Trace amounts of propionic acid was also produced. The production and consumption of organic acids is given in Table 3.22.

Table 3.22 The concentrations of the organic acids in co-cultivation of *R. palustris* (DSM 127) and *R. sphaeroides* O.U.001 (DSM 5864).

Hydrogen production media	*R. palustris*		*R. sphaeroides*		Co-culture (*R. palustris*-*R. sphaeroides*)	
Organic acids	C_0 (mM)	C_f (mM)	C_0 (mM)	C_f (mM)	C_0 (mM)	C_f (mM)
Acetic acid	0.181	7.98	0.129	1.028	0.408	5.516
Formic acid	1.58	4.68	0.50	15.54	1.56	5.75
Lactic acid	0.36	9.47	0.23	1.28	0.64	4.84

The consumption of sucrose (%) was about same and 50% by the single cell cultures and the co-culture of *R. palustris* (DSM 127) and *R. sphaeroides* O.U.001 (DSM 5864).

Table 3.23 The sucrose consumption (%) in co-cultivation of *R. palustris* (DSM 127) and *R. sphaeroides* O.U.001 (DSM 5864).

(PBRs) Sucrose (5 mM)	C_0 (mM)	C_f (mM)	Sucrose consumption (%)
R. palustris	4.97	2.40	50.07
R. sphaeroides	5.10	2.48	51.3
Co-culture (*R. palustris*-*R. sphaeroides*)	4.89	2.53	50.07

3.6.2 Co-cultivation of *R. capsulatus* YO3 (Hup$^-$) and *R. sphaeroides* O.U.001 (DSM 5864)

The bacterial inoculation (5% from each strain) was done with freshly grown cultures of *R. capsulatus* YO3 (Hup$^-$) and *R. sphaeroides* O.U.001 (DSM 5864) when their optical density (OD660) reached 2.0. Photobiological hydrogen production was followed in 150 ml PBRs on molasses medium with 5 mM of sucrose. The process was continued for 240 hours.

The range of pH was between 7.4-6.0 in all the PBRs. Figure 3.46 shows the pH changes in co-cultivation of *R. capsulatus* YO3 (Hup$^-$) and *R. sphaeroides* O.U.001 (DSM 5864).

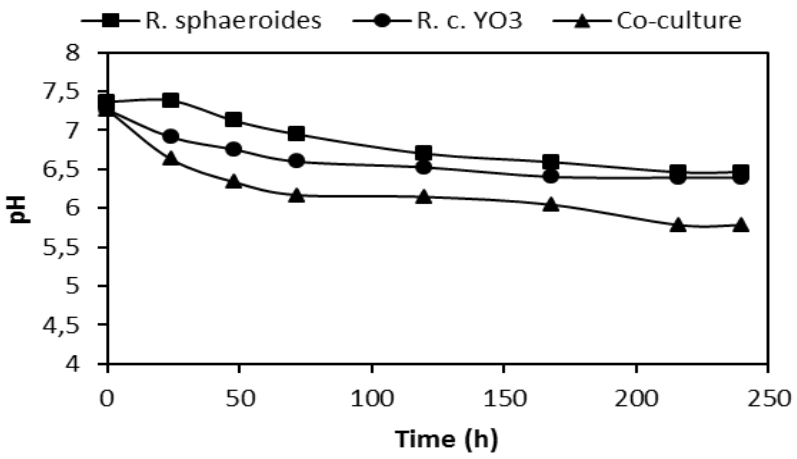


Figure 3.46 The pH variation in single cell and co-cultures of *R. capsulatus* YO3 (Hup-) and *R. sphaeroides* O.U.001 (DSM 5864) on molasses medium with 5 mM sucrose.

R. capsulatus YO3 (Hup^-) had the lowest biomass growth as 0.61 gdcw/l_c. The growth of *R. sphaeroides* O.U.001 (DSM 5864) was the greatest (1.18 gdcw/l_c) of all the PBRs. Co-culture of these two strains had a maximum growth as 1.02 gdcw/l_c. Figure 3.47 shows the growth curves of single cell and co-cultures of *R. capsulatus* YO3 (Hup^-) and *R. sphaeroides* O.U.001 (DSM 5864).

Hydrogen production of the co-culture were promising and the greatest of all the PBRs. The maximum hydrogen was 1.25 l/ l_c, 0.70 l/ l_c and 0.47 l/ l_c in order of co-culture, *R. capsulatus* YO3 (Hup^-) and *R. sphaeroides* O.U.001 (DSM 5864). Given in Figure 3.44 is the cumulative hydrogen production in co-cultivation of *R. capsulatus* YO3 (Hup^-) and *R. sphaeroides* O.U.001 (DSM 5864).

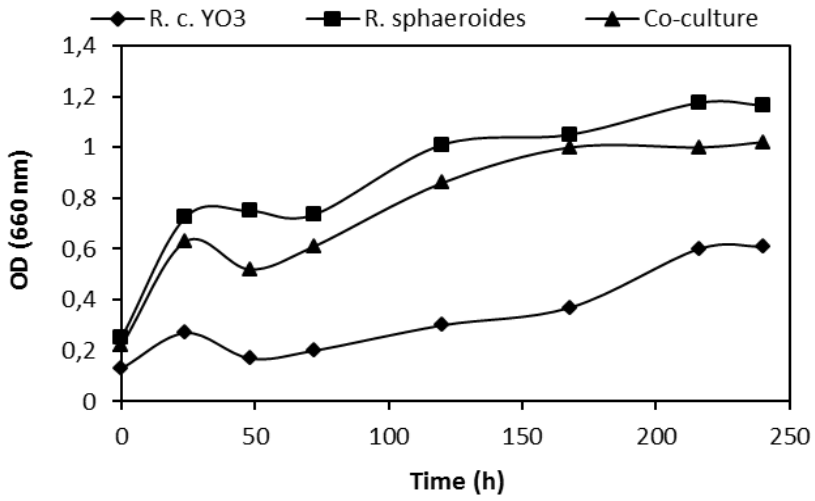


Figure 3.47 The growth of single cell and co-cultures of *R. capsulatus* YO3 (Hup-) and *R. sphaeroides* O.U.001 (DSM 5864) on molasses medium with 5 mM sucrose.

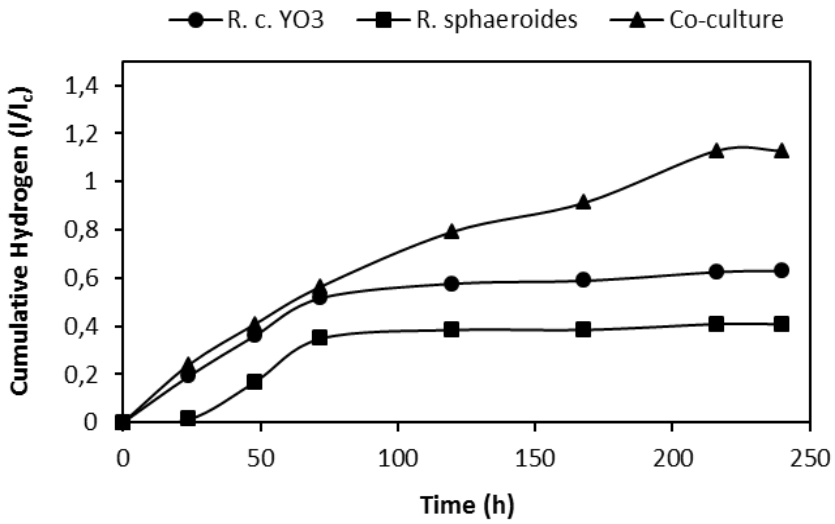


Figure 3.48 The cumulative hydrogen production of single cell and co-cultures of *R. capsulatus* YO3 (Hup-) and *R. sphaeroides* O.U.001 (DSM 5864) on molasses medium with 5 mM sucrose.

Formic acid were produced at high amounts especially in single cell cultures. The production of acetic acid was greater in co-culture than the single cell cultures. Propionic acid amount was greater in fermentation medium than lactic acid and butyric acid. Table 3.24 is given for the concentrations of organic acids in co-cultivation of *R. capsulatus* YO3 (Hup$^-$) and *R. sphaeroides* O.U.001 (DSM 5864).

50.9% of the initial sucrose was consumed through 240 hours. *R. capsulatus* YO3 (Hup$^-$) utilized sucrose at the highest rate as 94.9 and shown in Table 3.25.

Table 3.24 The concentrations of the organic acids in co-cultivation of *R. capsulatus* YO3 (Hup$^-$) and *R. sphaeroides* O.U.001 (DSM 5864).

Hydrogen production media	*R. capsulatus* YO3		*R. sphaeroides*		Co-culture (*R. capsulatus* YO3-*R. sphaeroides*)	
Organic products	C_0 (mM)	C_f (mM)	C_0 (mM)	C_f (mM)	C_0 (mM)	C_f (mM)
Acetic acid	0.394	4.676	0.138	1.18	1.274	8.422
Formic acid	1.414	14.657	0.56	12.46	3.604	6.645
Lactic acid	0.24	1.63	0.25	1.23	1.00	1.34

Table 3.21 The sucrose consumption (%) in co-cultivation of *R. capsulatus* YO3 (Hup$^-$) and *R. sphaeroides* O.U.001 (DSM 5864).

(PBRs) Sucrose (5 mM)	C_0 (mM)	C_f (mM)	Sucrose consumption (%)
R. capsulatus YO3	4.55	0.23	94.9
R. sphaeroides	5.08	2.4	52.7
Co-culture (*R. capsulatus* YO3-*R. sphaeroides*)	5.120	2.512	50.9

3.6.4 Mixed-cultures of *R. capsulatus* YO3 (Hup$^-$), *R. palustris* (DSM 127) and *R. sphaeroides* O.U.001 (DSM 5864)

The bacterial inoculum (3.33% from each strains) was done with freshly grown cultures of *R. capsulatus* YO3 (Hup$^-$), *R. palustris* (DSM 127) and *R. sphaeroides* O.U.001 (DSM 5864) when their optical density (OD660) reached 2.0. Photobiological hydrogen production was followed in 150 ml PBRs on molasses medium with 5 mM of sucrose. The process was continued for 240 hours.

The pH of the multiple-culture decreases in a rapid way during the process. The change of pH is given in Figure 3.49. The maximum biomass growth was 1.03 gdcw/ l_c and the cumulative hydrogen production was 1.02 l/l_c. The hydrogen production resembled a proportional relation with the growth curve. Figure 3.50 shows the hydrogen production and biomass (OD) versus time.

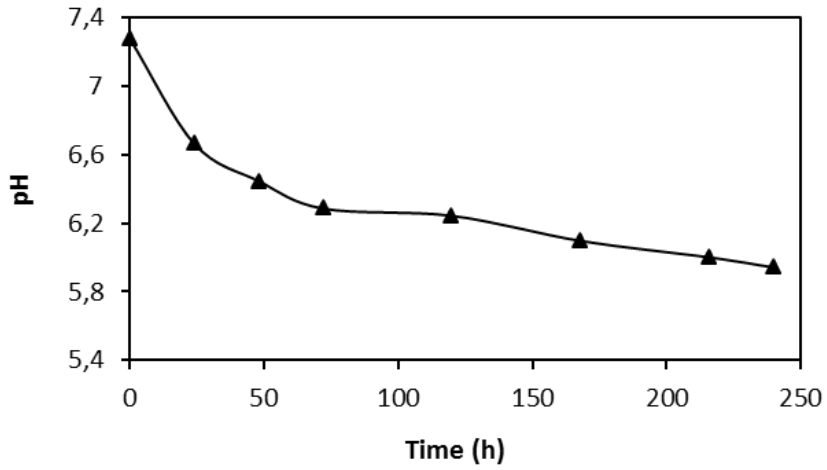


Figure 3.49 The changes of pH in mixed-cultures of *R. capsulatus* YO3 (Hup-), *R. palustris* (DSM 127) and *R. sphaeroides* O.U.001 (DSM 5864) on molasses medium with 5 mM sucrose.

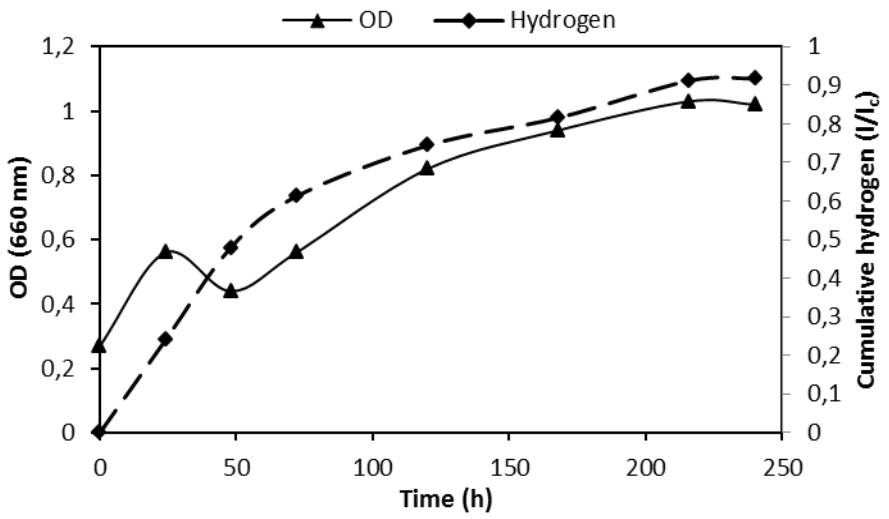


Figure 3.50 The cumulative hydrogen production and growth curves in mixed-culture of *R. capsulatus* YO3 (Hup-), *R. palustris* (DSM 127) and *R. sphaeroides* O.U.001 (DSM 5864).

The concentrations of acetic acid, formic acid and lactic acid increased at high amounts through the 192 hours time duration. The consumption of the organic acids could occur but the culture metabolism generally shifted in production mode. Table 3.25 and 3.26 indicate the concentrations of the organic acids and sucrose consumption by *R. capsulatus* YO3 (Hup$^-$), *R. palustris* (DSM 127) and *R. sphaeroides* O.U.001 (DSM 5864) respectively.

Table 3.25 The concentrations of the organic acids in mixed-culture of *R. capsulatus* YO3 (Hup^-), *R. palustris* (DSM 127) and *R. sphaeroides* O.U.001 (DSM 5864).

Hydrogen production media	Multi-culture (*R. capsulatus* YO3- *R. palustris*- *R. sphaeroides*)	
Organic acids	C_0 (mM)	C_f (mM)
Acetic acid	1.12	6.45
Formic acid	3.93	11.09
Lactic acid	0.99	12.84

Table 3.26 The sucrose consumption (%) in mixed-culture of *R. capsulatus* YO3 (Hup^-), *R. palustris* (DSM 127) and *R. sphaeroides* O.U.001 (DSM 5864).

(PBRs) Sucrose (5 mM)	C_0 (mM)	C_f (mM)	Sucrose consumption (%)
Co-culture (*R. capsulatus* YO3- *R. palustris*- *R. sphaeroides*)	4.957	2.484	50

The maximum growth was observed as 1.15 OD at 660 nm in co-cultivation of *R. sphaeroides* O.U.001 (DSM 5864) and *R. palustris* (DSM 127). The pH range of the co-cultures were between 6.0-7.4. The pHs of the all co-cultures decreased from ana initial pH of 7.4±1.0.

The cumulative hydrogen production was the highest with the co-cultivation of *R. capsulatus* YO3 and *R. sphaeroides* O.U.001 (DSM 5864) as 8.37 mmol/H_2 for 240 hours photofermentation time. Table 3.23 is given for total hydrogen production as mmol hydrogen.

The pH range of the same culture was between 6.0-7.4. The maximum biomass growth was 1.02 gdcw/l_c. The substrate conversion efficiencies and hydrogen

productivities were the greatest with the co-culture of *R. sphaeroides* O.U.001 (DSM 5864) and *R. palustris* (DSM 127).

The highest percentage of sucrose consumption was of 97.6% with the co-culture of *R. capsulatus* YO3 (Hup^-) and *R. sphaeroides* O.U.001 (DSM 5864). The other co-cultures and multiple cultivation had a consumption rate of approximately 50.0%.

Light conversion efficiency was maximal and 0.19% in co-cultivation of *R. sphaeroides* O.U.001 (DSM 5864) with *R. capsulatus* YO3 (Hup^-). The light conversion efficiencies of different co-cultures are given in Table 3.24.

Table 3.23 Duration of the processes and total hydrogen production of different PNS bacteria in 150 ml bioreactors.

Microorganisms	**Duration of process (h)**	**Total Hydrogen (mmol H_2)**
R. capsulatus YO3	240	4.67
R. palustris	192	11.5
R. sphaeroides	240	3.0
R. capsulatus YO3 and *R. palustris*	192	6.13
R. sphaeroides and *R. palustris*	240	7.83
R. capsulatus YO3 and *R. sphaeroides*	240	8.37
R. capsulatus YO3, *R. sphaeroides* and *R. palustris*	240	6.81

Table 3.24 Summary of the co-cultivation of different PNS bacteria on 5 mM sucrose from molasses.

Microorganisms	**Biomass (gdcw/l_c)**	**Hydrogen productivity (mmol/l_c.h)**	**Substrate conversion efficiency(%)**	**Sucrose consumption (%)**	**Light conversion efficiency(%)**
R. capsulatus YO3	0.61	0.35	30.2	94.9	0.10
R. palustris	0.94	1.07	42.5	53.3	0.32
R. sphaeroides	1.15	0.28	32.1	51.3	0.06
R. capsulatus YO3 and *R. palustris*	0.95	0.57	34.0	97.6	0.17
R. sphaeroides and *R. palustris*	1.10	1.0	92.0	50.0	0.18
R. capsulatus YO3 and *R. sphaeroides*	1.02	0.40	76.2	50.09	0.19
R. capsulatus YO3, *R. sphaeroides* and *R. palustris*	1.03	0.42	76.8	50.0	0.15

Efficiency of the Hydrogen Production

For practical application of hydrogen production from molasses, a global cost estimation has been done only considering the price of molasses and the efficiency of hydrogen production for each strain was given below;

Total molasses production of Ankara Sugar Factory in a year = 19520 tons = 19520.000 kg

The cost of molasses = 1kg = 0.279 TL

The price of total molasses = 5.446.080 TL

Energy content of hydrogen = 142.000 kj / kg

Total estimated hydrogen production in a year from sugar beet molasses;

Using *R. capsulatus* (DSM 1710) = 0.00222 g H_2/1.5 g molasses = 28.8 x 10^6 g H_2 / g molasses = 4.08 x 10^9 kj/kg H_2

R. capsulatus YO3 (Hup^-) = 0.00138 g H_2/1.5 g molasses = 17.9 x 10^6 g H_2 / g molasses = 2.51 x 10^9 kj/kg H_2

R. palustris (DSM 127) = 0.0062 g H_2/1.5 g molasses = 80.6 x 10^6 g H_2 / g molasses = 11.4 x 10^9 kj/kg H_2

R. sphaeroides O.U.001 = 0.00268 g H_2/1.5 g molasses = 34.8 x 10^6 g H_2 / g molasses = 4.94 x 10^9 kj/kg H_2

Co-cultivation of *R. palustris* (DSM 127) and *R. sphaeroides* O.U.001 = 0.016 g H_2/2.26 g molasses = 13.8 x 10^7 g H_2 / g molasses = 1.9 x 10^{10} kj/kg H_2.

CHAPTER 4

CONCLUSIONS

The growth and hydrogen production was performed by PNS bacteria with the utilization of sucrose in defined and diluted-molasses media in all photobioreactors through direct photofermentation. The concluded remarks were summarized below.

Sucrose is a good substrate especially for the growth of purple non-sulphur bacteria. The PNS bacteria have grown better in sucrose media than acetate media.

Molasses can also be considered as a potential source for bacterial growth and hydrogen production by PNS bacteria.

Hydrogen production capacities depend on the concentration of sucrose, pH and type of PNS bacteria.

Acetic acid, formic acid, lactic acid, propionic acid and butyric acid are end products of the photofermentation of PNS bacteria on sucrose and molasses. The amount and type of the organic acids varied between the strains of PNS bacteria. All of these organic substrates can be used for the bacteria while they are producing simultaneously. Propionic acid is produced mostly by *R. capsulatus* YO3 (Hup^-) in both single cell and co-cultures.

Formic acid was produced predominantly at higher amounts through the utilization of sucrose.

The intermediate products of the sucrose and organic acids decreased the pHs of hydrogen production media. This may inhibit or prevent hydrogen production metabolism of photosynthetic bacteria.

The evolved gases in PBRs was mostly hydrogen in a range of 70-90%. The rest was carbondioxide. Carbondioxide production was observed mainly after the third day of the photofermentation in 50 ml bioreactors. The amount of produced carbondioxide was about 5-10% of the total gas in 150 ml co-cultured bioreactors.

Rhodopseudomonas palustris (DSM 127) was found to be the most suitable PNS bacteria for hydrogen production.

The co-cultivation of *Rhodopseudomonas palustris* (DSM 127) and *R. sphaeroides* O.U.001 was more suitable for hydrogen production from molasses comparing with single cell cultures.

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http://www.iet.uniduesseldorf.de/Frameseiten/Photobiotechnology&topframenav.htm (R. capsulatus (a), Institut für Molekulare Enzymetechnologie)

http://microbewiki.kenyon.edu/images/a/a7/Rsphaeroides.gif, *R. Sphaeroides* (b) (from Institute of Doe Joint Genome)

http://microbewiki.kenyon.edu/images/6/6e/Palustris.jpg *R. palustris* (c), Dr. Gert-Wieland Kohring, University of Saarland, Germany

APPENDIX A

COMPOSITION OF THE MEDIA AND SOLUTIONS

Table A.1 Composition of the growth medium (20 mM Acetate/10 mM Glutamate, MERCK CHEMICALS)

Components	Amount	Unit
KH_2PO_4 (20 mM)	2.73	g/l
$MgSO_4.7H_2O$	0.5	g/l
$CaCl_2.2H_2O$	0.05	g/l
Vitamin Solution (1X)	1	ml/l
Fe-Citrate Solution (50X)	0.5	ml/l
Trace Element Solution (10X)	0.1	ml/l
Monosodium Glutamate (10 mM)	1.8	g/l
Acetic acid (20 mM)	1.15	ml/l

Table A.2 Composition of sucrose-acetate growth medium (20 mM Sucrose/10 mM Acetate)

Components	Amount	Unit
KH_2PO_4 (20 mM)	2.73	g/l
$MgSO_4.7H_2O$	0.5	g/l
$CaCl_2.2H_2O$	0.05	g/l
Vitamin Solution (1X)	1.0	ml/l
Fe-Citrate Solution (50X)	0.5	ml/l
Trace Element Solution (10X)	0.1	ml/l
Monosodium Glutamate (2 mM)	0.36	g/l
Acetic acid (20 mM)	1.15	ml/l
Sucrose (10 Mm)	3.42	g/l

Table A.3 Composition of sucrose-acetate growth medium (10 mM Sucrose)

Components	Amount	Unit
KH_2PO_4 (20 mM)	2.73	g/l
$MgSO_4.7H_2O$	0.5	g/l
$CaCl_2.2H_2O$	0.05	g/l
Vitamin Solution (1X)	1.0	ml/l
Fe-Citrate Solution (50X)	0.5	ml/l
Trace Element Solution (10X)	0.1	ml/l
Monosodium Glutamate (2 mM)	0.36	g/l
Sucrose (20 Mm)	3.42	g/l

Table A.4 Hydrogen production media with different sucrose (5*, 7.5**, 10*** mM) concentrations

Components	Amount	Unit
KH_2PO_4 (30 mM)	4.09	g/l
$MgSO_4.7H_2O$	0.5	g/l
$CaCl_2.2H_2O$	0.05	g/l
Vitamin Solution (1X)	1.0	ml/l
Fe-Citrate Solution (50X)	0.5	ml/l
Trace Element Solution (10X)	0.1	ml/l
Monosodium Glutamate (2 mM)	0.36	g/l
*Sucrose (5 Mm)	1.71	g/l
**Sucrose (7.5 Mm)	2.56	g/l
***Sucrose (10 Mm)	3.42	g/l

A 5. Composition of trace elements solution (1X) components.

Component	Amount/Unit
HCl (25% v/v)	1 ml/l
$ZnCl_2$	70 mg/l
$MnCl_2.4H_2O$	100 mg/l
H_3BO_3	60 mg/l
$CoCl_2.6H_2O$	200 mg/l
$CuCl_2.2H_2O$	20 mg/l
$NiCl_2.6H_2O$	20 mg/l
$NaMoO_4.2H_2O$	40 mg/l

APPENDIX B

DRY CELL WEIGHT CURVES

B1. Dry Cell Weight Determination of PNS bacteria

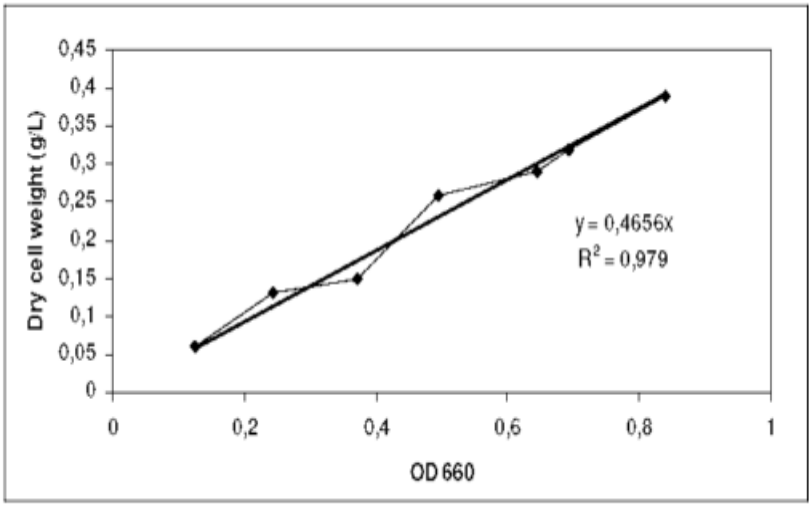


Figure B1. Calibration curve of *R. capsulatus* YO3. (Öztürk, 2005).

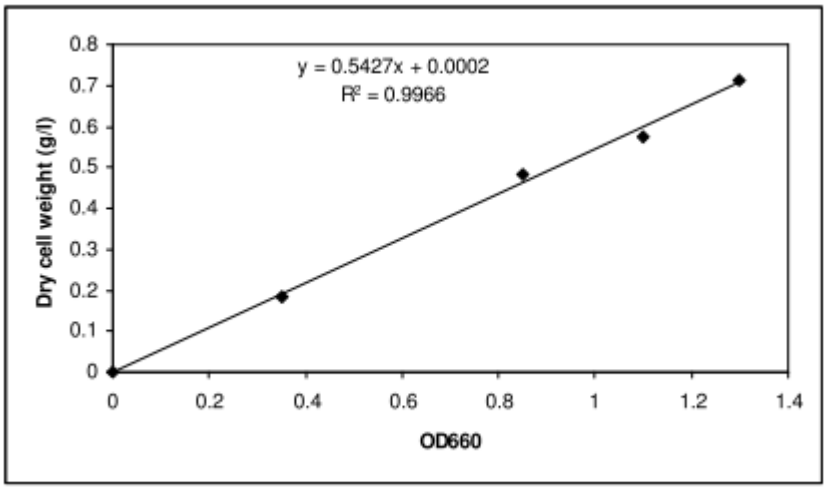


Figure B2. Calibration curve of *R. capsulatus* DSM 1710. (Uyar, 2008).

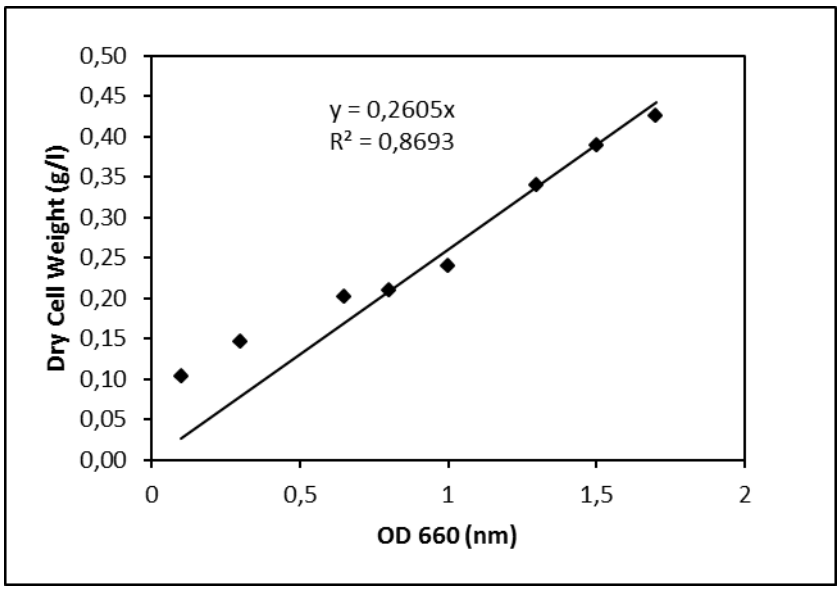


Figure B3. Calibration curve of *R. palustris* (DSM 127).

APPENDIX C

SAMPLE SUGAR ANALYSIS

C1. Sample Sugar HPLC Analysis Chromatogram

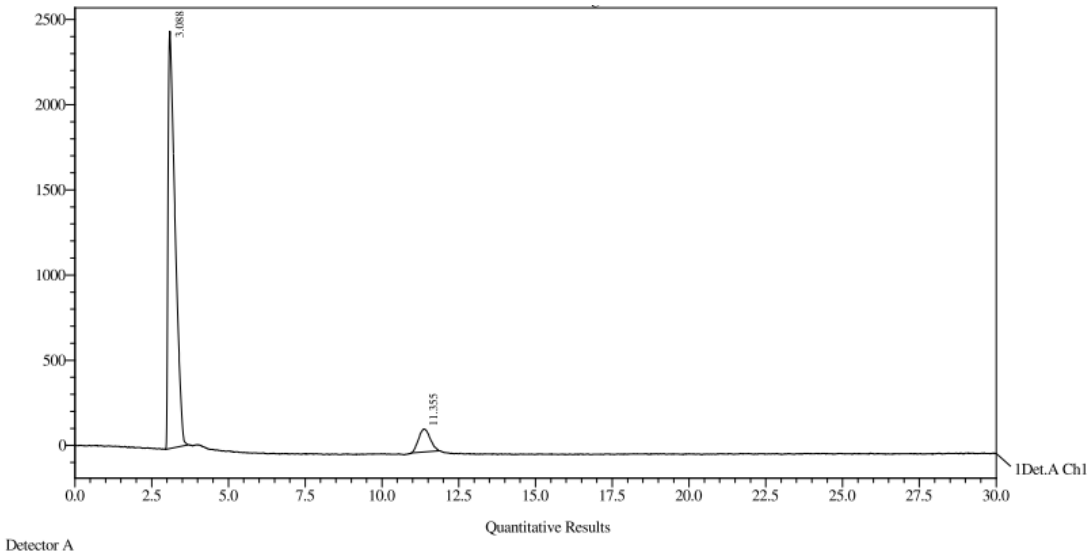


Figure C1. Sample sugar HPLC chromatogram. Sucrose peak is at 11.3 seconds. Acetonitrile mobile phase peak is at 3.0 seconds. (NH2 sugar column and Shimadzu Agilent 10A series HPLC, RID detector)

C2. Sample HPLC Calibration Curve for Sucrose

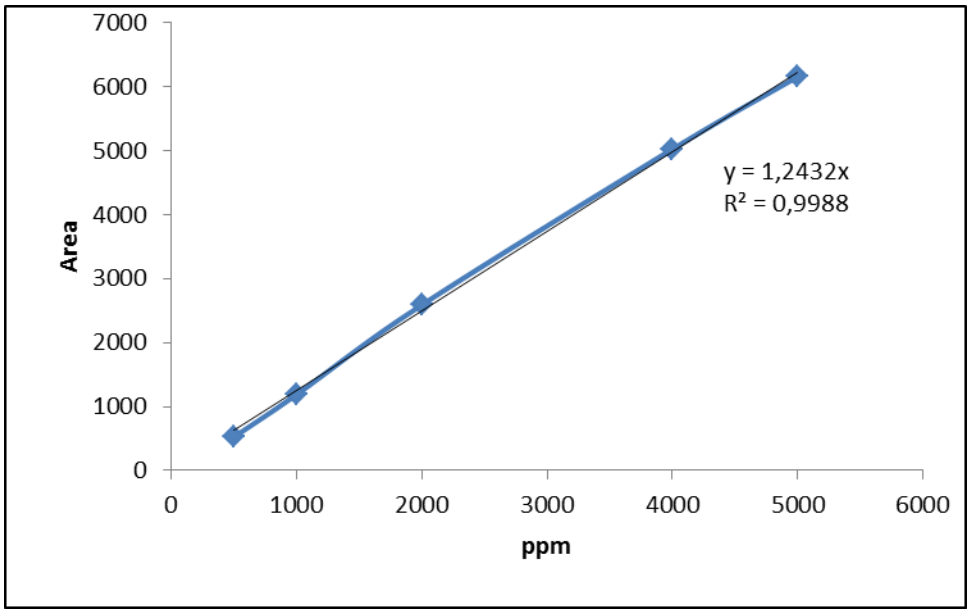


Figure C.2 Sample standard sucrose calibration curve (HPLC, Agilent 10A series).

APPENDIX D

ORGANIC ACID ANALYSIS

D 1. Sample organic acid chromatogram

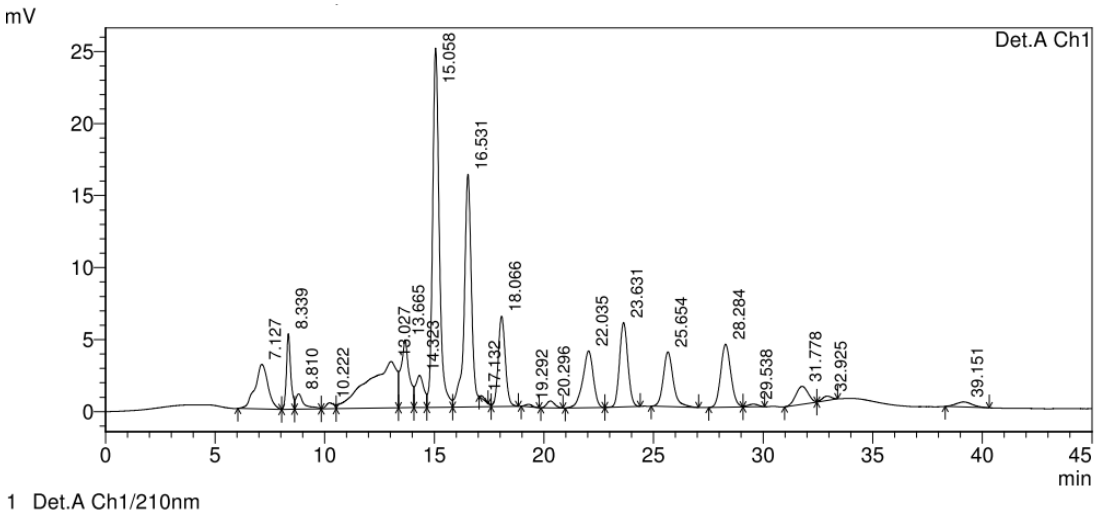


Figure D1. Sample HPLC organic acid chromatogram (Acetic acid peak in 25.654 min and Formic acid peak is in 23.631 min, Shimadzu Agilent 10A series HPLC, UV 210 nm detector).

D 2. Sample chromatogram of Acetic Acid Calibration

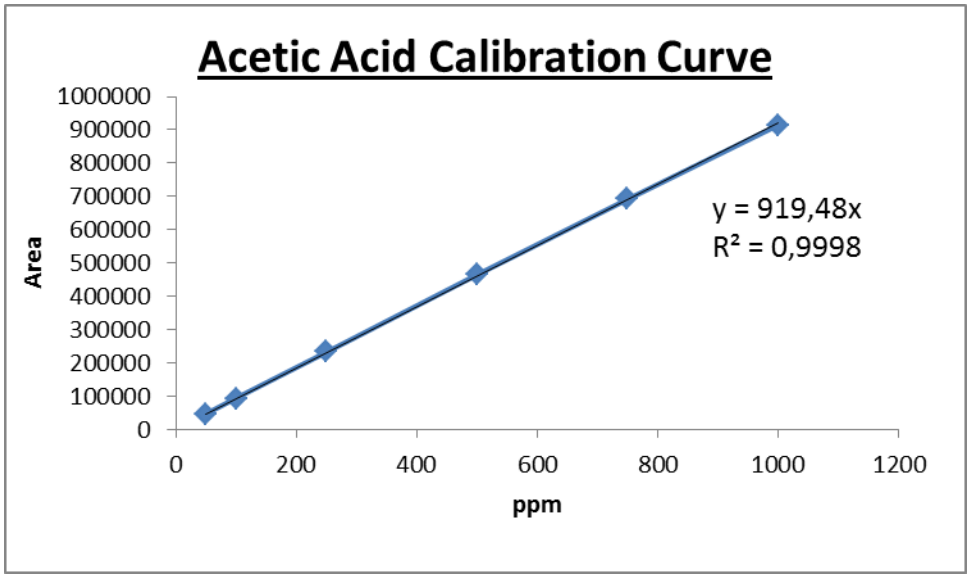
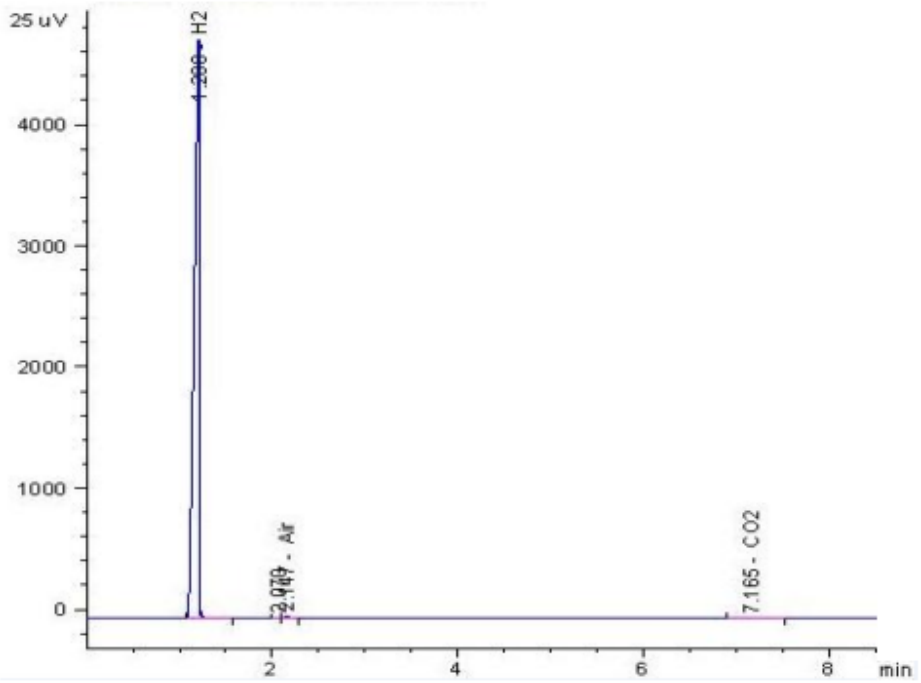


Figure D.1 Calibration curve of sample acetic acid.

APPENDIX E

SAMPLE GAS CHROMATOGRAM



RetTime [min]	Type	Area [25 uV*s]	Amt/Area	Norm %	Grp	Name
1.208	BB	1.78157e4	5.36760e-3	92.790983		H2
2.147	VB	104.16668	4.30476e-2	4.351104		Air
7.165	BB	52.88443	5.56929e-2	2.857913		CO2

Figure E. Sample gas chromatogram (Agilent Technologies 6890 N gas chromatography) (Androga, 2009).

APPENDIX F

COMPOSITION OF MOLASSES

Table F. Composition of Undiluted Molasses

Parameters	Units	Results of Chemical Analysis
Sucrose	mM	1.16
Alcohol(ethyl alcohol)	% (v/v)	0.16
Phenol	mg/L	25.4
Iron (Fe)	µg/L	516.9
Molybdenum (Mo)	µg/L	99.7
Sulphur (S)	mg/L	187.1
Potassium (K)	mg/L	5365
Total amino acids	g/100 g	
Aspartic acid	g/100 g	0.164
Glutamic acid	g/100 g	0.729
Asparagine	g/100 g	< 0.02[(1)]
Serine	g/100 g	0.06
Histidine	g/100 g	< 0.02[(1)]
Glycine	g/100 g	0.042
Threonine	g/100 g	< 0.01[(1)]
Citrulline	g/100 g	< 0.02[(1)]

Arginine	g/100 g	< 0.02(1)
Alanine	g/100 g	0.076
Tyrosine	g/100 g	0.027
Cystine	g/100 g	0.052
Valine	g/100 g	0.020
Methionine	g/100 g	< 0.02(1)
Tryptophan	g/100 g	0.048
Phenylalanine	g/100 g	< 0.02(1)
Isoleucine	g/100 g	0.030
Ornithine	g/100 g	0.025
Leucine	g/100 g	0.037
Lysine	g/100 g	< 0.02(1)
Hydroxyproline	g/100 g	< 0.3(1)
Sarcosine	g/100 g	< 0.02(1)
Proline	g/100 g	< 0.01(1)
Total of total amino acids	g/100 g	1.3
(1) MDL, Method detection limit		

APPENDIX G

EXPERIMENTAL DATA

Table G1 OD, pH, dry cell weight and hydrogen production of 5 mM sucrose containing molasses medium by *R. capsulatus* DSM 1710

Time (h)	OD (660 nm)	Dry Cell Weight ($gdcw/l_c$)	pH	Cumulative Hydrogen (ml)	Cumulative Hydrogen (l/l_c)
0	0,34	0,17	7,292	0	0,00
24	0,98	0,49	7,012	2	0,04
48	1,04	0,52	6,923	4	0,08
72	1,15	0,58	6,707	7	0,14
96	1,1	0,55	6,73	8	0,16
120	0,83	0,42	6,757	9	0,18
144	0,83	0,42	6,658	10	0,20
180	0,75	0,38	6,463	10	0,20

Table G2 Organic acid concentrations (mM) in 5 mM sucrose medium by *R. capsulatus* DSM 1710.

Time (h)	Acetic acid (mM)	Lactic acid (mM)	Formic acid (mM)	Propionic acid (mM)	Butyric acid (mM)
0	0,12	0,43	0,64	-	-
72	0,31	0,84	1,98	-	-
144	3,32	0,08	16,40	-	-

Table G3 OD, pH, dry cell weight and hydrogen production of 7.5 mM sucrose defined medium by *R. capsulatus* DSM 1710

Time (h)	OD (660 nm)	Dry Cell Weight (gdcw/l_c)	pH	Cumulative Hydrogen (ml)	Cumulative Hydrogen (l/l_c)
0	0,5	0,25	7,255	0	0,00
24	0,7	0,35	7,1	25	0,50
48	1,05	0,53	6,855	33	0,66
72	1,19	0,60	6,625	35	0,70
96	1,08	0,54	6,51	37	0,74
120	0,9	0,45	6,394	40	0,80
144	0,88	0,44	6,381	43	0,86
180	0,88	0,44	6,362	43	0,86

Table G4 Organic acid concentrations (mM) in 7.5 mM sucrose medium by *R. capsulatus* DSM 1710.

Time (h)	Acetic acid (mM)	Lactic acid (mM)	Formic acid (mM)	Propionic acid (mM)	Butyric acid (mM)
0	0,04	0,23	0,39	-	-
72	0,62	1,47	3,51	-	-
144	1,18	0,99	2,57	-	-

Table G5 OD, pH, dry cell weight and hydrogen production of 10 mM sucrose defined medium by *R. capsulatus* DSM 1710

Time (h)	OD (660 nm)	Dry Cell Weight (gdcw/l_c)	pH	Cumulative Hydrogen (ml)	Cumulative Hydrogen (l/l_c)
0	0,42	0,21	7,295	0	0,00
24	0,88	0,44	7,127	10	0,20
48	1,08	0,54	6,866	17	0,34
72	1,26	0,63	6,667	22	0,44
96	1,03	0,52	6,519	23	0,46
120	0,88	0,44	6,424	25	0,50
144	0,75	0,38	6,107	27	0,54
180	0,83	0,42	6,05	30	0,60

Table G6 Organic acid concentrations (mM) in 10 mM sucrose medium by *R. capsulatus* DSM 1710.

Time (h)	Acetic acid (mM)	Lactic acid (mM)	Formic acid (mM)	Propionic acid (mM)	Butyric acid (mM)
0	0,02	0,04	0,26	-	-
72	0,48	1,28	2,70	-	-
144	4,17	0,00	6,11	-	-

Table G7 OD, pH, dry cell weight and hydrogen production of 5 mM sucrose defined medium by R. capsulatus YO3

Time (h)	OD (660 nm)	Dry Cell Weight (gdcw/l_c)	pH	Cumulative Hydrogen (ml)	Cumulative Hydrogen (l/l_c)
0	0,23	0,13	7,59	0	0
24	0,58	0,32	7,11	16	0,32
48	0,74	0,41	6,72	16	0,32
72	0,76	0,42	6,26	16	0,32
96	0,88	0,48	6,14	40	0,8
120	0,96	0,53	6,09	40	0,8
168	0,89	0,49	5,75	40	0,8
212	0,85	0,47	5,75	40	0,8

Table G8 Organic acid concentrations (mM) in 5 mM sucrose medium by R. capsulatus YO3

Time (h)	Acetic acid (mM)	Lactic acid (mM)	Formic acid (mM)	Propionic acid (mM)	Butyric acid (mM)
0	3,14	-	2,11	-	-
72	2,22	-	2,73	-	-
144	3,09	-	3,48	-	-

Table G9 OD, pH, dry cell weight and hydrogen production of 7.5 mM sucrose defined medium by R. capsulatus YO3

Time (h)	OD (660 nm)	Dry Cell Weight ($gdcw/l_c$)	pH	Cumulative Hydrogen (ml)	Cumulative Hydrogen (l/l_c)
0	0,27	0,15	7,35	0	0,00
24	0,62	0,34	7,12	4,06	0,08
48	0,75	0,41	7	8,7	0,17
72	0,78	0,43	6,3	12,76	0,26
96	0,89	0,49	6,2	14,96	0,30
120	0,95	0,52	6,02	17,6	0,35
168	0,95	0,52	5,8	23,32	0,47
212	0,98	0,54	5,87	25,08	0,50

Table G10 Organic acid concentrations (mM) in 7.5 mM sucrose medium by *R. capsulatus* YO3

Time (h)	Acetic acid (mM)	Lactic acid (mM)	Formic acid (mM)	Propionic acid (mM)	Butyric acid (mM)
0	0,32	0,2	3,12	-	-
72	1,2	0,1	3,85	-	-
144	4,54	0	4,32	-	-

Table G11 OD, pH, dry cell weight and hydrogen production of 10 mM sucrose defined medium by *R. capsulatus* YO3

Time (h)	OD (660 nm)	Dry Cell Weight (gdcw/l_c)	pH	Cumulative Hydrogen (ml)	Cumulative Hydrogen (l/l_c)
0	0,31	0,17	7,3	0	0,00
24	0,69	0,38	7,23	2,08	0,04
48	0,78	0,43	7,05	5,76	0,12
72	0,82	0,45	6,4	9,12	0,18
96	1,00	0,55	6,34	10,32	0,21
120	1,09	0,6	6	12,32	0,25
168	1,13	0,62	5,99	18,72	0,37
212	1,16	0,64	5,8	20,72	0,41

Table G12 Organic acid concentrations (mM) in 10 mM sucrose medium by *R. capsulatus* YO3

Time (h)	Acetic acid (mM)	Lactic acid (mM)	Formic acid (mM)	Propionic acid (mM)	Butyric acid (mM)
0	0,23	0,12	3,45	-	-
72	3,37	0,4	4,2	-	-
144	5,65	0,08	5,32	-	-

Table G13 OD, pH, dry cell weight and hydrogen production of 5 mM sucrose defined medium by *R. palustris* (DSM 127)

Time (h)	OD (660 nm)	Dry Cell Weight ($gdcw/l_c$)	pH	Cumulative Hydrogen (ml)	Cumulative Hydrogen (l/l_c)
0	0,21	0,05	7,07	0	0
24	1,14	0,30	7,25	18,9	0,378
48	1,05	0,27	6,72	42,3	0,846
72	0,92	0,24	6,68	63,3	1,266
120	0,79	0,21	6,72	66,9	1,338
144	0,75	0,20	6,85	67,5	1,35
168	0,73	0,19	6,69	67,8	1,356
192	0,58	0,15	6,92	68,1	1,362

Table G14 Organic acid concentrations (mM) in 5 mM sucrose medium by *R. palustris* (DSM 127)

Time (h)	Acetic acid (mM)	Lactic acid (mM)	Formic acid (mM)	Propionic acid (mM)	Butyric acid (mM)
0	0,07	0,15	0,42	-	-
72	2,17	1,86	2,32	-	-
144	0,00	0,00	3,06	-	-

Table G15 OD, pH, dry cell weight and hydrogen production of 7.5 mM sucrose defined medium by *R. palustris* (DSM 1710)

Time (h)	OD (660 nm)	Dry Cell Weight ($gdcw/l_c$)	pH	Cumulative Hydrogen (ml)	Cumulative Hydrogen (l/l_c)
0	0,21	0,05	7,22	0	0
24	1,25	0,32	7,18	16,2	0,324
48	1,03	0,27	6,69	36	0,72
72	1,46	0,38	6,54	55,8	1,116
120	1,21	0,31	6,61	63,6	1,272
144	1,20	0,31	6,75	69,6	1,392
168	0,82	0,21	6,66	71,4	1,428
192	0,67	0,17	6,74	75	1,5

Table G16 Organic acid concentrations (mM) in 7.5 mM sucrose medium by *R. palustris* (DSM 127)

Time (h)	Acetic acid (mM)	Lactic acid (mM)	Formic acid (mM)	Propionic acid (mM)	Butyric acid (mM)
0	0,09	0,14	0,43	0,00	-
72	2,31	0,00	2,88	0,00	-
144	12,06	0,00	10,47	0,21	-

Table G17 OD, pH, dry cell weight and hydrogen production of 10 mM sucrose defined medium by *R. palustris* (DSM 127)

Time (h)	OD (660 nm)	Dry Cell Weight ($gdcw/l_c$)	pH	Cumulative Hydrogen (ml)	Cumulative Hydrogen (l/l_c)
0	0,20	0,05	7,25	0	0
24	1,16	0,30	7,19	13,5	0,27
48	1,20	0,31	6,74	25,2	0,504
72	1,34	0,35	6,58	37,2	0,744
120	1,18	0,31	6,54	45	0,90
144	0,97	0,25	6,60	50,4	1,008
168	0,98	0,25	6,56	52,2	1,044
192	0,69	0,18	6,60	55,2	1,104

Table G18 Organic acid concentrations (mM) in 10 mM sucrose medium by *R. palustris* (DSM 127)

Time (h)	Acetic acid (mM)	Lactic acid (mM)	Formic acid (mM)	Propionic acid (mM)	Butyric acid (mM)
0	0,21	0,19	0,58	0,00	-
72	2,69	0,00	2,69	0,77	-
144	19,15	0,00	5,76	0,14	-

Table G19 OD, pH, dry cell weight and hydrogen production of 5 mM sucrose containing molasses medium by *R. sphaeroides* O.U.001

Time (h)	OD (660nm)	Dry Cell Weight ($gdcw/l_c$)	pH	Cumulative Hydrogen (ml)	Cumulative Hydrogen (l/l_c)
0	0,28	0,15	7,57	0,00	0,00
24	-	-	-	-	-
48	1,13	0,62	7,56	22,00	0,44
72	1,17	0,64	7,16	30,00	0,60
96	1,02	0,56	7,09	38,50	0,77
120	0,85	0,47	7,07	40,20	0,80
144	0,88	0,48	6,87	42,00	0,84
180	0,94	0,52	6,94	43,00	0,86

Table G20 Organic acid concentrations (mM) in 5 mM sucrose medium by *R. sphaeroides* O.U.001

Time (h)	Acetic acid (mM)	Lactic acid (mM)	Formic acid (mM)	Propionic acid (mM)	Butyric acid (mM)
0	0,04	0,10	0,04	-	-
72	--	-	-	-	-
144	0,10	0,68	0,10	-	-

Table G21 OD, pH, dry cell weight and hydrogen production of 7.5 mM sucrose containing molasses medium by *R. sphaeroides* O.U.001

Time (h)	OD (660nm)	Dry Cell Weight (gdcw/l_c)	pH	Cumulative Hydrogen (ml)	Cumulative Hydrogen (l/l_c)
0	0,28	0,15	7,59	0,00	0,00
24	-	-	-	-	-
48	1,24	0,68	7,46	18,00	0,36
72	1,23	0,68	7,14	26,00	0,52
96	1,01	0,56	7,14	27,00	0,54
120	1,07	0,59	7,00	30,00	0,60
144	0,82	0,45	6,89	30,00	0,60
180	1,03	0,57	6,76	30,00	0,60

Table G22 Organic acid concentrations (mM) in 7.5 mM sucrose medium by *R. sphaeroides* O.U.001

Time (h)	Acetic acid (mM)	Lactic acid (mM)	Formic acid (mM)	Propionic acid (mM)	Butyric acid (mM)
0	0,00	0,00	0,00	-	-
72	-	-	-	-	-
144	0,06	1,07	0,06	-	-

Table G23 OD, pH, dry cell weight and hydrogen production of 10 mM sucrose containing molasses medium by *R. sphaeroides* O.U.001

Time (h)	OD (660nm)	Dry Cell Weight ($gdcw/l_c$)	pH	Cumulative Hydrogen (ml)	Cumulative Hydrogen (l/l_c)
0	0,32	0,18	7,53	0,00	0,00
24	-	-	-	-	-
48	1,22	0,67	7,49	13,00	0,26
72	1,22	0,67	7,17	24,00	0,48
96	1,00	0,55	7,09	32,00	0,64
120	1,12	0,62	7,10	35,00	0,70
144	0,92	0,51	6,87	36,00	0,72
180	0,92	0,51	6,84	37,00	0,74

Table G24 Organic acid concentrations in 10 mM sucrose medium by *R. sphaeroides* O.U.001

Time (h)	Acetic acid (mM)	Lactic acid (mM)	Formic acid (mM)	Propionic acid (mM)	Butyric acid (mM)
0	0,05	0,02	0,05	-	-
72	-	-	-	-	-
144	0,07	1,30	0,07	-	-

Table G25 OD, pH, dry cell weight and hydrogen production of 5 mM sucrose containing molasses medium by R. capsulatus DSM 1710

Time (h)	OD (660nm)	Dry Cell Weight ($gdcw/l_c$)	pH	Cumulative Hydrogen (ml)	Cumulative Hydrogen (l/l_c)
0	0,30	0,17	7,32	0,00	0,00
24	0,75	0,41	6,62	0,94	0,02
72	1,04	0,57	6,246	1,88	0,04
96	1,15	0,63	6,177	5,64	0,11
120	0,94	0,52	6,054	13,16	0,26
144	0,92	0,51	5,96	13,16	0,26
168	0,83	0,45	5,927	13,16	0,26
192	0,82	0,45	5,73	13,16	0,26

Table G26 Organic acid concentrations (mM) in 5 mM molasses (sucrose) medium by *R. capsulatus* DSM 1710.

Time (h)	Acetic acid (mM)	Lactic acid (mM)	Formic acid (mM)	Propionic acid (mM)	Butyric acid (mM)
0	0	0	0	0	0
24	0,25	0,3	0,3	0	0
48	0,5	0,3	0,4	0,9	0,1
96	1,4	1,4	1,5	2	0,3
144	7,85	2,8	0,75	2,1	1,25

Table G27 OD, pH, dry cell weight and hydrogen production of 7.5 mM sucrose containing molasses medium by *R. capsulatus* DSM 1710

Time (h)	OD (660nm)	Dry Cell Weight ($gdcw/l_c$)	pH	Cumulative Hydrogen (ml)	Cumulative Hydrogen (l/l_c)
0	0,41	0,23	7,184	0	0,00
24	0,78	0,43	6,98	1,65	0,03
72	1,19	0,65	6,672	4,95	0,10
96	1,23	0,67	6,425	7,7	0,15
120	1,06	0,58	6,146	9,9	0,20
144	1,01	0,56	5,954	10,45	0,21
168	0,96	0,53	5,858	10,45	0,21
192	0,97	0,53	5,75	10,45	0,21

Table G28 Organic acid concentrations (mM) in 7.5 mM molasses (sucrose) medium by *R. capsulatus* DSM 1710.

Time (h)	Acetic acid (mM)	Lactic acid (mM)	Formic acid (mM)	Propionic acid (mM)	Butyric acid (mM)
0	0	0	0	0	0
24	0,35	1,1	0,9	0	0
48	0,1	0,3	6	0,7	0,15
96	3,1	0,9	1	1,75	0,3
144	8,5	1,8	0,75	0	2,5

Table G29 OD, pH, dry cell weight and hydrogen production of 10 mM sucrose containing molasses medium by *R. capsulatus* DSM 1710

Time (h)	OD (660nm)	Dry Cell Weight ($gdcw/l_c$)	pH	Cumulative Hydrogen (ml)	Cumulative Hydrogen (l/l_c)
0	0,40	0,22	7,195	0	0,00
24	0,97	0,53	6,67	3.0	0,06
72	1,17	0,65	6,516	15,5	0,31
96	1,23	0,67	6,082	19,5	0,39
120	1,07	0,59	5,999	22,5	0,45
144	1,04	0,57	5,991	24,5	0,49
168	2,22	1,22	5,85	25	0,50
192	0,99	0,54	5,740	25	0,50

Table G30 Organic acid concentrations (mM) in 10 mM molasses (sucrose) medium by *R. capsulatus* DSM 1710.

Time (h)	Acetic acid (mM)	Lactic acid (mM)	Formic acid (mM)	Propionic acid (mM)	Butyric acid (mM)
0	0,00	0,00	0,00	0,00	0,00
24	0,65	0,21	2,51	0,00	0,00
48	2,41	0,56	5,31	1,32	0,22
96	3,00	1,55	0,96	1,21	1,42
144	4,86	0,60	0,66	0,00	3,32

Table G31 OD, pH, dry cell weight and hydrogen production of 5 mM molasses (sucrose) medium by *R. capsulatus* YO3

Time (h)	OD (660 nm)	Dry Cell Weight ($gdcw/l_c$)	pH	Cumulative Hydrogen (ml)	Cumulative Hydrogen (l/l_c)
0	0,30	0,17	7,42	0	0,00
24	0,61	0,34	6,48	13,86	0,28
48	1,09	0,60	6,42	20,79	0,42
72	1,10	0,61	6,36	33,66	0,67
120	1,24	0,68	6,34	34,65	0,69
144	2,25	1,24	6,12	34,64	0,70
168	2,29	1,26	5,90	35,0	0,70
192	2,29	1,26	5,87	35,64	0,71

Table G32 Organic acid concentrations in 5 mM molasses (sucrose) medium by *R. capsulatus* YO3

Time (h)	Acetic acid (mM)	Lactic acid (mM)	Formic acid (mM)	Propionic acid (mM)	Butyric acid (mM)
0	0	0	0	0	0
24	0,2	0,35	1,1	0	0
48	0,1	0,2	3	0,2	0
72	0	0,1	2,6	0,4	0
96	1,95	0,15	3,65	2	0,1

Table G33 OD, pH, dry cell weight and hydrogen production of 7.5 mM molasses (sucrose) medium by *R. capsulatus* YO3

Time (h)	OD (660 nm)	Dry Cell Weight ($gdcw/l_c$)	pH	Cumulative Hydrogen (ml)	Cumulative Hydrogen (l/l_c)
0	0,28	0,15	7,32	0	0,00
24	0,74	0,41	7,05	10,56	0,21
48	1,01	0,56	6,72	20,10	0,40
72	1,07	0,59	6,56	20,16	0,40
120	1,10	0,60	6,44	20,16	0,40
144	1,65	0,91	6,10	25,92	0,52
168	1,81	1,00	5,97	26,88	0,54
192	1,82	1,00	5,65	26,88	0,54

Table G34 Organic acid concentrations (mM) in 7.5 mM molasses (sucrose) medium by *R. capsulatus* YO3

Time (h)	Acetic acid (mM)	Lactic acid (mM)	Formic acid (mM)	Propionic acid (mM)	Butyric acid (mM)
0	0	0	0	0	0
24	0,5	0,6	2,1	0	0
48	0,3	0,25	3,6	0	0
72	0,35	0,2	4,65	1	0
96	0,2	0,1	3,85	0,8	0

Table G35 OD, pH, dry cell weight and hydrogen production of 10 mM molasses (sucrose) medium by *R. capsulatus* YO3

Time (h)	OD (660 nm)	Dry Cell Weight ($gdcw/l_c$)	pH	Cumulative Hydrogen (ml)	Cumulative Hydrogen (l/l_c)
0	0,30	0,17	7,32	0	0,00
24	0,90	0,49	7,05	1,8	0,04
48	1,25	0,69	6,72	3,6	0,07
72	1,42	0,78	6,56	4,8	0,10
120	1,86	1,02	6,44	6,5	0,13
144	2,11	1,16	6,10	12	0,24
168	2,14	1,18	5,97	13,8	0,28
192	2,13	1,17	5,65	15,6	0,31

Table G36 Organic acid concentrations (mM) in 10 mM molasses (sucrose) medium by *R. capsulatus* YO3

Time (h)	Acetic acid (mM)	Lactic acid (mM)	Formic acid (mM)	Propionic acid (mM)	Butyric acid (mM)
0	0	0	0	0	0
24	0,8	0,9	3,7	0,1	0
48	0,15	0,35	5,5	0	0
72	0,5	0,15	6,4	0,5	0
96	0,1	0	3,5	0,7	0

Table G37 OD, pH, dry cell weight and hydrogen production of 5 mM molasses (sucrose) medium by *R. palustris* (DSM 127)

Time (h)	OD (660 nm)	Dry Cell Weight ($gdcw/l_c$)	pH	Cumulative Hydrogen (ml)	Cumulative Hydrogen (l/l_c)
0	0,20	0,05	7,40	0,00	0,00
24	0,58	0,15	7,10	11,70	0,23
48	0,54	0,14	6,75	33,30	0,67
72	0,78	0,20	6,59	44,10	0,88
120	0,93	0,24	6,72	44,70	0,89
144	1,12	0,29	6,65	46,50	0,93
168	1,02	0,26	6,53	49,50	0,99
192	0,64	0,17	6,36	53,70	1,07

Table G38 Organic acid concentrations (mM) in 5 mM molasses (sucrose) medium by *R. palustris* (DSM 127)

Time (h)	Acetic acid (mM)	Lactic acid (mM)	Formic acid (mM)	Propionic acid (mM)	Butyric acid (mM)
0	0,97	1,04	3,15	-	-
72	-	-	-	-	-
144	1,62	0,92	6,88	-	-

Table G39 OD, pH, dry cell weight and hydrogen production of 7.5 mM molasses (sucrose) medium by *R. palustris* (DSM 127)

Time (h)	OD (660 nm)	Dry Cell Weight ($gdcw/l_c$)	pH	Cumulative Hydrogen (ml)	Cumulative Hydrogen (l/l_c)
0	0,17	0,04	7,31	0,00	0,00
24	0,72	0,19	7,04	14,40	0,29
48	0,64	0,17	6,77	36,90	0,74
72	1,01	0,26	6,51	37,50	0,75
120	1,03	0,27	6,98	45,30	0,91
144	1,17	0,30	6,54	54,30	1,09
168	1,08	0,28	6,43	57,30	1,15
192	0,82	0,21	6,36	62,10	1,24

Table G40 Organic acid concentrations (mM) in 7.5 mM molasses (sucrose) medium by *R. palustris* (DSM 127)

Time (h)	Acetic acid (mM)	Lactic acid (mM)	Formic acid (mM)	Propionic acid (mM)	Butyric acid (mM)
0	1,43	1,34	4,23	0,03	-
72	-	-	-	-	-
144	1,25	0,81	9,37	0,00	-

Table G41 OD, pH, dry cell weight and hydrogen production of 10 mM molasses (sucrose) medium by *R. palustris* (DSM 127)

Time (h)	OD (660 nm)	Dry Cell Weight ($gdcw/l_c$)	pH	Cumulative Hydrogen (ml)	Cumulative Hydrogen (l/l_c)
0	0,21	0,05	7,44	0,00	0,00
24	1,07	0,28	6,95	19,80	0,40
48	0,97	0,25	6,36	30,60	0,61
72	1,33	0,35	6,15	43,80	0,88
120	1,44	0,37	6,09	55,20	1,10
144	1,53	0,40	6,24	63,60	1,27
168	1,43	0,37	5,98	66,60	1,33
192	1,35	0,35	5,86	69,60	1,39

Table G42 Organic acid concentrations (mM) in 10 mM molasses (sucrose) medium by *R. palustris* (DSM 127)

Time (h)	Acetic acid (mM)	Lactic acid (mM)	Formic acid (mM)	Propionic acid (mM)	Butyric acid (mM)
0	0,69	1,15	3,73	0,00	-
72	-	-	-	-	-
144	6,54	5,37	3,74	0,05	-

Table G43 OD, pH, dry cell weight and hydrogen production of 5 mM sucrose containing molasses medium by *R. sphaeroides* O.U.001

Time (h)	OD (660nm)	Dry Cell Weight ($gdcw/l_c$)	pH	Cumulative Hydrogen (ml)	Cumulative Hydrogen (l/l_c)
0	0,25	0,14	7,25	0,00	0,00
24	-	-	-	-	-
48	0,99	0,54	7,22	7,00	0,14
72	1,24	0,68	6,97	10,00	0,20
96	1,31	0,72	6,20	22,00	0,44
120	0,70	0,39	6,16	28,00	0,56
144	0,95	0,52	6,00	30,00	0,60
180	0,52	0,29	5,98	33,00	0,66

Table G44 Organic acid concentrations (mM) in 5 mM molasses (sucrose) medium by *R. sphaeroides* O.U.001

Time (h)	Acetic acid (mM)	Lactic acid (mM)	Formic acid (mM)	Propionic acid (mM)	Butyric acid (mM)
0	0,24	0,41	0,96	0,00	-
72	0,08	0,00	0,00	0,00	-
144	3,30	2,37	5,37	0,25	-

Table G45 OD, pH, dry cell weight and hydrogen production of 7.5 mM sucrose containing molasses medium by *R. sphaeroides* O.U.001

Time (h)	OD (660nm)	Dry Cell Weight ($gdcw/l_c$)	pH	Cumulative Hydrogen (ml)	Cumulative Hydrogen (l/l_c)
0	0,21	0,12	7,25	0,00	0,00
24	-	-	-	-	-
48	1,38	0,76	6,73	7,00	0,14
72	1,07	0,59	6,61	9,00	0,18
96	1,11	0,61	6,53	10,00	0,20
120	1,20	0,66	6,60	15,00	0,30
144	1,10	0,61	6,42	18,00	0,36
180	1,12	0,62	6,38	20,00	0,40

Table G46 Organic acid concentrations (mM) in 7.5 mM molasses (sucrose) medium by *R. sphaeroides* O.U.001

Time (h)	Acetic acid (mM)	Lactic acid (mM)	Formic acid (mM)	Propionic acid (mM)	Butyric acid (mM)
0	0,36	0,59	1,51	0,04	-
72	0,78	0,03	7,56	0,00	-
144	0,78	1,07	11,41	0,02	-

Table G47 OD, pH, dry cell weight and hydrogen production of 7.5 mM sucrose containing molasses medium by *R. sphaeroides* O.U.001

Time (h)	OD (660nm)	Dry Cell Weight (gdcw/l_c)	pH	Cumulative Hydrogen (ml)	Cumulative Hydrogen (l/l_c)
0	0,27	0,15	7,22	0,00	0,00
24	-	-	-	-	-
48	1,21	0,67	7,14	7,00	0,14
72	1,25	0,69	7,19	11,00	0,22
96	1,50	0,83	7,21	15,00	0,30
120	1,30	0,72	7,01	23,00	0,46
144	1,32	0,73	6,98	27,00	0,54
180	1,30	0,72	6,82	30,00	0,60

Table G48 Organic acid concentrations (mM) in 10 mM molasses (sucrose) medium by *R. sphaeroides* O.U.001

Time (h)	Acetic acid (mM)	Lactic acid (mM)	Formic acid (mM)	Propionic acid (mM)	Butyric acid (mM)
0	1,13	1,22	3,42	0,09	-
72	0,41	0,06	0,00	0,00	-
144	0,31	0,03	4,94	0,00	-

G49-G53 Experimental data for co-cultivation studies in 150 ml bioreactors.

Table G49 OD, pH, dry cell weight and hydrogen production of 5 mM molasses sucrose medium by *R. palustris* (DSM 127)

Time (h)	OD (660 nm)	Dry Cell Weight (gdcw/l_c)	pH	Cumulative Hydrogen (ml)	Cumulative Hydrogen (l/l_c)
0	0,21	0,05	7,29	0,0	0,0
24	0,41	0,11	7,16	28,8	0,2
48	0,42	0,11	6,81	109,8	0,7
72	0,64	0,17	6,74	156,6	1,0
120	0,75	0,20	6,67	198,9	1,3
144	0,95	0,25	6,56	227,7	1,5
168	0,80	0,21	6,63	242,1	1,6
192	0,77	0,20	6,48	260,1	1,7

Table G50 OD, pH, dry cell weight and hydrogen production of 5 mM molasses sucrose medium by *R. capsulatus* YO3 (Hup-)

Time (h)	OD (660 nm)	Dry Cell Weight (gdcw/l_c)	pH	Cumulative Hydrogen (ml)	Cumulative Hydrogen (l/l_c)
0	0,22	0,10	7,34	0	0
24	0,33	0,15	7,23	21,6	0,144
48	0,37	0,16	6,91	80,1	0,534
72	0,61	0,27	6,79	98,1	0,654
120	0,61	0,27	6,81	117,9	0,786
144	0,69	0,31	6,75	130,5	0,87
168	0,42	0,19	6,64	134,1	0,894
192	0,47	0,21	6,52	137,7	0,918

Table G51 OD, pH, dry cell weight and hydrogen production on 5 mM molasses sucrose medium by the co-cultivation of *R. palustris* (DSM 127) *and R. capsulatus* YO3 (Hup-)

Time (h)	OD (660 nm)	Dry Cell Weight (gdcw/l_c)	pH	Cumulative Hydrogen (ml)	Cumulative Hydrogen (l/l_c)
0	0,22	0,10	7,34	0	0
24	0,33	0,15	7,23	21,6	0,144
48	0,37	0,16	6,91	80,1	0,534
72	0,61	0,27	6,79	98,1	0,654
120	0,61	0,27	6,81	117,9	0,786
144	0,69	0,31	6,75	130,5	0,87
168	0,42	0,19	6,64	134,1	0,894
192	0,47	0,21	6,52	137,7	0,918

Table G52 OD, pH, dry cell weight and hydrogen production of 5 mM molasses sucrose medium by *R. sphaeroides* O.U.001

Time (h)	OD (660 nm)	Dry Cell Weight (gdcw/l_c)	pH	Cumulative Hydrogen (ml)	Cumulative Hydrogen (l/l_c)
0	0,25	-	7,37	0,00	0,00
24	0,73	-	7,38	2,00	0,01
48	0,75	-	7,12	28,00	0,19
72	0,74	-	6,95	58,00	0,39
120	1,01	-	6,70	64,00	0,43
168	1,05	-	6,59	64,00	0,43
216	1,18	-	6,46	68,00	0,45
240	1,17	-	6,46	68,00	0,45

Table G53 OD, pH, dry cell weight and hydrogen production of 5 mM molasses sucrose medium by *R. palustris* (DSM 127)

Time (h)	OD (660 nm)	Dry Cell Weight ($gdcw/l_c$)	pH	Cumulative Hydrogen (ml)	Cumulative Hydrogen (l/l_c)
0	0,19	0,05	7,35	0,00	0,00
24	0,64	0,17	6,29	48,00	0,32
48	0,53	0,14	6,00	84,00	0,56
72	0,60	0,16	6,01	124,00	0,83
120	0,88	0,23	5,93	172,00	1,15
168	0,85	0,22	5,85	177,00	1,18
216	0,91	0,24	5,54	178,00	1,19
240	0,92	0,24	5,55	178,00	1,19

Table G53 OD, pH, dry cell weight and hydrogen production of the co-cultivation of *R. sphaeroides* O.U.001 and *R. palustris* (DSM 127) on 5 mM molasses sucrose medium.

Time (h)	OD (660 nm)	Dry Cell Weight ($gdcw/l_c$)	pH	Cumulative Hydrogen (ml)	Cumulative Hydrogen (l/l_c)
0	0,21	-	7,33	0,00	0,00
24	0,68	-	6,57	40,00	0,27
48	0,57	-	6,46	64,00	0,43
72	0,58	-	6,22	96,00	0,64
120	0,95	-	6,06	136,00	0,91
168	1,08	-	6,03	148,00	0,99
216	1,12	-	5,93	176,00	1,17
240	1,11	-	5,94	176,00	1,17

APPENDIX H

H1-H24. Organic Acid Concentrations of PNS Bacteria on Different Sucrose and Molasses Media with Different Sucrose Concentrations in 50 ml bioreactors.

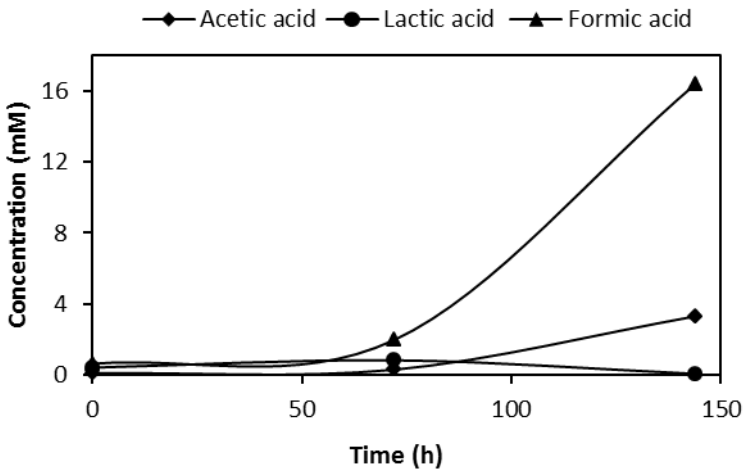


Figure H1 Concentrations of organic acids in photofermentation of *R.capsulatus* DSM 1710 on 5 mM sucrose hydrogen production media.

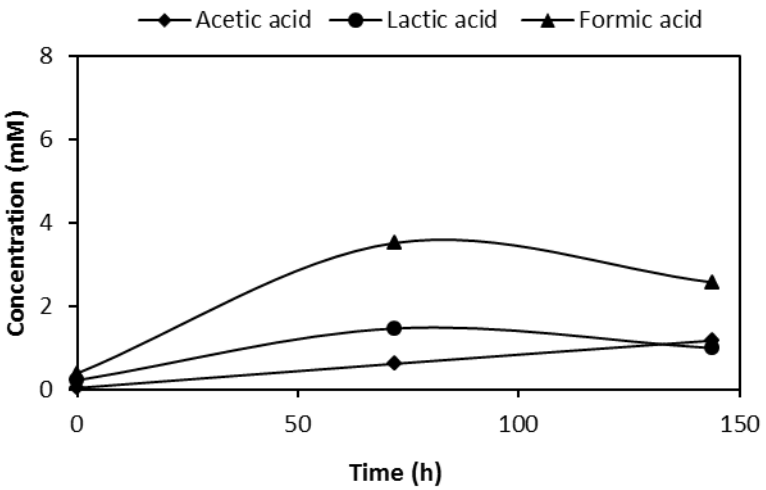


Figure H2 Concentrations of organic acids in photofermentation of *R.capsulatus* DSM 1710 on 7.5 mM sucrose hydrogen production media.

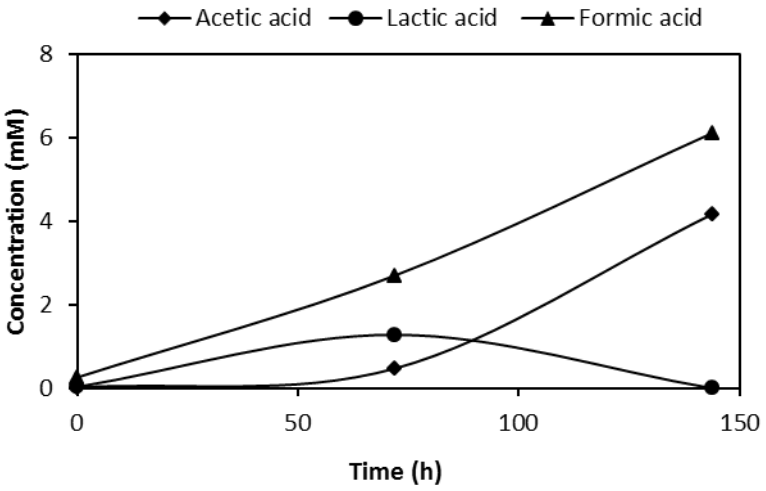


Figure H3 Concentrations of organic acids in photofermentation of *R.capsulatus* DSM 1710 on 10 mM sucrose hydrogen production media.

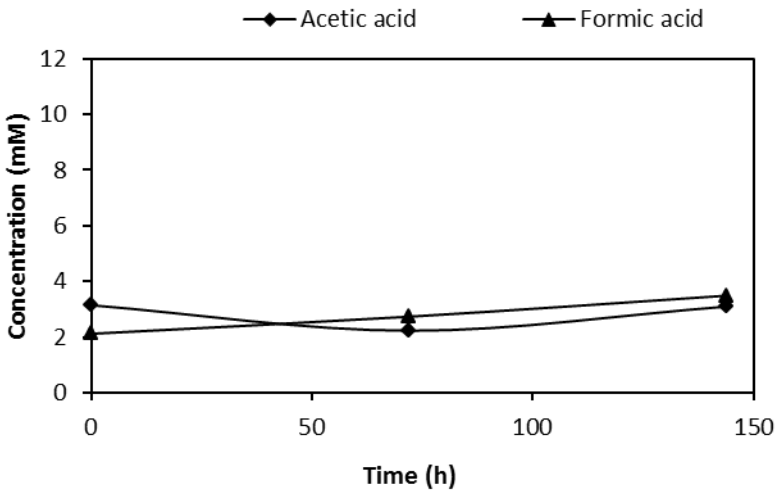


Figure H4 Concentrations of organic acids in photofermentation of *R.capsulatus* YO3 (Hup^-) on 5 mM sucrose hydrogen production media.

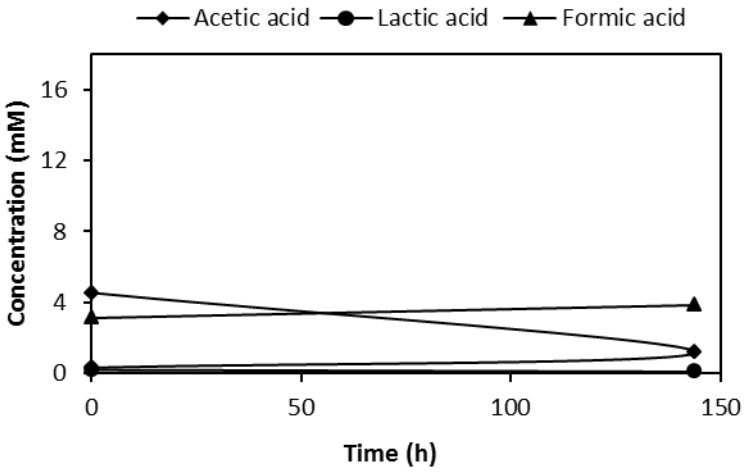


Figure H5 Concentrations of organic acids in photofermentation of *R.capsulatus* YO3 (Hup^-) on 7.5 mM sucrose hydrogen production media.

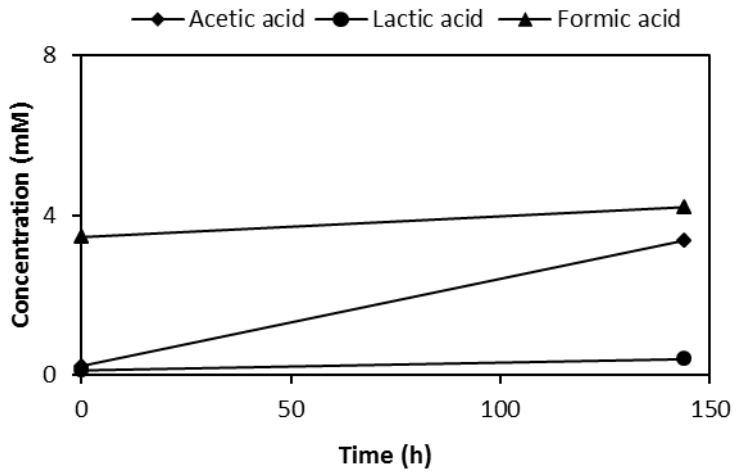


Figure H6 Concentrations of organic acids in photofermentation of *R.capsulatus* YO3 (Hup-) on 10 mM sucrose hydrogen production media.

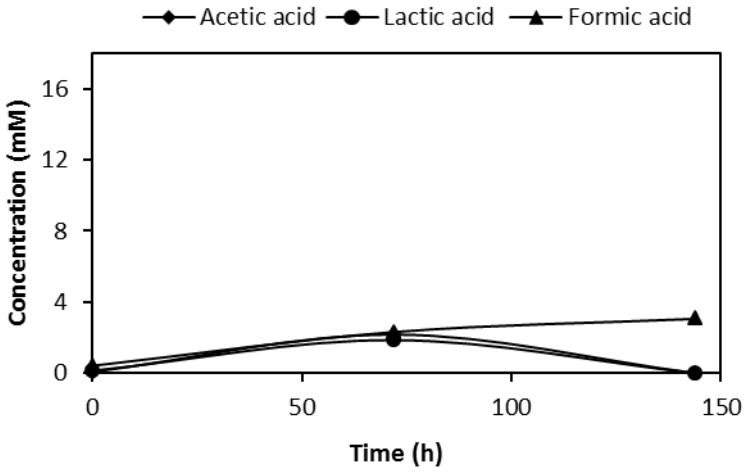


Figure H7 Concentrations of organic acids in photofermentation of *R. palustris* (DSM 127) on 5 mM sucrose hydrogen production media.

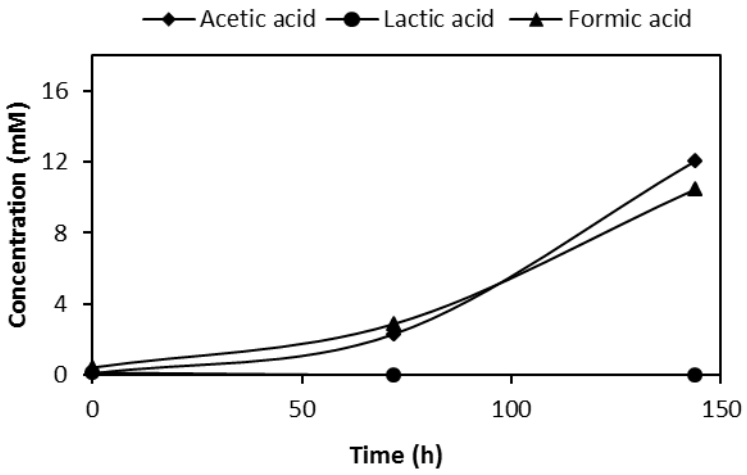


Figure H8 Concentrations of organic acids in photofermentation of *R. palustris* (DSM 127) on 7.5 mM sucrose hydrogen production media.

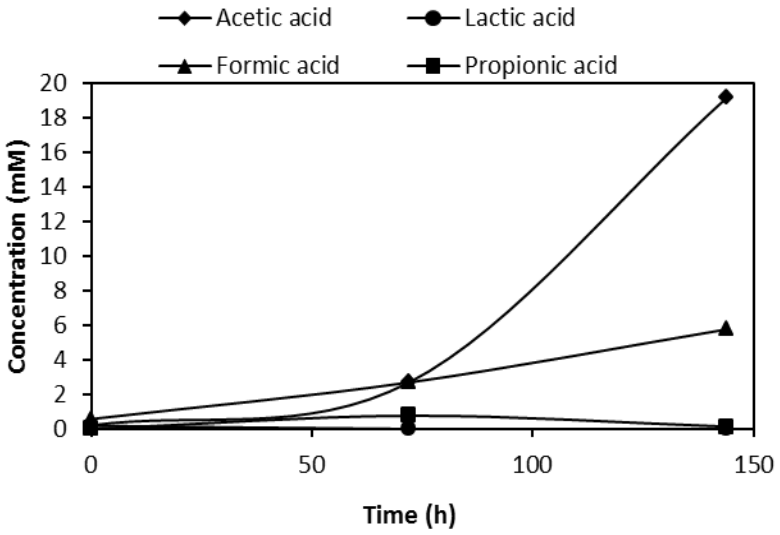


Figure H9 Concentrations of organic acids in photofermentation of *R. palustris* (DSM 127) on 10 mM sucrose hydrogen production media.

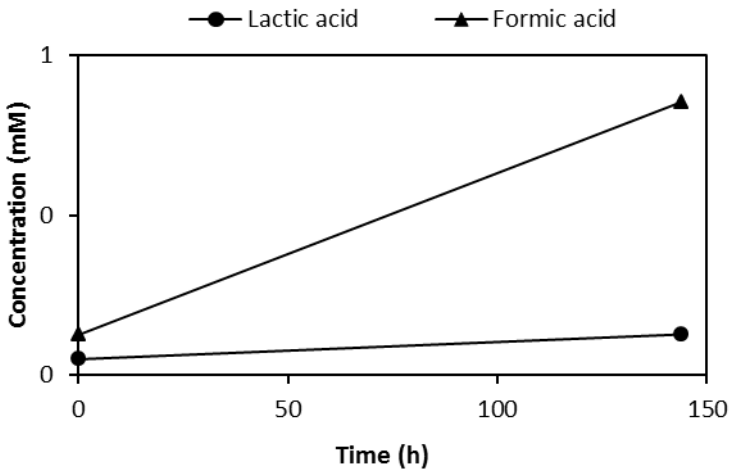


Figure H10 Concentrations of organic acids in photofermentation of *R. sphaeroides* O.U.001 on 5 mM sucrose hydrogen production media.

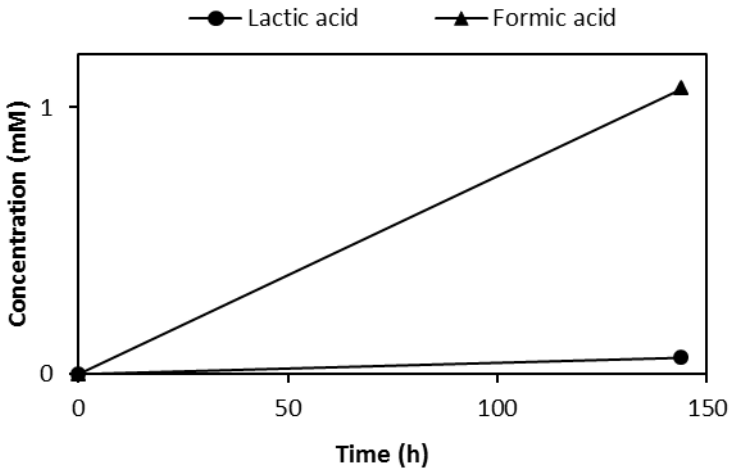


Figure H11 Concentrations of organic acids in photofermentation of *R. sphaeroides* O.U.001 on 7.5 mM sucrose hydrogen production media.

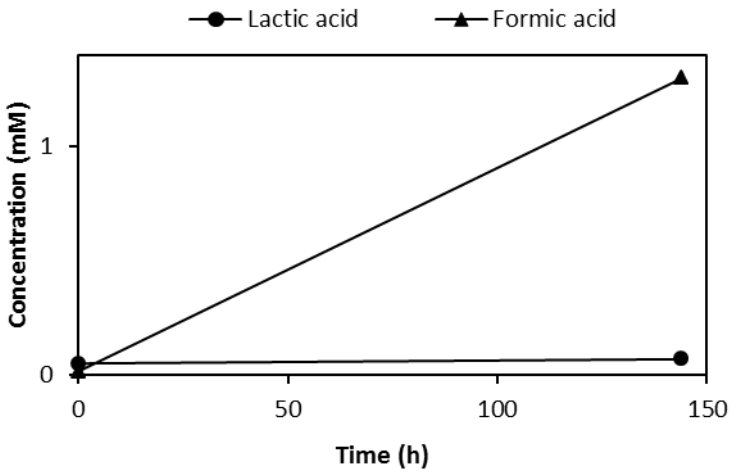


Figure H12 Concentrations of organic acids in photofermentation of *R. sphaeroides* O.U.001 on 10 mM sucrose hydrogen production media.

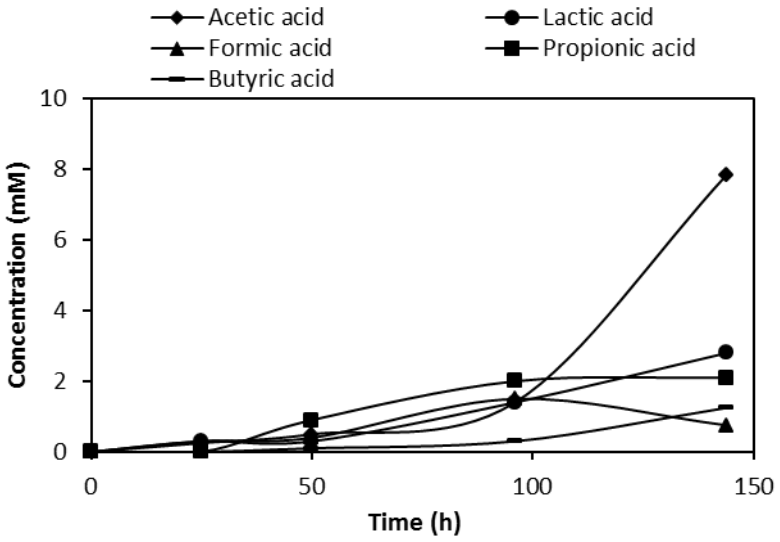


Figure H13 Concentrations of organic acids in photofermentation of *R. capsulatus* DSM 1710 on molasses medium with 5 mM sucrose hydrogen production media.

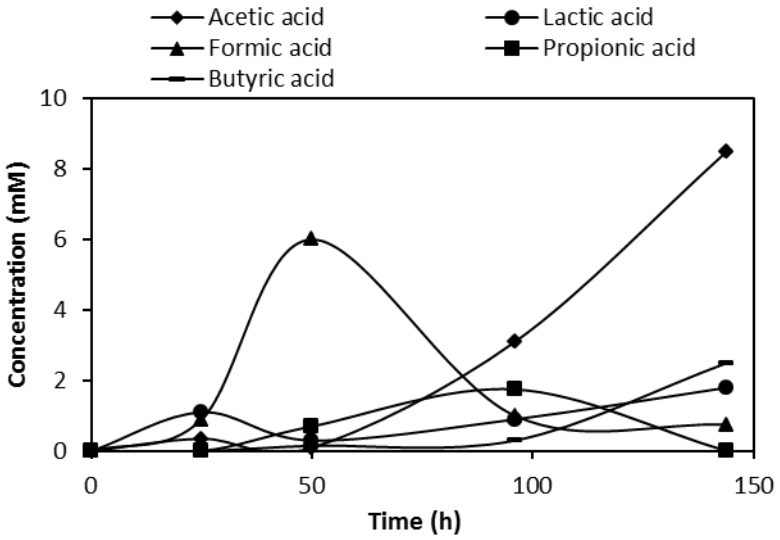


Figure H14 Concentrations of organic acids in photofermentation of *R. capsulatus* DSM 1710 on molasses medium with 7.5 mM sucrose hydrogen production media.

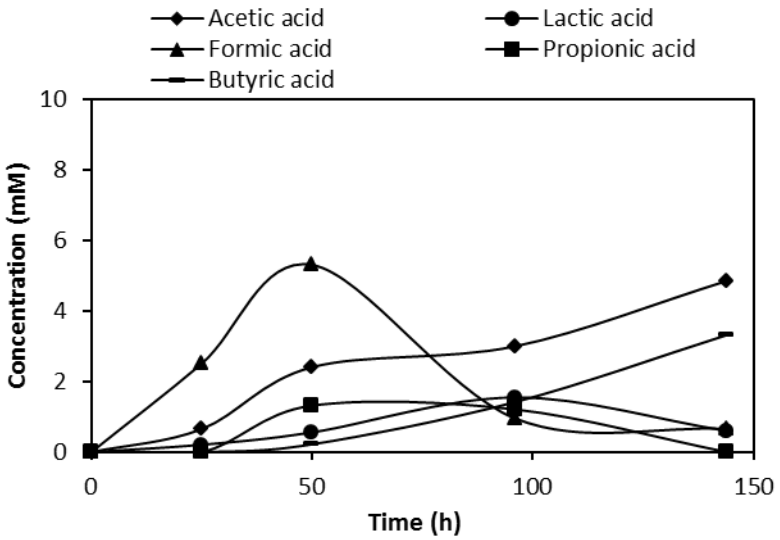


Figure H15 Concentrations of organic acids in photofermentation of *R. capsulatus* (DSM 1710) on molasses medium with 10 mM sucrose hydrogen production media.

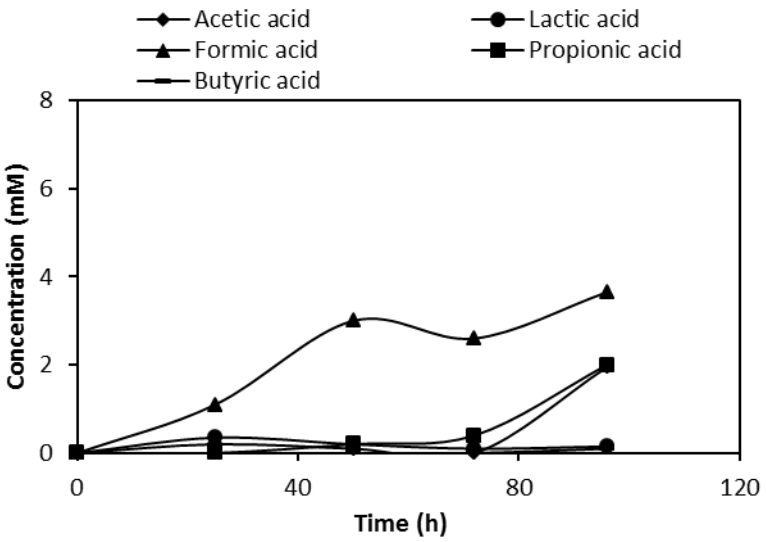


Figure H16 Concentrations of organic acids in photofermentation of *R. capsulatus* YO3 (Hup-) on molasses medium with 5 mM sucrose hydrogen production media.

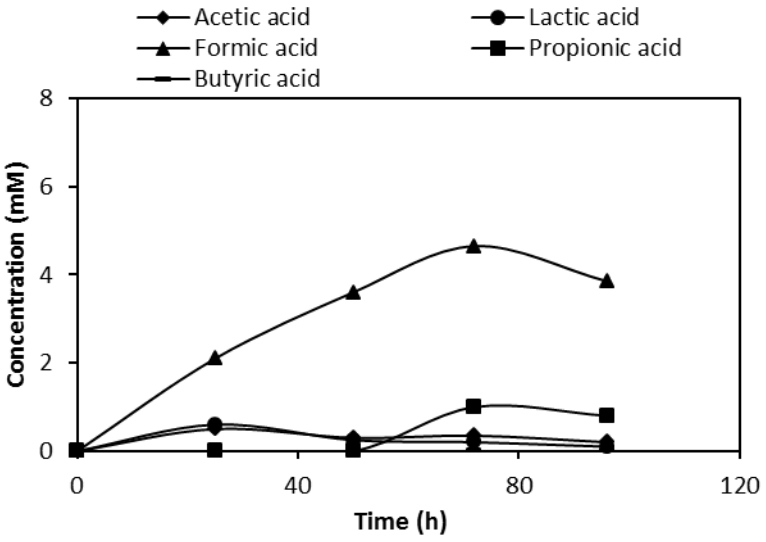


Figure H17 Concentrations of organic acids in photofermentation of *R. capsulatus* YO3 (Hup-) on molasses medium with 7.5 mM sucrose hydrogen production media.

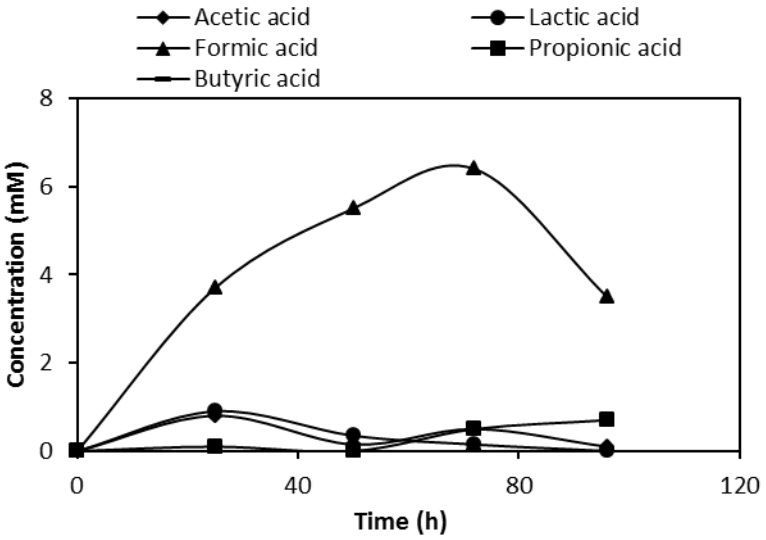


Figure H18 Concentrations of organic acids in photofermentation of *R. capsulatus* YO3 (Hup-) on molasses medium with 10 mM sucrose hydrogen production media.

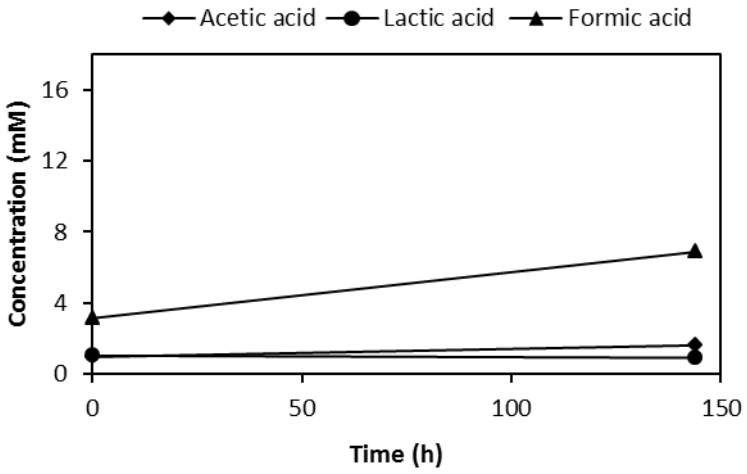


Figure H19 Concentrations of organic acids in photofermentation of *R. palustris* (DSM 127) on molasses medium with 5 mM sucrose hydrogen production media.

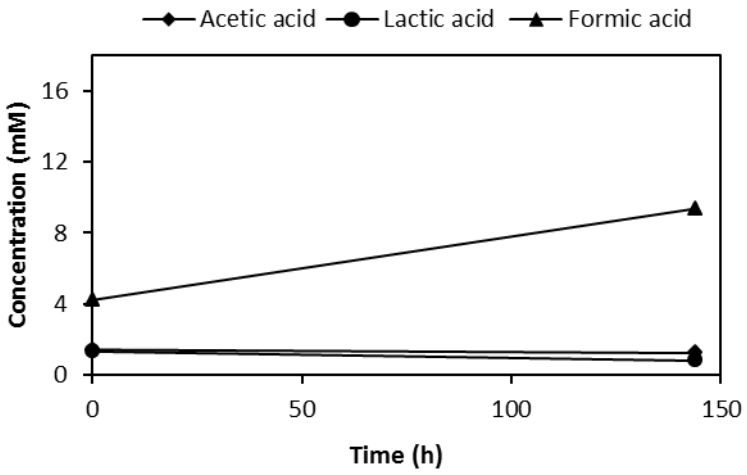


Figure H20 Concentrations of organic acids in photofermentation of *R. palustris* (DSM 127) on molasses medium with 7.5 mM sucrose hydrogen production media.

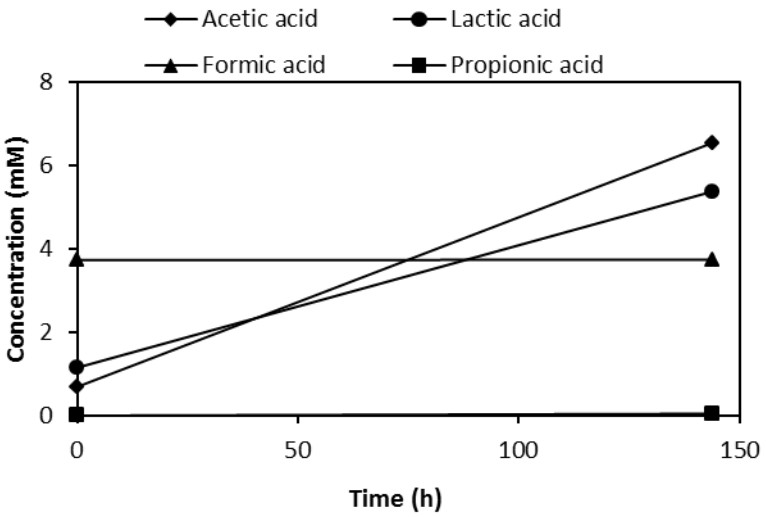


Figure H21 Concentrations of organic acids in photofermentation of *R. palustris* (DSM 127) on molasses medium with 10 mM sucrose hydrogen production media.

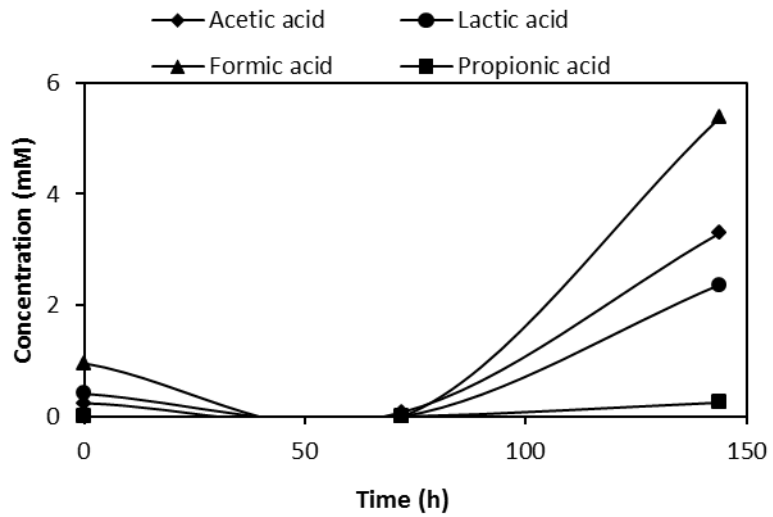


Figure H22 Concentrations of organic acids in photofermentation of *R. sphaeroides* O.U.001 on molasses medium with 5 mM sucrose hydrogen production media.

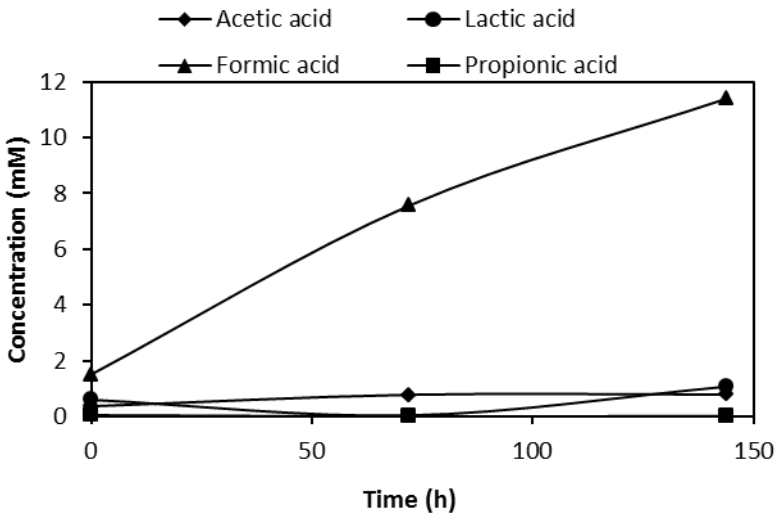


Figure H23 Concentrations of organic acids in photofermentation of *R. sphaeroides* O.U.001 on molasses medium with 7.5 mM sucrose hydrogen production media.

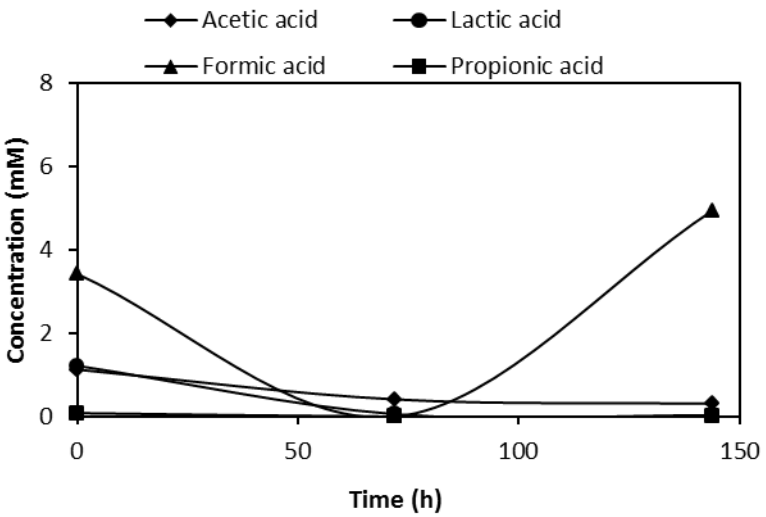


Figure H24 Concentrations of organic acids in photofermentation of *R. sphaeroides* O.U.001 on molasses medium with 10 mM sucrose hydrogen production media.

Figure H25-H33. Concentrations of organic acids of co-cultivation studies of PNS bacteria in 150 ml bioreactors.

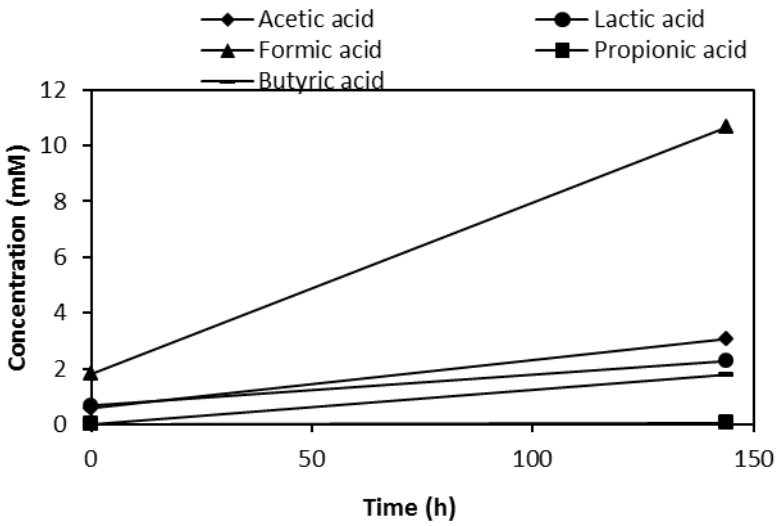


Figure H25 Concentrations of organic acids in photofermentation of *R. palustris* (DSM 127) on molasses medium with 5 mM sucrose hydrogen production media

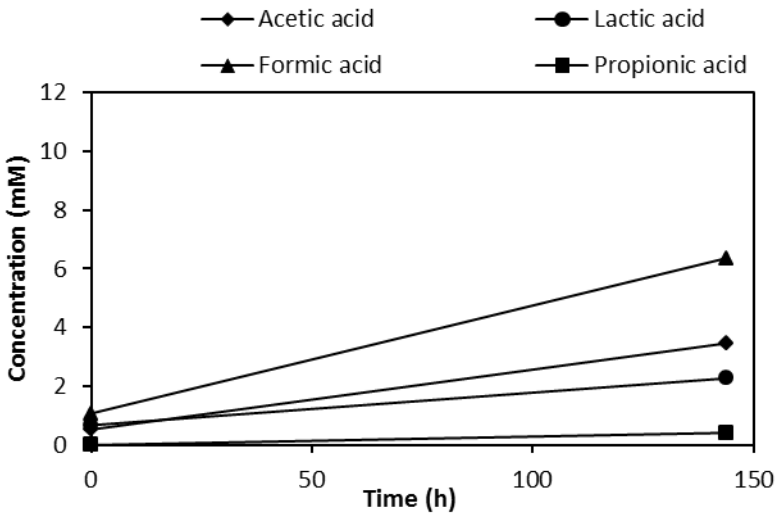


Figure H26 Concentrations of organic acids in photofermentation of *R. capsulatus* YO3 (Hup-) on molasses medium with 5 mM sucrose hydrogen production media.

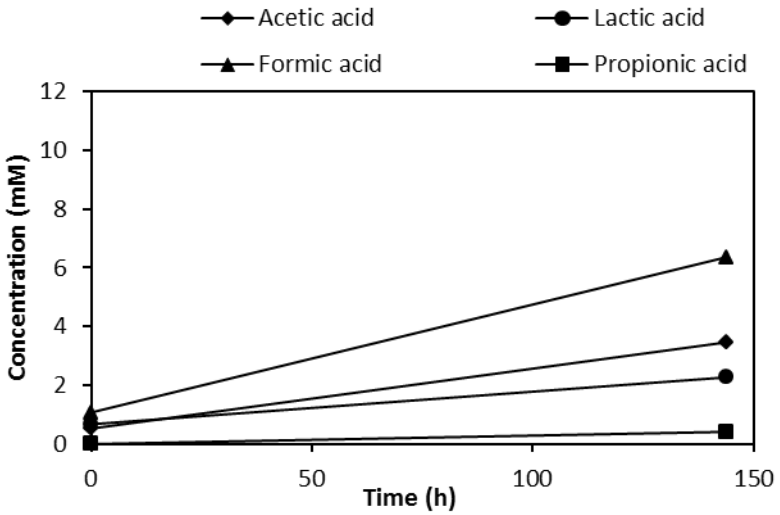


Figure H27 Concentrations of organic acids in photofermentation of the co-cultivation of *R. palustris* (DSM 127) and *R. capsulatus* YO3 (Hup-) on molasses medium with 5 mM sucrose hydrogen production media.

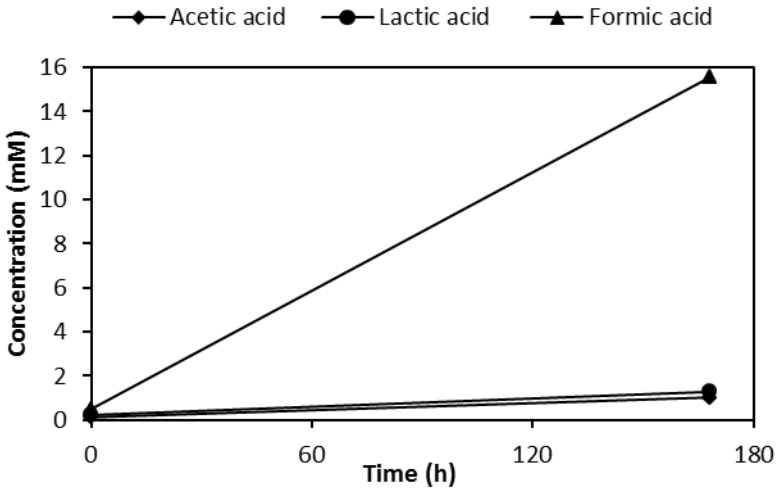


Figure H28 Concentrations of organic acids in photofermentation of *R. sphaeroides* O.U.001 on molasses medium with 5 mM sucrose hydrogen production media.

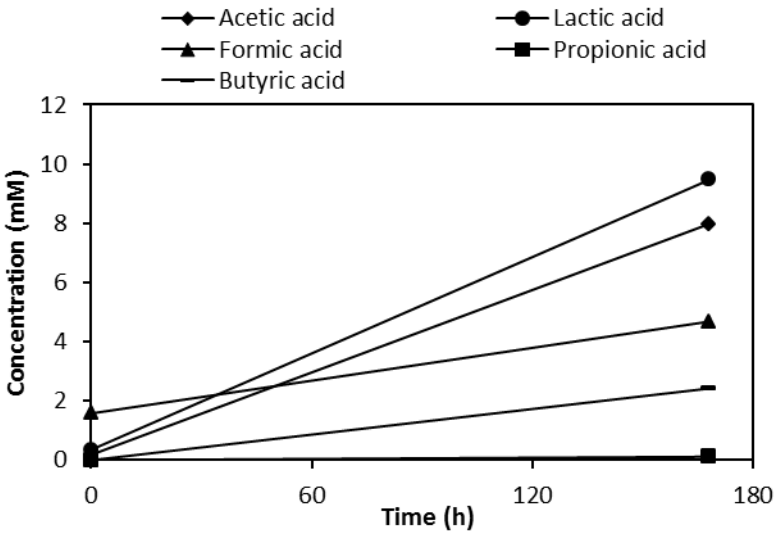


Figure H29 Concentrations of organic acids in photofermentation of *R. palustris* (DSM 127) on molasses medium with 5 mM sucrose hydrogen production media.

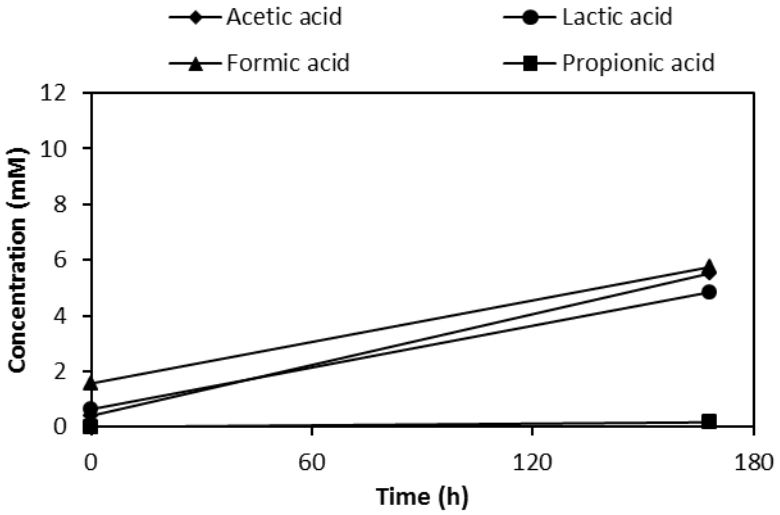


Figure H30 Concentrations of organic acids in photofermentation of the co-cultivation of *R. palustris* (DSM 127) and *R. sphaeroides* on molasses medium with 5 mM sucrose hydrogen production media.

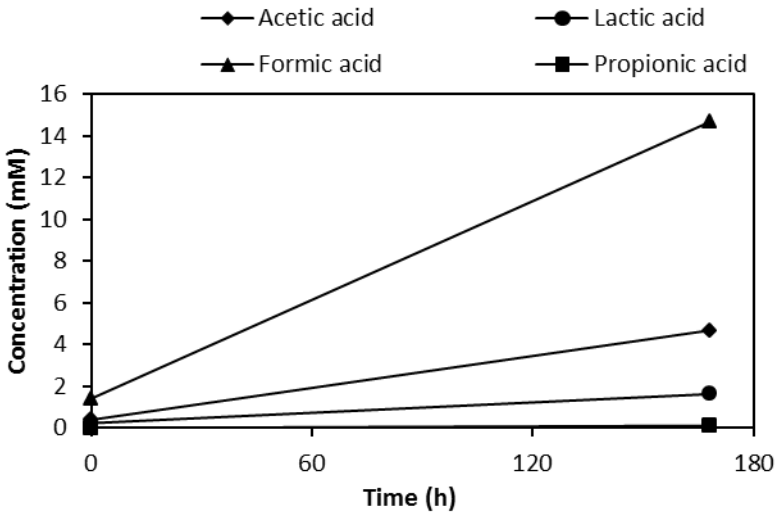


Figure H31 Concentrations of organic acids in photofermentation of *R. capsulatus* YO3 (Hup-) on molasses medium with 5 mM sucrose hydrogen production media.

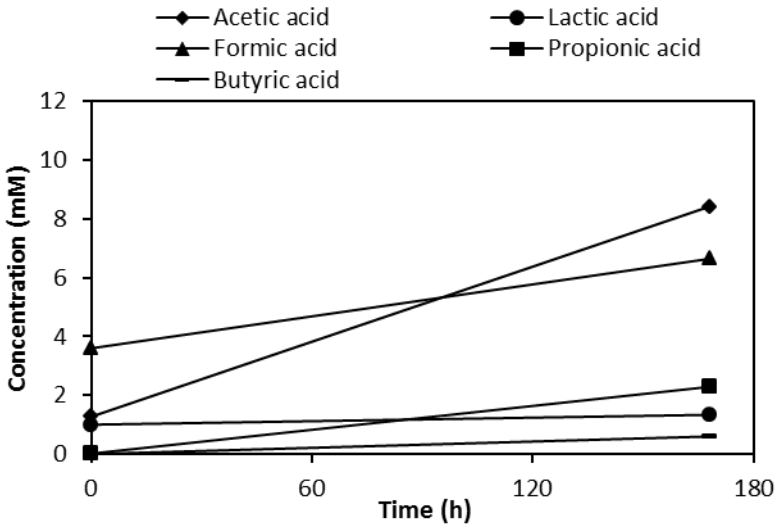


Figure H32 Concentrations of organic acids in photofermentation of the co-cultivation of *R. capsulatus* YO3 (Hup-) and *R. sphaeroides* on molasses medium with 5 mM sucrose hydrogen production media.

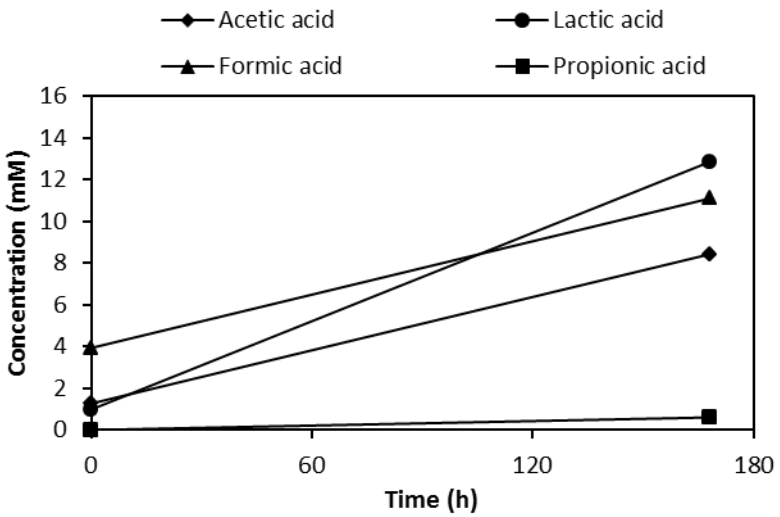


Figure H33 Concentrations of organic acids in photofermentation of the co-cultivation of *R. palustris* (DSM 127), *R. capsulatus* YO3 (Hup-) and *R. sphaeroides* on molasses medium with 5 mM sucrose hydrogen production media.

APPENDIX I

I1 Calculation of substrate conversion efficiency (%)

For calculation of *R. capsulatus* YO3 on 5 mM molasses medium.

Substrate conversion efficiency (%) = (Theoretical mole of H_2 over consumed substrate) / (Experimental mole H_2) x 100

Volume of reactor = 50 ml = 0.05 l

Theoretical mole of hydrogen on sucrose = 24

C_f = Final concentration of sucrose (mM) = 5.1

C_o = Initial concentration of sucrose (mM) = 2.7

Theoretical H_2 = (C_f - C_o) x Volume of reactor (l)

= (5.1-2.7) x (0.05) = 0.12 moles x 24 =2.88 moles H_2

Experimental Produced H_2 = 35.64 ml / 22.4 x 10^{-3} = 1.59 mmol

Substrate conversion efficiency (%) = (1.59)/(2.88) x 100 = 55.2%

I2 Calculation of hydrogen productivity

The formula for calculation of hydrogen productivity is given as an example for calculation of *R. capsulatus* YO3 on 5 mM molasses medium.

t = Duration of hydrogen production (hour) =72

v = Volume of culture = 0.05 l

V_{H2} = Produced hydrogen = 33.66 ml

Hydrogen productivity (mmol H_2/l_c.h) = [33.66 ml / 22.4 l] / [0.05 x 72] = 0.41

I3 Calculation of light conversion efficiency (%)

Using the data of R. sphaeroides on 5 mM sucrose medium.

V_{H2} = 0.86 l

I = 114.286 Watt/m^2

d_{H2} = 0.089 g/l

A = 0.002 m^2

t=Duration of H_2 production

$$\eta(\%) = \frac{33.6 \times \rho_{H_2} \times V_{H_2}}{I \times A \times t} \times 100$$

light conversion efficiency (%) = (33.6 x 0.089 x 0.86) / (114.286 x 0.002 x 180) x 100

= 6.25%