HYDROGEN GAS PRODUCTION BY *ESCHERICHIA COLI* IN A BIOREACTOR

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ABSTRACT

HYDROGEN GAS PRODUCTION BY *ESCHERICHIA COLI* IN A BIOREACTOR

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A bioreactor and a gas collection system were designed for the production of H_2 gas using *Escherichia coli*.

In order to optimize the conditions for *E. coli*, the salt tolerant *E. coli* DS 1576 strain was grown anaerobically at various conditions. It was observed that H_2 gas was produced only under anaerobic conditions in LB Broth containing glucose, formate and different metal ions (Ni, Mo, Se).

The maximum hydrogen gas evolution was observed at the exponential growth phase of anaerobically grown *E. coli* with a rate of 0.858 $L_{H_2}/L_{culture}$. h (4.35 μmole H_2 / mg cell. h). Optimum operating temperature and stirring rate were determined as 39°C and 360 rpm, respectively. In such systems the gas evolution

ceased down after 24 hours when the pH of the medium became slightly acidic (around 4.5).

Production of H_2 restarted by addition of formate to the medium with almost the same evolution rate.

It was observed that *E. coli* strains loose their H_2 productivity when they are transferred through passages for a long time. When they were stored at -20°C, H_2 productivity of the cells did not decreased.

It was also concluded that, for the continuous operation, the reasons of the decrease in H_2 productivity of *E. coli* must be well understood.

Key words: *E. coli*, Biological hydrogen production, Anaerobic bioreactor

ÖZ

ESCHERICHIA COLI İLE BİYOREAKTÖRDE HİDROJEN GAZI ÜRETİMİ

Bora, Kerem

Yüksek Lisans, Biyoteknoloji Bölümü

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E. coli ile H_2 gazı üretmek için, bir fotobiyoreaktör ve gaz toplama ünitesi tasarlandı.

E. coli sisteminin koşullarını optimize etmek için, tuza dayanıklı *E. coli* DS 1576 suşu anaerobik olarak çeşitli koşullarda büyütüldü. Hidrojen gazının sadece anaerobik şartlar altında, LB medyumuna ek olarak glukoz, format, ve farklı metal iyonları (Ni, Mo, Se) içeren besi ortamında büyüdüğü gözlendi.

Hidrojen gazının en yüksek çıkış hızı, anaerobik koşullarda büyütülmüş *E. coli*'nin logaritmik büyüme fazında 0.858 L_{H_2}/Lkültür. saat (4.35 μmol H_2 / mg hücre. saat) olarak bulundu. Hidrojen gazının üretiminde optimum koşullar 39 derece ve 360 dev/dak olarak bulundu.

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Bu tip sistemlerde gaz çıkışı 24 saat sonra ortam pH'ı asidik değerlere (yaklaşık 4.5) ulaştığında tamamen durdu.

Buna karşılık, ortama format eklenmesi ile yaklaşık aynı hızda H_2 üretimi tekrar başladı.

E. coli suşlarının uzun bir süre boyunca transferi sonucunda H_2 üretkenliğini kaybettiği gözlendi. Aerobik koşullarda -20 derecede altı ay boyunca saklanan kültürün H_2 üretkenliği azalmadı.

Sürekli sistemin geliştirilebilmesi için öncelikle *E. coli* bakterisinin H_2 üretkenliğindeki düşüşün nedeninin anlaşılması gerektiğine karar verildi.

Anahtar kelimeler: *E. coli*, Biyolojik hidrojen üretimi, Anaerobik biyoreaktör.

To My Family

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INTRODUCTION

Fossil fuels have a great importance on the economic growth of modern industrialized society. These fuels are readily available and convenient to use. They are storable, transportable and concentrated. On the other hand, consumption of fossil fuels became a danger to the environment. It is locally because of emissions, spills and leaks, and mining; regionally because of pollutant dispersion and globally because of acid rains. It is questionable whether the present energy system has outgrown the range of its effectiveness and whether it provides any net benefits to society (Barlar *et al.*, 1993; Tsygankov *et al.*, 1993; Nakada *et al.*, 1993).

Problems with the present fossil fuel based energy system, such as pollution, depletion of natural sources, international conflicts due to unequal distribution of fossil fuels etc., are growing and will probably get worse in the future. Fossil fuels supply more then 90% of the today's energy and the rest of the energy demand is provided by nuclear fuels, by the kinetic energy of rivers and by traditional fuels (wood, crop wastes, dung). Between 1932 and 1974, the consumption of fossil fuels grew at 4.5% per year (Barlar *et al.*, 1993). After the oil shocks in the early 1970's the growth in fossil fuel consumption has decreased but in the most recent years it has started to accelerate again (Barlar *et al.*, 1993). It is necessary to develop a new

energy system; a system that can be based on energy conversion (Tsygankov *et al.*, 1993; Taqui Khan and Bhatt, 1989; Miyake and Asada, 1993).

Hydrogen is a well-known, clean and powerful energy carrier. It has the highest energy to weight ratio of all fuels. Hydrogen can be produced in a variety of ways. Nearly 90% of H_2 is obtained from the steam reforming of natural gas or light oil at high temperature (Markov *et al.*, 1995). Another used method is the electrolysis of water in which water is split into hydrogen and oxygen.

Biological H_2 production is one of the promising areas of research for future. Microorganisms are able to produce molecular H_2. The ability is distributed both among chemotrophs and phototrophs. Most H_2 producing chemotrophic microorganisms belong to strict and facultative anaerobes. The strict anaerobes are particularly numerous. Most chemotrophs evolve H_2 under anaerobic conditions. Amounts of H_2 produced by several chemotrophic bacteria are shown in Table 1.1. (Kontratieva and Gogotov, 1982). Among other microorganisms *E. coli* produces H_2 at a high rate and in rather high amounts. Metabolism of glucose and some other sugars by the bacteria is connected with the production of H_2 and CO_2 in the ratio of 2.3-3.0; this ratio is higher than in other bacteria (Kondratieva and Gogotov, 1982).

1.1. *Escherichia coli*

E. coli is the major species of *Enterobacteria* family, it is facultatively anaerobic, gram-negative and rod shaped.

Table 1.1. Amounts of H_2 (μmoles/100 μmoles of substrate) produced by several chemotrophic bacteria

Genera	Species	Substate	H_2 produced (μmole/100 μmole substrate)
	STRICT ANAEROBES		
Clostridium	*C.acetobutylicum*	Starch	135.00
	C.butyricum	Glucose	235.00
	C. butylicum	Starch	78.00
	C.kluyvei	Ethanol + acetate	1.10
	C.perfringons	Glucose	214.00
	C.thermocellum	Cellulose	122.00
	C. thermohydrosulfuricum	Starch	11.00
Eubacterium	*Eub. Limosum*	Glucose	74.00
Peptococcus	*P.aerogenes*	Serine, threonine Adenine, uracil	49.0 - 96.0
Ruminococcus	*Rum. Albus*	Carbohydrate	257.00
Sarcina	*Sar. Maxima*	Carbohydrate	230.00
	Sar. Ventriculi	Hexose	41.00
Thermoanaerobacter	*T.brackii*	Hexose	50.00
Veillonella	*V.alcalescens*	Lactate	14.00
	FACULTATIVE ANAEROBES		
Bacillus	*B.polymyxa*	Glucose + mannitol	70.9 - 170.0
Esherichia	*E. coli*	Glucose	75.00
Photobacterium	*Ph. Phosphoreum*	Glucose	54.00

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Like many other enteric bacteria, it has evolved the ability to grow on a variety of different carbon compounds including many mono- and disaccharides, amino acids, fatty acids and short chain alcohols or acids. It can derive energy via electron transport linked phosphorylation reactions during aerobic as well as during anaerobic conditions when any of the alternative electron acceptors are available. The cell can also perform simple fermentation of sugars in order to yield ATP by substrate level phosphorylation when no electron acceptors are present in the cell environment. Thus, cell growth and survival are possible over a wide range of conditions. Depending on the presence of the alternative substrates, the bacterium switch the appropriate pathway on or off. So that, unnecessary enzymes are not synthesized, while the needed ones are present in sufficient amounts. By this way cellular energy generation for macromolecular biosynthesis, assembly and cell division are optimized (Ingledew and Poole, 1984; Gunsalus and Park, 1994; Vignais and Toussant, 1994).

1.1.1. Adjustment of cellular respiration of *E. coli*

During aerobic cell growth, glucose and other mono and disaccharides are directly channelled into the major pathway for sugar utilization, the Embden-Meyerhof-Parnas pathway, which converts them to pyruvate with the net generation of two ATP and two NADH per mole of glucose. Pyruvate is than converted to acetyl-CoA and CO_2 by pyruvate dehydrogenase. Acetyl-CoA is subsequently oxidized by the tricarboxylic acid cycle (TCA cycle) enzymes to CO_2. Depending on

the type of substrate(s) that are available to the cell, one or more additional enzymes may be synthesized to convert the substrate to an intermediate of the above pathways, so that it can be subsequently degraded.

When oxygen becomes limiting or absent from the cell environment; the cell can utilize any of a number of alternative electron acceptors (nitrate, nitrite, trimethyl-amide-N-oxide, dimethyl-sulphoxide, or fumarate) as a respiratory substrate instead of oxygen for electron transport-linked phosphorylation reactions (Gunsalus and Park, 1994; Tseng *et al.*, 1994).

If neither oxygen nor any of the alternative anaerobic electron acceptors are present, *E. coli* must resort to a fermentative mode of carbon catabolism. This relatively complex fermentation is also called as mixed acid fermentation because four of the seven end products are organic acids (Kontratieva and Gogotov, 1982) as shown in Table 1.2, Figure 1.1. Thus accumulation of ethanol, lactate and succinate results, along with the accumulation of the other oxidation products that include acetate, formate, hydrogen and CO_2. The energy obtained by the cell using this mode of metabolism is limited to the 2 ATP per mole glucose (Gunsalus and Park, 1994). Since the yield of ATP from fermentation is relatively low, large quantities of substrate are used during fermentative growth.

1.1.2. The production of molecular hydrogen by *E. coli* and regulation of the synthesis of hydrogenase systems

Microbial production of molecular hydrogen has been extensively studied during this century (Hörnsten *et al.*, 1986). Today the knowledge increases rapidly in

many different disciplines, from investigations of enzymes involved hydrogen transfer, to net yield of molecular hydrogen from different organisms on various substrates.

E. coli produces molecular hydrogen during mixed acid fermentation (Figure 1.1; Table 1.3) (Gunsalus and Park, 1994). In fact *E. coli* metabolizes hydrogen in two distinct pathways, both of which involve hydrogenase activity.

In the formate hydrogen lyase pathway, which oxidizes endogenously produced formate to CO_2 and H_2, hydrogenase passes electrons derived from formate oxidation via unidentified electron carriers to protons to yield molecular hydrogen.

Table 1.2. Products of the mixed acid fermentation of glucose by *E. coli*

Product	Moles formed / 100 moles glucose fermented
Formate	2.4
Acetate	36.5
Lactate	79.5
Succinate	10.7
Ethanol	46.8
2, 3 Butanodiol	0.3
CO_2	88.0
H_2	75.0

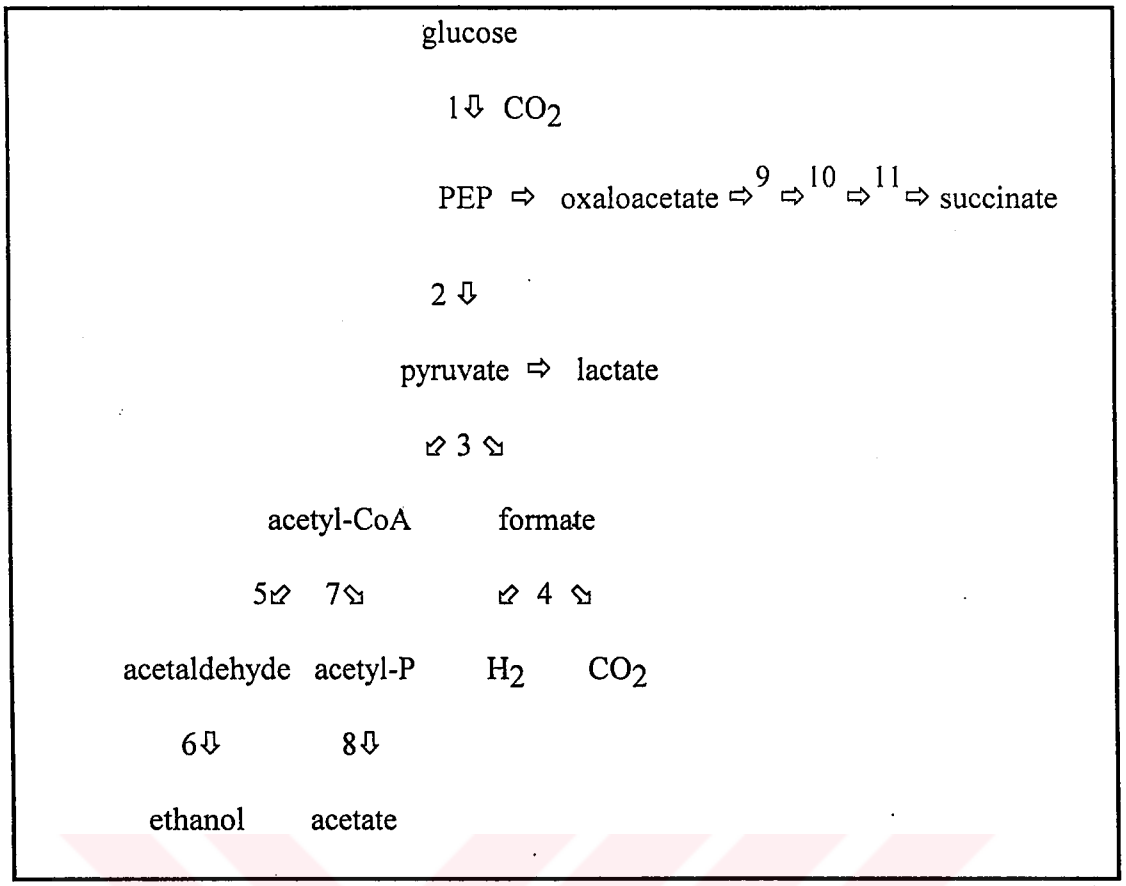

Figure 1.1. Carbon flow during anaerobic cell growth of *E. coli*.

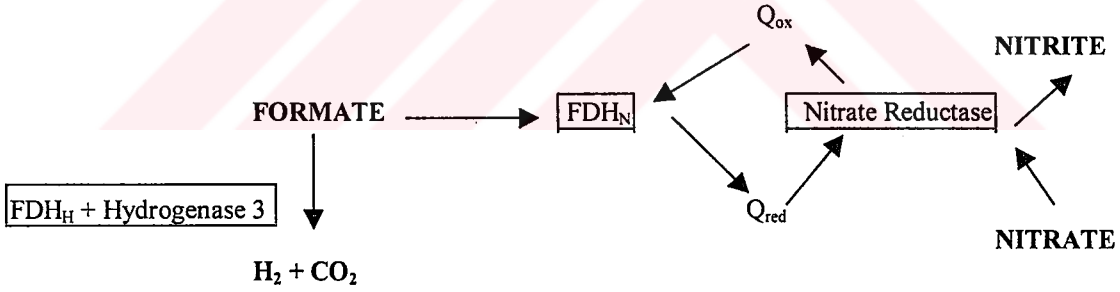

Figure 1.2. Utilization of formate in two different ways; by formate dehydrogenase nitrate reductase linking (FDH-N), by formate dehydrogenase hydrogenase 3 linking (FDH-H)

Table 1.3. Enzymes involved in anaerobic carbon flow (numbers indicate the corresponding enzymes in Figure 1.1)

Numbers	Enzymes
1	PEP carboxylase
2	Lactate dehydrogenase (anaerobic)
3	Pyruvate formate lyase
4	Formate hydrogen lyase
5	Acetaldehyde dehydrogenase
6	Alcohol dehydrogenase
7	Phosphotransacetylase
8	Acetate kinase
9	Malate dehydrogenase
10	Fumarase B (anaerobic)
11	Fumarate reductase

This later step is catalyzed by hydrogenase component. The whole pathway functions when the organism grows anaerobically in the absence of an exogenous electron acceptor (Ballantine and Boxer, 1985). The overall reaction is scalar, non-energy conserving and functions both to remove redox equivalents exchangeable with formate and to help offset acidification of the growth medium during fermentative growth.

Alternatively, the organism can utilize hydrogen as an energy source to allow anaerobic growth on a nonfermentable carbon source that also functions as an electron acceptor, i.e., fumarate. In this case, hydrogenase oxidizes hydrogen to protons and passes electrons directly or indirectly in a proton translocating, energy conserving manner to the quinone pool (Ballantine and Boxer, 1985; Sankar and Shanmugam, 1988; Sankar and Shanmugam, 1985; Sawers and Boxer, 1986; Sawers *et al.*, 1985).

Multiple hydrogenase isoenzymes have been identified in the membrane fraction of anaerobically grown *E. coli* (Ballantine and Boxer, 1985; Sawers and Boxer, 1986; Sawers *et al.*, 1985). The organism possesses at least three hydrogenase isoenzymes, termed hydrogenases 1,2 and 3 (Sawers *et al.*, 1985).

The physiological role of hydrogenases 1 and 2 appear to be catalysis of hydrogen oxidation coupled to the energy conserving reduction of electron acceptors like fumarate (Sawers and Boxer, 1986). Both hydrogenases 1 and 2 have been purified and extensively characterized at the biochemical level, (Sawers and Boxer, 1986, Ballantine and Boxer, 1986) and recently the cloning and nucleotide sequence of an operon coding for components of hydrogenase1 have been reported (Lutz *et al.*, 1991).

The physiological function of isoenzyme 3 is that of a gas evolving hydrogenase. It forms part of the formate hydrogen lyase (FHL) complex that catalyses formate oxidation coupled to H^+ reduction. Biochemically, hydrogenase 3 is the least well characterized isoenzyme, mainly because it has proved to be difficult to purify as a result of its inherent instability. However, an operon has been cloned

which contains genes coding for structural components of the enzyme (Lutz *et al.*, 1991).

Recently, Sode et al. (1998) reported that, formate dehydrogenase takes role in utilization of formate by linking to either nitrate reductase or hydrogenase 3 as represented in Figure 1.2.

In the hydrogen-producing pathway, the regulation of pyruvate formate lyase/ formate hydrogen lyase complex is an interesting example for the expression of anaerobically induced enzymes. The synthesis of formic dehydrogenase and hydrogenase are repressed by oxygen, anaerobically by nitrate and/or fumarate and induced in the presence of formate. The effects of oxygen, nitrate and formate occur at the transcriptional level (Zinani *et al.*, 1984).

The metabolism of formate and hydrogen is complex as there are three distinct hydrogenase activities and two formate dehydrogenases plus associated proteins require nickel, selenium and molybdenium for cofactor processing (Gunsalus and Park, 1994).

Hydrogenase restoration of *E. coli* hydrogenase deficient mutant HK7, which carries a mutation at *hyd B* locus was studied. Anaerobic growth of HK7 in the presence of iron chloride or vanadium chloride resulted in the restoration of hydrogen uptake activity of hydrogenase, but not hydrogen evolution activity. The growth of HK7 in the presence of nickel chloride restored total hydrogenase activity as Sode *et al.* (1986) reported.

The expression of isoenzymes is differentially influenced by nutritional and genetic factors. Using crossed immunoelectrophoresis, Sawers and coworkers

(Sawers *et al.*, 1985) found that the cellular content of hydrogenase2 is enhanced during anaerobic growth in the presence of either hydrogen and fumarate or glycerol and fumarate. The hydrogenase1 content is negligible under these conditions but is enhanced by exogenous formate. It was found by Sawers *et al.* (1985) that, the expression of hydrogenase1 was greatly reduced in a mutant that was unable to synthesize formate, but was restored to normal levels when the growth medium included formate. Therefore hydrogenase1 content does not correlate with formate hydrogenlyase activity and its role is unclear. Moreover hydrogenase isoenzyme1 constitutes about 8% of the hydrogenase (H_2 uptake) activity, the remainder being attributable to hydrogenase isoenzyme 2 (Sawers and Boxer, 1986). In addition the cellular content of hydrogenase isoenzyme 2 correlates reasonably with the cellular H_2 uptake capacity (Sawers *et al.*, 1985). Therefore it can be concluded that, isoenzyme 2 probably catalyses the H_2 uptake reaction.

1.1.3 Properties of the Isoenzymes

Hydrogenases of various microorganisms differ in molecular weight, electron donor or acceptor they interact with, cellular localization and in other properties. Some microorganisms like *E. coli* contain more than one hydrogenase differing in various properties and functions. On the basis of metal content two classes of hydrogenases are distinguished by Corremans *et al.* (1992);

(i) The iron hydrogenases which contain only iron-sulfur clusters,

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(ii) The [NiFe] hydrogenases, which contain a nickel centre in addition to iron-sulfur clusters. The [NiFeSe] hydrogenases, which contain nickel with selenium ligand plus iron-sulfur clusters, can be considered as subclass of [NiFe] hydrogenases (Corremans *et al.*, 1992)

Both isoenzyme1 and 2 are in the second class. Nickel is the hydrogen-binding site and iron-sulfur clusters are probably involved in electron transfer to or from the active site. These metalloenzymes are also membrane bound (Corremans *et al.*, 1992) Sawers and Boxer (1986) found that hydrogenase isoenzyme1 contains minimally two immunologically distinct polypeptides of M_r 64,000 and M_r 35,000. They also reported that, the enzyme is stable over a broad pH range (4-10). The H_2 uptake activity was still 60% active after 4 hours at pH 4 or at pH 10. H_2 evolution activity showed a similar profile to that of the H_2 uptake activity at the high pH range but it was more susceptible to inactivation at low pH.

H_2 uptake activity was stable at 70°C for 15 min but was lost irreversibly at higher temperatures. H_2 evolution activity was less temperature stable since the enzyme retained only the 40% of its capacity to evaluate H_2 from reduced methyl viologen after 15 min at 70°C (Sawers and Boxer, 1986)

The membrane bound form of hydrogenase isoenzyme2 consists of minimally two kinds of polypeptides; one of M_r 61,000 and the other of M_r 35,000. In contrast to isoenzme1, exposure to high pH irreversibly inactivates isoenzyme2. The effect of pH variation on hydrogenase activity was investigated, by Ballantine and Boxer (1986) and they reported that; 75% of the enzyme activity present after incubation at pH 7 was progressively lost between pH 8 and pH 10.

Both hydrogenase isoenzyme1 and 2 contain nickel. In fact, they are likely to be the only significant nickel containing proteins in the membrane. The majority of membrane bound nickel is attributable to hydrogenase isoenzyme1. A smaller proportion was found to be associated with hydrogenase2 (Ballantine and Boxer, 1985).

Both isoenzymes have a low K_m for H_2 when catalysing H_2 dependent reduction of benzyl viologen but isoenzyme 2 has the greater catalytic capacity for this reaction. While both isoenzymes are able to catalyse H_2 evolution with reduced methyl viologen as the electron donor, the rates found in each case are much lower than those for the H_2 uptake reaction. On the basis of these results, it is difficult to think either of these isoenzymes participating physiologically, in the evolution of H_2. However, since the catalysis of neither enzyme has been examined employing likely physiological electron carriers as substrates, only tentative conclusions concerning their physiological roles based on such studies can be drawn (Ballantine and Boxer, 1986).

Since the cellular content of neither of these isoenzymes can be correlated with formate hydrogen lyase, which is thought to be the sole hydrogen evolving pathway in *E. coli*, a further hydrogenase isoenzyme which is a structural unit of this pathway must be present in the organism.

According to Sawers *et al.* (1985); immunoelectrophoretic and gel electrophoretic analysis of both soluble and membranous subcellular fractions fail to reveal further components with substantial hydrogenase activity other than isoenzymes 1 and 2. They also reported that; the additional hydrogenase may exhibit

very poor activity in the activity stain performed on the electrophoretograms, and therefore it may correspond to one of the poorly discerned which are observed. The enzyme though reasonably stable in broken cell preparations, may be labile under those analytical conditions. Indeed the lability of formate hydrogen lyase after cell disruption is a major obstacle to its molecular characterization.

1.2. Recent studies on H_2 Production by *E. coli*

The anaerobic decomposition of formate into H_2 and CO_2 by *E. coli* was studied extensively (Nandi and Sengupta, 1998). Immobilization of FHL system of *E. coli* and sustained stoichiometric conversion of formate into H_2 and CO_2 were reported by Nandi and Sengupta (1998). They reported H_2 production from 1.15 M formate over a 96 hr cycle with loss of 25% efficiency per cycle. It was found possible to achieve 1.2 stoichiometry of H_2/glucose by using immobilized whole cells of *E. coli.*

Kanayama *et al.* (1987) reported biochemical energy conversion by immobilized *E. coli.* In a small-scale bioreactor (17 ml), the obtained maximum hydrogen evolution rate was 1.312 μmol H_2/mg cell. h. A recombinant *E. coli* (over expressed in hydrogenase activity) was used in the study. They also achieved hydrogen evolution using wild type *E. coli* in a 150-L reactor. During seven hours operation 157 L (1 atm, 37 °C, 6.18 mol) hydrogen was evolved with a rate of 0.735 μmol H_2/mg cell. h). Produced hydrogen was transferred to a fuel cell system and ca. 10-12 W power with a current of 10-12 A was obtained. Kanayama *et al.* remarked

the importance of precultivation temperature in hydrogen productivity. They reported that recombinant *E. coli* precultured at a temperature lower than the optimum for cell growth (30-37°C) showed a higher hydrogen evolution rate from glucose than cells precultured at 30-37°C.

Based on available molecular genetics data on the regulations and enzymes in the *E. coli* anaerobic metabolism, Sode *et al.* (1998) examined approaches for the enhancement of hydrogen production by *E. coli* through metabolic engineering.

1.3. Hydrogen gas production using coupled enzyme systems from *E. coli* and *H. halobium*

1.3.1. *Halobacterium halobium*

H. halobium is an extremely halophilic bacteria that lives in hyper saline ponds and salty lakes of Middle East region. The cell envelope of halobacteria is made up of a cell membrane surrounded by a glycoprotein cell wall. The lipids contained in the cell membrane carry exclusively dihydrophytol chains in ether linkage to glycerol. At low salt concentration (less than 3 M NaCl) most cellular enzymes are irreversibly denatured and cell wall rigidity is completely lost (Oesterhelt and Stoeckenius, 1974). The organism can grow with very high yield in aerobic conditions and use oxygen to oxidize fuel molecules. However, the natural habitat of halobacteria consists of warm, low-oxygen, highly concentrated salt solutions. Surviving under these extreme conditions required the development of a

system of photosynthesis equivalent to that found eg. in the higher plant species. The key element of bacterial photosynthesis is a retinal protein Bacteriorhodopsin (BR), which is similar to the human visual protein rhodopsin. In the purple membrane of the bacteria, the retinal protein Bacteriorhodopsin (BR), acts as a light driven proton pump (Lanyi, 1993; Oesterhelt *et al.*, 1991).

1.3.2. Properties of BR as light driven proton pump.

Bacteriorhodopsin (BR) is the only protein present in the purple membrane of *H. halobium* acting as a light-driven proton pump. All the molecules are unidirectional oriented within the bacterial membrane. Irradiation causes BR molecules to pump protons from interior of the cell (cytoplasm) to the surrounding medium, thereby generating proton gradient between the interior and exterior of the cell membrane. The energy content of the proton gradient is used by a membrane bound enzyme, an ATPase to convert adenosine diphosphate (ADP) to adenosine triphosphate (ATP), which is the universal fuel of all living cells. By this way BR converts light energy into chemical energy.

The basic biochemical and photophysical properties of BR are summarized in Table 1.4. (Kaya, 1995)

The retinal protein BR is very stable and may retain its activity for several years even in isolated form. This property has attracted the attention of the researchers (Birge, 1990; Trissl, 1990). In *H. halobium* packed cells, or as purple

Table 1.4. Biochemical and photophysical properties of bacteriorhodopsin

Biochemical properties		Photophysical properties	
Molecular weight	26,000 Dalton	initial absorbtion	ε_{570}=63,000 $M^{-1}cm^{-1}$
Structure		quantum efficiency	$\Phi \geq 64$ %
-primary	248 amino acids		
-secondary	7 α-helical domains	angle to retinal to	20^{o}
-tertiary	cage with proton core	membrane	
-quarternary	trimers, 2-D crystalline		
lipid bilayer	trans-membrane, 10	photoactive	at least 4
	lipidmolecules per BR	intermediates	
Chromophoric	retinal	Photochromism	cis-*trans* isomerisation
group			
Biological function	light-driven proton	refractory period	none
	pump	after relaxation	
Stability	- constant illumination	Instabilities	UV-light
	- oxygen + light		organic solvents
	- temperatures > $80^{o}C$		
	- pH 3-10		
	- most proteases		

membrane fragments, the role of BR is to give protons to the medium. As it is shown in Figure 1.3., BR undergoes its cyclic series of transformations in light and in dark (Zabut, 1990).

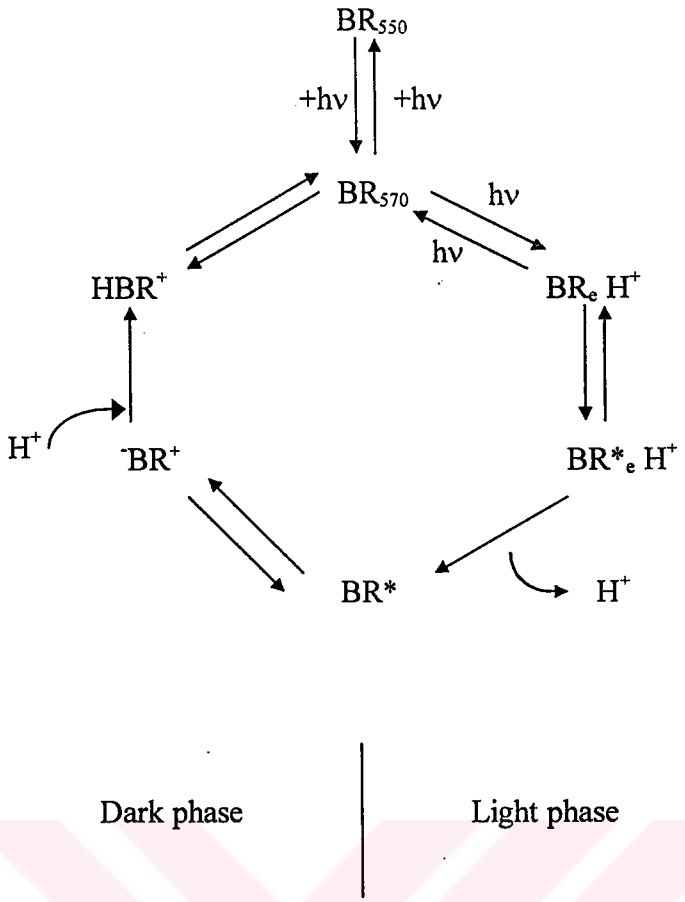

Figure 1.3. The photochemical cycle of BR (Zabut,1990)

El-Bashiti (1998) isolated a new strain of *H. halobium* MILTU from Tuz Gölü in central Anatolia, Turkey. He immobilized either packed cells or isolated purple membrane fragments in polyacrylamide gel (PAG). He observed the enhancement of photoelectrochemical hydrogen production by the biocatalytical effect of BR in immobilized systems.

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1.3.3. Hydrogen gas production by *E. coli* coupled to BR reconstituted systems

In recent studies, systems containing *H. halobium* or separated BR have been used for the photo production of hydrogen. Although protons are generated upon illumination in these microorganisms, protons cannot be converted to molecular hydrogen because they normally lack hydrogenase enzymes. A proton reduction system should be coupled to *H. halobium* to enable the hydrogen production. This has been achieved by coupling *H. halobium* to a salt tolerant *Escherichia coli* (Khan and Bhatt, 1989; Patel and Madamwar, 1994; Kaya, 1995). *H. halobium* packed cells coupled to an electrochemical system was also found to be very promising and advantageous, since nutrients are not required (Khan and Bhatt, 1990, 1992; Sediroğlu *et al.*, 1996a). The mechanism of hydrogen evolution is presented in Figure 1.4. Electrons are donated by hydrogenase to the protons released from BR in purple membrane fragments with the evolution of molecular hydrogen.

There are numerous advantages of the biological H_2 production by coupled systems over the thermochemical and physical methods (Taqui Khan and Bhatt, 1989).

i) The biological system could be operated at low physiological temperature (i.e. 10-40^{o}C) as opposed to the normally high temperatures required for chemical or physical production of H_2 (400-1000^{o}C).

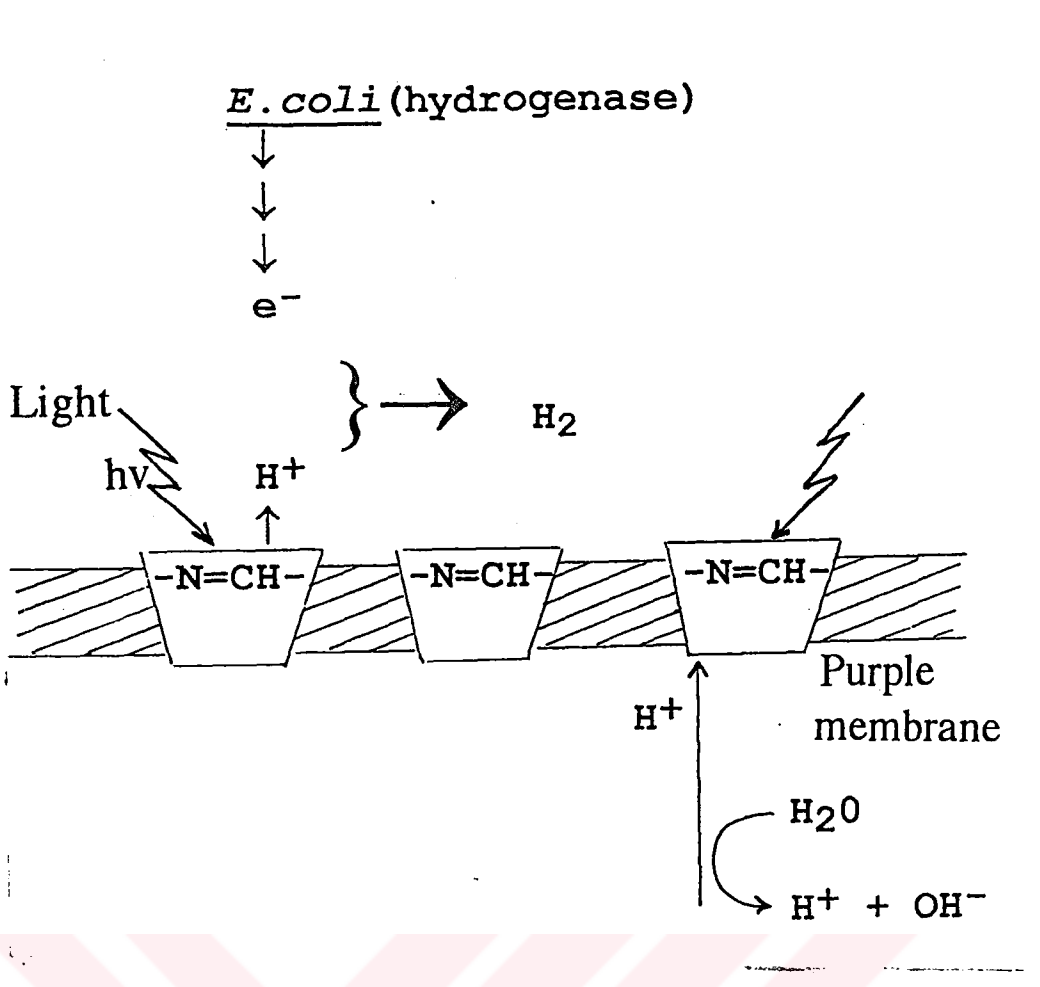

Figure 1.4. Mechanism of Hydrogen evolution by *H. halobium* coupled to *E. coli*

ii) The major inputs to the system would be solar energy and a hydrogen donor, water (salt water) which are plenty and readily available. This is very important since availability of fresh water is a problem in many countries.

iii) It is economical to construct large-scale systems using combination of photosynthetic and anaerobic microorganisms for production of hydrogen.

iv) Intact cells of *H. halobium* as well as the isolated purple membrane can be efficiently used in biotechnology. This together with the stability of the quantum

capture system of *Halobacterium* makes it an ideal system for exploitation at industrial level.

It will take time for hydrogen generating bacterial systems to be put into practice. It will gradually replace the existing energy sources with new ones. The rate at which the new energy system could be introduced without causing economic stresses is yet to be determined. Analysis of the historical trends in the energy market could provide the information necessary for forecasting and evaluation of the future energy options.

Table 1.5. illustrates the summary of the recent studies on hydrogen production by *E. coli* combined systems. Patel and Madamwar (1994) could not achieve hydrogen evolution using only *E. coli* cells. However, when *E. coli* was coupled to *H. halobium* and *P. valdorianum*, H_2 evolution was observed. Bagai and Madamwar (1998) achieved continuous and stable hydrogen production by immobilizing the combined system of *E. coli, H. halobium* and *P. valdorianum* in a PVA-alginate film. The intermittent supply of nitrogen was found to be essential to retain cellular metabolic activities which in turn showed prolonged production of hydrogen. The immobilized system was far more stable than the free cells and produced hydrogen continuously over 60 days under on/off light (6 h) and dark (18 h) cycles (Bagai and Madamwar, 1999).

Taqui Khan and Bhatt (1991) could not achieve H_2 evolution with only *E. coli.* They achieved photochemical evolution of hydrogen by *H. halobium* packed cells and *E. coli* coupled system (1989). Their system produced maximum 2406 μmol. H_2/mg cell. h for about only 30 minutes. The same group also used

immobilized *E. coli* systems for enhancement of hydrogen production (Taqui Khan and Bhatt, 1990a) and reported the same rate of hydrogen evolution, but five times higher yield due to the prolonged hydrogen evolution.

Kaya *et al.* (1996) have studied hydrogen gas production using coupled enzyme systems from salt tolerant *E. coli* DS 1576 and *H. halobium*. Under anaerobic growth conditions, the salt resistant *E. coli* DS 1576 induced the over-expression of hydrogenase isoenzymes, which catalyzed the reduction H^+ ion to H_2 gas. Hydrogen gas production was enhanced when packed cells of *H. halobium* and *E. coli* were used together or isolated BR and *E. coli* packed cells were used together. The purity of H_2 gas obtained was 98%.

1.4. Aim of the study

The aim of this study is to produce hydrogen gas by *E. coli*. Following steps are considered to reach this goal.

i) Optimization of the anaerobic growth conditions of *E. coli*.

ii) Construction of a one-liter photo-bioreactor and optimizing the bioreactor conditions for H_2 production with different *E. coli* strains.

iii) Coupling *E. coli* to immobilized *H. halobium* packed cells for production of hydrogen gas.

Table 1.5. Summary of the studies about H_2 production by *E. coli*

Study	*E. coli* strain	Production time	H_2 production (μmol H_2 / mg cell. h)
H_2 production by *E. coli.* Kanayama *et al.* (1987)	*E. coli* C 600 *E. coli* (over-expressed in H_2 productivity)	7 h 20 days	0.74 (Large-scale) 1.31
H_2 production by *P. valdorianum* (1), *H. halobium* (2) and *E. coli* (3). Patel & Madamvar (1994)	*E. coli* NLC 2065 ATCC (salt tolerant)	144 h	4 (1) 4.30 (1 + 3) 5.10 (1 + 2) 13.6 (1 + 2 + 3)
H_2 production by *H. halobium* (1) coupled to *E. coli* (2). Kaya (1995)	*E. coli* DS 1576 (salt tolerant)	14 h	1.67 (2) 2.16 (1+2)
H_2 production by *H. halobium* coupled to *E. coli.* Khan and Bhatt (1989)	*E. coli* NLC 2065 ATCC (salt tolerant)	0.5 h 0.5 h	3 (*H. h.* MMT_{19}) 2406 (*H. h.* MMT_{22})
H_2 production by immobilized cells of *H. halobium* and *E. coli.* Khan and Bhatt (1989)	*E. coli* NLC 2065 ATCC (salt tolerant)	25 h	180

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CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

The common reagents used were all of reagent quality and purchased from Merck (Germany) and Oxoid (U.K) or Sigma (U.S.A) companies. A list is presented in Appendix A.

E. coli DS 1576 was salt tolerant strain obtained from Germany. *E. coli* C600 and *E. coli* W677 were the gifts of Prof. Dr. Gülay Özcengiz. *H. halobium* S-9 strain originally obtained from Dr. Khorana's laboratory (MIT, USA) and was a gift of Dr. Mehmet Şimşek. Transferring them into freshly prepared liquid growth medium activated the bacteria and activated cultures were continuously transferred into new medium to keep them active. All the strains that were used during experiments were inoculated from active cultures. The composition of the medium used for cultivation is described in Appendix B.

2.2. Cultivation techniques

Three different *E. coli* strains were used in this study; DS 1576, C 600 and W 677. They all require the same cultivation conditions for optimal growth. *H. halobium* cells were grown as described by Oesterhelt and Stoeckenius (1974). In the following sections, aerobic and anaerobic cultivation in liquid medium, aerobic growth in solid media, and storage of bacteria were considered and explained.

2.2.1. Aerobic cultivation

Aerobic cultivation of *E. coli* strains was carried out in 5-ml test tubes. Growth medium contained 2.5%(w/v) Luria Broth (LB) medium, glucose (1.5%, w/v), sodium formate (0.2%, w/v) and metal salts. Additional metal salts that are briefly given in Appendix B. Medium components were homogeneously distributed in distilled water, and pH was adjusted to neutral values (6.6-7.3) by addition of NaOH. Then, 5 ml of medium was distributed into each of test tubes. Test tubes were sterilized in an autoclave for 15 min. at 121°C. 0.1 ml of active bacteria was inoculated into sterilized medium and test tubes were incubated at 37°C.

H. halobium was grown at 39°C and 7.0 pH for seven days under illumination in a sterilized medium containing 250 g NaCl, 20 g $MgSO_4.2H_2O$, 3.64 g trisodium citrate

5.5H_2O, 2 g of KCl and 10 g of bacteriological peptone L-37 in 1000 ml distilled water.

2.2.2. Anaerobic cultivation

Anaerobic cultivation was performed in test tubes. After the tubes were filled with 5 ml of medium having neutral pH, 2 ml of liquid vaseline was poured into the tubes in order to set anaerobic conditions. Then, test tubes were sterilized in autoclave for 15 min. at 121°C. Two percent inoculum of bacteria from active culture was transferred into prepared anaerobic liquid media. Bacteria were incubated at 37°C.

2.2.3. Long term storage of bacteria

Bacteria were stored under aerobic conditions using liquid or solid media. When liquid medium was preferred for long time storage, sterile glycerol was added to prevent freezing of culture. 0.5 ml of grown cells and 0.5 ml of sterile glycerol were injected into the eppendorf tubes. All the contents were mixed by a vortex. Tubes were stored at –20°C. Stocks was renewed in every 3 months.

Storage was also performed in agar slant tubes. Solid media contain agar as a solidifying agent in addition to the liquid media components. Agar (1.5-2%) was dissolved in the growth medium, mixed and was boiled for 10 minutes until solution become clear. Then solution was poured into test tubes. Tubes were put into autoclave

almost horizontally, having greater surface werea for bacterial growth and sterilized for 15 min. at 121°C. When the bacteria reach the exponential growth phase, a loopful of bacteria was taken and streaked onto the surface of agar slant. Then, these slants were incubated at 37°C (*E. coli* strains) or 39°C (*H. halobium* cells). When colonies appear on the surface of the slants, they were taken out and stored at –20°C for 3 months.

2.3. Optimization of growth conditions of *E. coli*

2.3.1. Aerobic growth of *E. coli*

Salt tolerant *E. coli* DS 1576 strain was grown in a 50-ml flask with a sampling outlet at the bottom, in a thermoshaker (Gerhardt thermoshaker) at 37°C, shaking at 120 rpm. Every two hours, samples were taken in order to follow the aerobic growth of bacteria. The absorbance of the samples was measured at 660 nm using an UV spectrophotometer (Shimatzu UV 1201).

2.3.2. Salt adaptation of E. coli DS 1576

E. coli DS 1576 was grown in aerobic conditions (as explained in Section 2.2.1.) in a growth medium containing different NaCl concentrations (0.03, 0.25, 0.5, 1 M). The test tubes having medium with different NaCl concentrations were inoculated with

0.1 ml of overnight grown bacterial culture. At the sixth hour of the growth, absorbance of the samples taken from the tubes was measured at 660 nm wavelength.

In order to adapt the bacteria to higher NaCl concentrations, *E. coli* cells were grown in 0.5 M NaCl containing growth media with passages through one week. These cells were inoculated to the test tubes having growth medium containing different NaCl concentrations as given above. At the sixth hour of these experiments, the absorbance of the samples taken from the tubes was measured at 660 nm wavelength.

2.3.3. Anaerobic growth of *E. coli*

E. coli DS 1576 was grown at 37°C in 50-ml flasks under anaerobic conditions by using three different inoculations as given below:

i) 1 ml aerobically grown bacterial culture,

ii) 5 ml aerobically grown bacterial culture,

iii) 1 ml anaerobically grown bacterial culture.

Liquid vaseline was placed above the growth medium to obtain anaerobic conditions during the growth. Samples were taken from the culture every two hours. Growth was followed by measuring the optical density at 660 nm (Hitachi UV spectrophotometer).

2.4. Experimental set up and procedure

2.4.1. Design and construction of the H_2 production system

H_2 production system consisted of three parts; the bioreactor, CO_2 trap and the gas collector (Figure 2.1., Figure 2.2.). Bioreactor was made of Pyrex having a capacity of two litres. A magnetic stirrer was used. A temperature controller (TC 1000 of New Brunswick Scientific) controlled the temperature. The inlet for the inert gas placed at the top of the bioreactor and it was connected to a sparger. There was an outlet for the gas evolved which was connected to the gas collector. The injection port at the top of the bioreactor was used for inoculation and sampling. However, in most of the experiments samples were not taken in order not to disturb the anaerobic condition in the bioreactor.

In some experiments a CO_2 trap was inserted between the reactor and gas collection system to remove CO_2 in the evolved gas. There were two traps connected in series containing 300 ml of 40% KOH solution.

Gas collector consisted of two parallel tanks with 1L volume each. The tanks were filled with water. Cumulative volume of the evolved gas was measured by the water replacement method. A gas tight Hamilton syringe was used for sampling through a septum which was placed on the top of a tank.

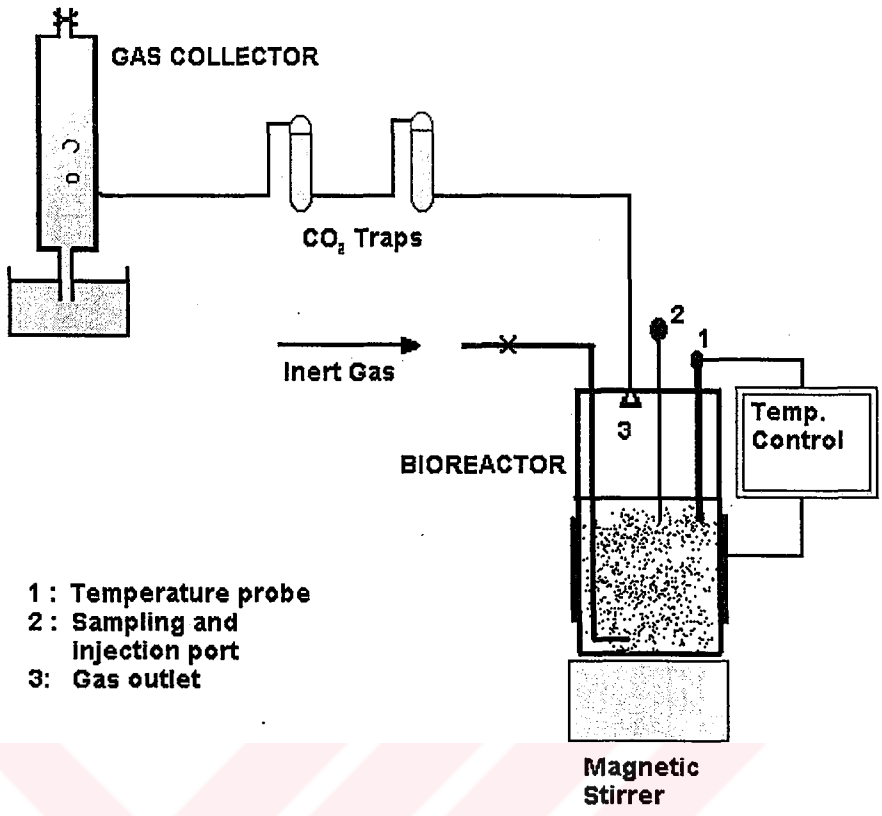

Figure 2.1. The experimental set up

2.4.2. Procedure

Growth medium of *E. coli* was inoculated by at least 5-8 times activated culture of aerobic or anaerobic *E. coli.* Initial pH of the medium was neutral (6.7-7.2) and working volume of the bioreactor was in between 500 to 2000 ml. The evolved gas was accumulated in gas collector. The purity of the H_2 gas evolved was analysed by the gas chromotography as described in section 2.8. Experiments were carried out under argon

or nitrogen atmosphere.

Experimental conditions were varied by changing: culture volume (500-2000 ml), temperature (37-40°C), stirring rate (120-360 rpm), initial pH (6.5-7.1), inoculation ratio (0.5 to 10 percent), precultivation technique (aerobic or anaerobic), and time.

Argon or nitrogen was used as inert gas to test the effect of the anaerobic environment. In some experiments liquid vaseline was placed on the culture as liquid membrane to provide anaerobic growth.

(a) (b)

Figure 2.2. The photographs of the experimental set up; (a) Bioreactor, (b) Bioreactor and the gas collectors.

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In some of the experiments Na-formate was added to the bioreactor two times at the 24^{th} and 48^{th} hours of the experiment. In order to obtain 0.2% (w/v) formate concentration in the bioreactor, 2.1 g of sodium formate was added to one litre of culture.

2.5.Tests with different *E. coli* strains

Three *E. coli* strains were cultivated under anaerobic conditions and tested for the gas productivity in test tubes. Anaerobic cultivation technique is described in Section 2.2.2. The first inoculum was 0.1 ml of overnight grown culture at 37°C under anaerobic condition. After inoculation, tubes were incubated at 37°C overnight. Growth was observed by watching the turbidity. Gas production was observed by the rising of gas bubbles. These experiments were repeated through bacterial transfers up to 3-4 times.

These tests were repeated at 21°C to investigate the effect of precultivation temperature on H_2 gas productivity of *E. coli*. The transfers were performed through two weeks.

Similar tests were held to detect the inhibitory effect of some species in the growth medium on H_2 productivity. *E. coli* strain which has no H_2 productivity, was cultivated in;

(i) LB medium (2.5% w/v LB),

(ii) Medium containing LB (2.5% w/v), glucose (1.5% w/v), NaCl (0.2% w/v)

and the half weight of the other compounds in growth medium (Appendix C),

(iii) Medium containing LB (2.5% w/v), glucose (1.5% w/v), NaCl (0.2% w/v) and NiCl (0.012% w/v).

2.6. Rapid screening for presence of hydrogenase

E. coli strains without H_2 productivity were examined whether they were lacking hydrogenase enzymes or not. The only screening test relatively specific for hydrogenase-containing colonies was the reduction of methyl viologen (0.2 % w/v, in 75 mM-phosphate buffer, pH 6.7) in soft agar (1% w/v) overlays with argon gas in a desiccator (Krasna, 1984). This assay was successfully applied to *R. sphaeroides* by Türkarslan (1999).

(i) Preparation of soft agar: For preparation of soft agar 1 % (w/v) of nutrient agar was dissolved in suitable amount of distilled water. It was boiled for few minutes until solution become clear. Then it was autoclaved at 121 °C for 15 minutes. After that, solution was cooled to temperature of 50°C or 60°C and then poured into petri plates under sterile conditions. Plates were incubated overnight at 37°C to test the contamination.

(ii) Preparation of methyl viologen solution: Firstly, 75 mM, pH 6.7 Phosphate buffer was prepared. 1.31 gr K_2HPO_4 was dissolved in some distilled water and 1.02 gr K_2HPO_4 was dissolved in 100 ml of distilled water. K_2HPO_4 was titrated with K_2HPO_4

until pH reaches to 6.7. After preparation of buffer 0.2 % (w/v) methyl viologen was added and mixed well. Solution was prepared freshly and stored at 4°C.

(iii) Experimental set up and screening test: When anaerobically grown bacteria reached the logarithmic phase, samples were taken and diluted with saline solution to 10^{-6} or 10^{-8}. 0.1 ml from the last dilution was dispensed onto the surface of soft agar and spread with glass spreader. Plates were then placed inside the desiccator that was modified for anaerobic cultivation. The top part of the desiccator has one inlet and one outlet. Argon was flushed from the inlet for 30 minutes and it was discharged from the outlet. This provided anaerobic conditions. Desiccator was incubated at 37°C and reaction was started by illumination with 150 W tungsten lamp. After 2-3 days, when colonies appeared on the surface of the soft agar, plates were removed from desiccator. Under sterile conditions, methyl viologen solution just enough to cover surface of the agar was added. Plates again placed in desiccator, flushed with argon and incubated. Hydrogenase containing colonies turned purple within 1 to 2 hours while colonies lacking hydrogenase did not reduce the dye for at least 6 to 8 hours.

2.7. *H. halobium* coupled to *E. coli* DS 1576

2.7.1. Large scale growth of *H. halobium* in the fermentor

Large-scale growth of *H. halobium* was performed as described by Sediroğlu (1997) and El-Bashiti (1998) by using BioFlo 1000 Adaptable Bioreactor having two litres capacity (New Brunswick Scientific).

BioFlo 1000 Adaptable Fermentor contains three modules consisting of vessel assembly, temperature measurement and control module and pH measurement and control module. Vessel assembly includes a two litered borosilicate glass vessel, a quick release headplate, a drive shaft assembly with stainless steel ball bearings and Viton PTFE lip seals, one 6-blade Rushton impeller, heater blancket, Pt-100 temperature probe, pH probe, flow meter glass tube and stainless steel headplate with 7 x 12 mm and 5 x 6.35 mm.

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

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

Growth of *H. halobium* in the fermentor was carried out using strain S-9 with a capacity of 2 L. Stirring rate was 300 rpm and aeration rate was 0.6 L/min according to the results of El-Bashiti (1998).

Two litres of the growth medium was inoculated with 60 ml of liquid culture from the stationary phase.

Growth was followed spectrophotometrically by measuring the optical density (by Hitachi U-2000 Double Beam, Japan) at 660 nm. The steady increase in biosynthesis of bacteriorhodopsin was observed by following the increase in absorbance at 570 nm.

2.7.2. Measurement of photoactivity of *H. halobium* packed cells

The photoactivity of *H. halobium* packed cells was measured as pH vs time by using a combined pH electrode connected to a Nel pH meter at 25°C as described by Yücel *et al.* (1995). The experimental set up is shown in Figure 2.3. *H. halobium* packed cells were suspended in a water jacketed vessel containing 3 ml of 4 M NaCl solution and the concentration of BR was adjusted to 5 μM. The system was incubated in dark until constant temperature and pH values were reached. Then, the light was turned on. The continuous illumination was achieved by a 300 W projector lamp from a distance of 15 cm apart from vessel. After steady pH values were attained, the illumination was stopped. During the experiments, pH was recorded continuously.

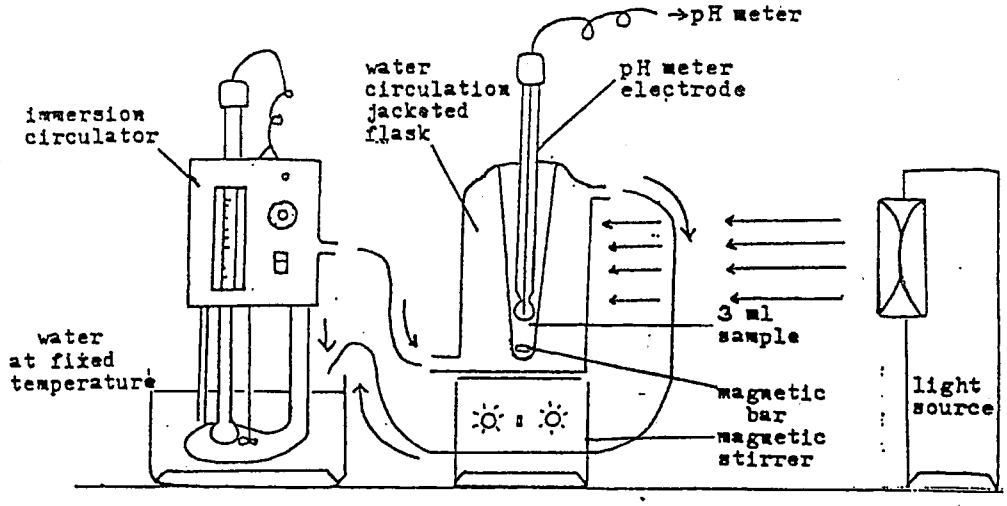

Figure 2.3. The experimental set up for measuring the photoactivity of BR.

2.7.3. Immobilization of *H. halobium* packed cells in polyacrylamide gel

Aydemir (1991) originally developed the procedure followed for the immobilization of *H. halobium* packed cells in polyacrylamide gel (PAG) membrane. The Polyacrylamide gel is formed from acrylamide and bisacrylamide. 100 ml stock solution (Acrylamide/Bis (30% T, 2.67% C)) was prepared by dissolving 29.2 g of acrylamide and 0.8 g bisacrylamide in distilled water. In order to prepare 15 ml 7.5 % PAG, 3.75 ml of stock solution was mixed with 75 µl of 10 % w/v of ammonium persulfate, 11.17 ml of *H. halobium* packed cells, and 8 µl TEMED (N, N, N, N tetramethyl ethylenediamine). The concentration of BR could be varied by the amount of *H. halobium* packed cells added.

Gel solution polymerized within the holes of the immobilization assembly shown in Figure 2.4. and nestled down in this niche by sticking to periphery of the hole. Immobilization assembly was made of Plexiglas, which had nine hollow places each, 2.5-cm in diameter and 0.35 cm in depth. The hollows contained small holes each 1-mm in diameter. Polymerization ended in about 30 minutes under anaerobic condition. Gel constituted assembly thus prepared were sank into 4 M NaCl solution to attain the uppermost swelling of gel and kept in this solution in a refrigerator, if it was not used. It should be noted that a perfect coverage of the hole by the gel without any leakage has been achieved by experience and a novel technique has been developed by Sediroğlu (1997).

2.7.4. Hydrogen production by *E. coli* coupled with *H. halobium*

H. halobium packed cells, which were immobilized in polyacrylamide gel (as explained in Section 2.7.3.) were put into the bioreactor having the sterilized growth

medium. *E. coli* cells were injected into the bioreactor and the experiment was started as described in Section 2.4.2. After the 12^{th} hour of the experiment, the bioreactor was illuminated by a 300 W projector lamp from a distance of 15 cm during the periods of 30 min light, 30 min dark.

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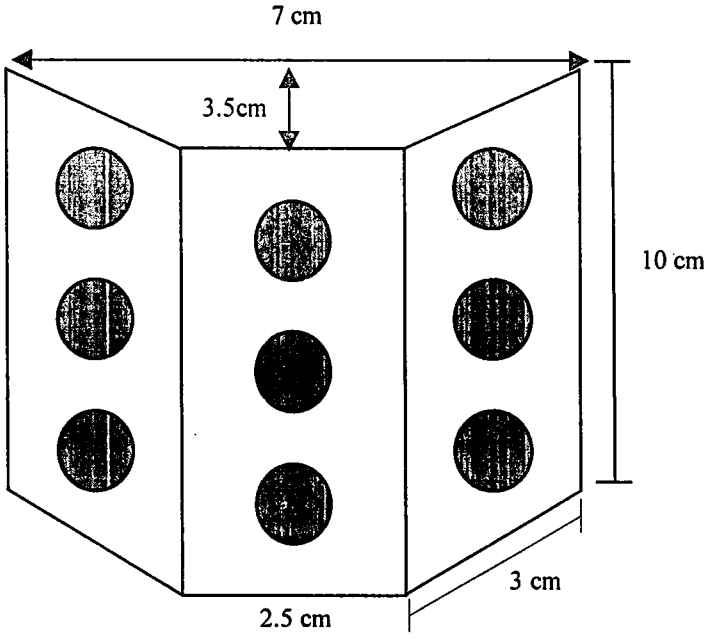

Figure 2.4. Immobilization assembly for the polyacrylamide gel membrane

2.8. Gas Analysis

The evolved gas was collected in the gas collectors that were tightly closed with a septum at the top. The bottom of the tube was immersed into water. The gas samples (2 ml) were taken by a gas tight Hamilton syringe, and the analysis of the evolved gas was carried out by Hewlett 5890 Packard Series II gas chromotograph. The working conditions were as follows;

Injection temperature : 35°C

Oven temperature : 30°C

Detector temperature : 40°C

Detector type : Thermal conductivity detector

Column : Propak Q (2 meters, 80/100 mesh packed column)

Carrier gas : N_2 (11 ml/min or 12 psi)

CHAPTER 3

RESULTS AND DISCUSSION

3.1. The optimization of growth conditions of *E. coli*

3.1.1. Aerobic growth curve of *E. coli*

Figure 3.1. illustrates the aerobic growth curve of *E. coli* DS 1576 obtained at 37°C in a 50-ml flask that was shaken at 120 rpm. Experimental procedure is explained in Section 2.3.1. Under these conditions lag phase ended in one hour and the bacteria reached stationary phase within seven hours.

3.1.2. Effect of salt concentration on growth of *E. coli*

Effect of different salt concentration on the growth of *E. coli* DS 1576 was investigated under aerobic conditions as explained in Section 2.3.2. Figure 3.2. illustrates the absorbance of the culture measured at 660 nm after six hours of incubation of *E. coli* DS 1576 with non-adapted and salt adapted cells. The absorbance measured at the 6^{th} hour of the growth curve given in Figure 3.1. was compared with the absorbance value measured for 0.1 M NaCl containing non-

adapted cells. It has been concluded that the bacterial growth in test tubes was lower than in 50-ml flask.

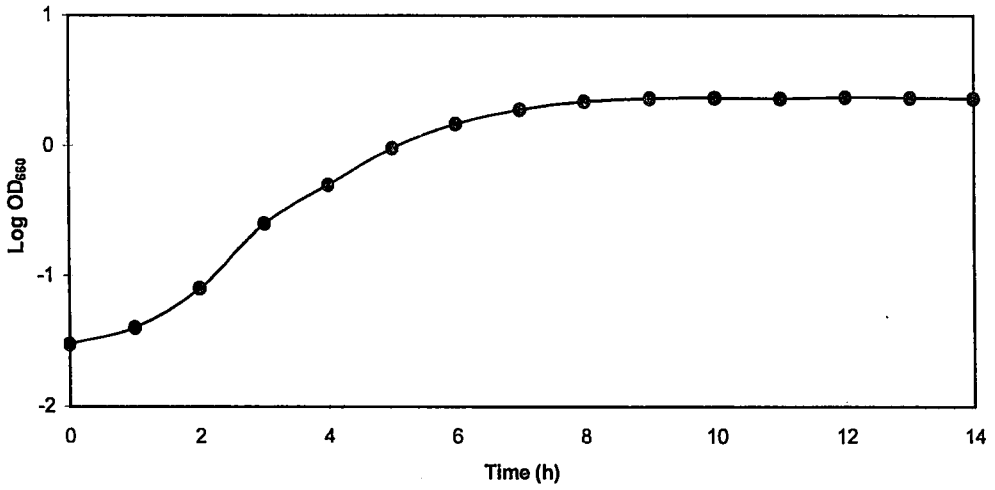

Figure 3.1. The aerobic growth curve of *E. coli* DS 1576 at 37°C and 120 rpm in a 50-ml flask.

Bacterial growth decreased as salt concentration increased as seen in Figure 3.2. (Gray columns). The growth of *E. coli* at 0.5 M salt concentration was significantly low and there was no *E. coli* grown at 1 M salt concentration. However, Kaya (1995) reported that *E. coli* DS 1576 tolerated 2 M salt concentration. When the bacteria was grown at 0.5 M NaCl, after some passages it was observed that, bacterial growth increased (Black columns). Moreover, strain was also adapted to tolerate 1 M NaCl concentration using the same method. This showed that the salt tolerance of *E. coli* DS 1576 could be improved by precultivation at increasing NaCl concentrations. It was also observed that, the salt tolerance of *E. coli* DS 1576 impaired when it was precultivated at low salt concentrations.

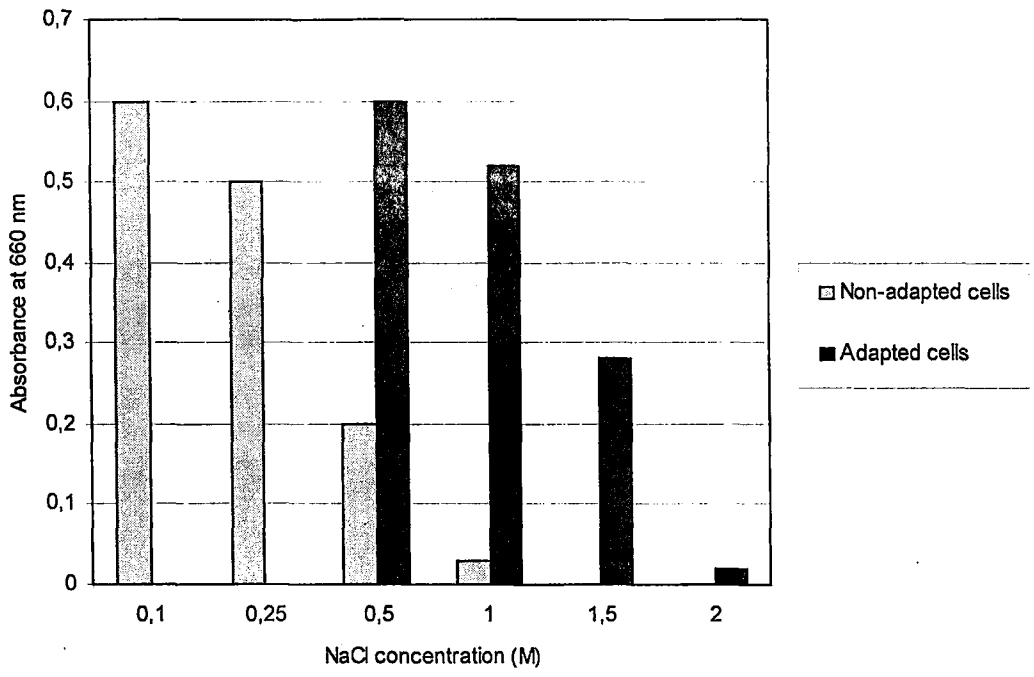

Figure 3.2. The absorbance of the culture measured at 660 nm after six hours of incubation of *E. coli* DS 1576 with non-adapted and salt adapted cells.

3.1.3. Anaerobic growth of *E. coli*

Anaerobic growth of *E. coli* C 600 was studied under various inoculation conditions as described in Section 2.3.3. Figure 3.3. illustrates the growth curves obtained. When 2% inoculation of aerobically grown cells was used, a long lag period was observed (about 3 hours). Increasing inoculation ratio to 10% shortened the lag phase to approximately 1 h. However, 2% inoculation of anaerobically grown cells decreased the lag period to half an hour.

Similarity of the growth curves is significant. The log periods are parallel which refers to the same specific growth rates. Inoculation conditions affected the length of lag phase, but not the growth rate.

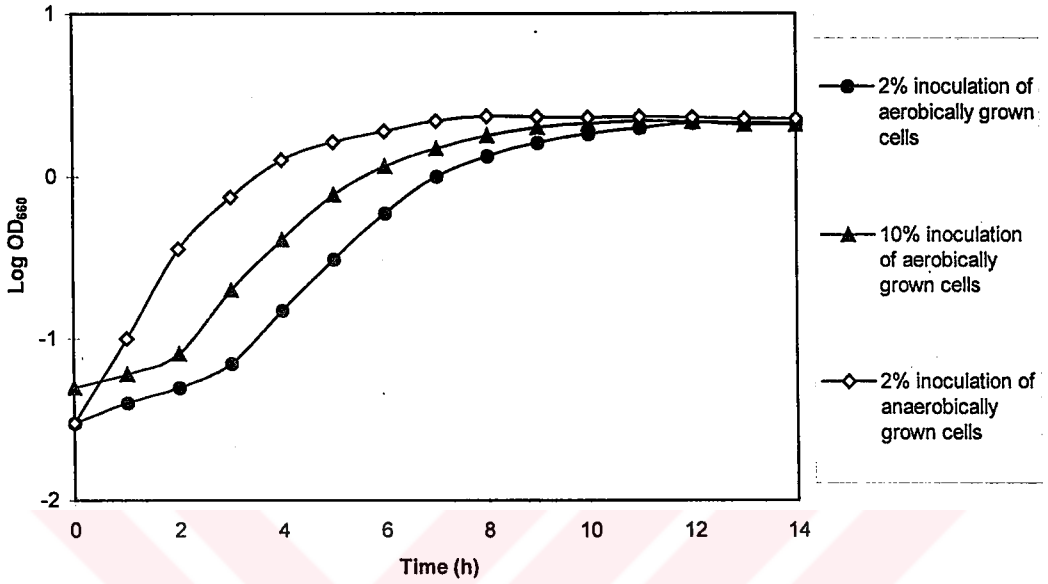

Figure 3.3. Anaerobic growth of *E. coli* C 600 under various inoculation conditions

3.2. Factors affecting H_2 production in the bioreactor

During anaerobic growth of *E. coli* strains H_2 gas was produced. The evolved gas was measured volumetrically and also the purity of the gas was analyzed by gas chromotography. Initial and final conditions of the experiments carried out in batch mode in the bioreactor are tabulated in Table 3.1. The total volume of the gas produced, the total time of the experiment, the maximum H_2 evolution rate and the

purity of H_2 in the evolved gas were also reported in Table 3.1. Initial pH was between 7.10-6.52. Final pH of the medium was also recorded. pH decreased in all of the experiments. However this decrease was not pronounced when there was no growth of bacteria (Run 16, 25, 28). In all the other experiments, pH was dropped down to 5.03-3.60.

The evolved gas was analysed by gas chromotography. The purity of hydrogen in inert free bases is reported in Table 3.1. The rest of the evolved gas was CO_2. According to the gas analysis, other runs are classified into two groups:

(i) *E. coli* cells grow and produce CO_2 gas only (Run 5, 6, 7, 14, 15, 17, 18, 21, 27, 30, 31, 32, 38, 40, 41). During these runs, the total volume of the gas evolved was between 10 to 220 ml. In some of the runs, the gas analysis was not available (Run 19, 22, 23, 24, 26, 29, 37, 39). Since the gas production in these runs was varied between 10 to 50 ml, it is expected that only CO_2 be produced during these runs.

(ii) *E. coli* cells grow and produce H_2 and CO_2 gas (Run 1, 8, 12, 13, 20, 34, 35, 36). During these runs, the total volume of the gas evolved was between 400 to 4530 ml. The evolved gas contained 85 to 99.6% H_2 in inert free bases and the rest was CO_2. When CO_2 trap was placed before the gas collector 100% pure H_2 gas was obtained. Appendix D includes the procedure for the rate calculations. Results are reported in Table 3.1.

That runs which are not included in Table 3.1. (Run 2, 3, 4, 9, 10, 11), were interrupted due to the problems caused by gas collection system or shut down due to electricity turn off.

Table 3.1. Summary of the bioreactor experiments

Run	Operating conditions[1] and temperature (°C)	Total vol. (ml)	Inoc. vol. (ml)	Initial pH	Final pH	Final OD_{660}	Total gas (ml)	Time (h)	Max. H_2 evol. Rate[2]	Purity of H_2 [3] (%)
1	IA,LS,DS,37	1000	10	6,85	4,52	1,71	2798	22	0,41	99,0
5	IA,LS,DS,37	1000	5	7,00	4,96	NA	101	81	0,00	0,0
6	IA,LS,DS,38	1000	30	7,08	3,90	1,96	50	23	0,00	0,0
7	IA,LS,DS,37.5	1000	10	7,00	4,12	NA	30	22	0,00	0,0
8	IA,HS,DS,37	1000	20	6,70	4,45	1,69	1600	22	0,42	99,6
12	IN,HS,DS,37	1000	25	7,00	4,33	1,78	1660	20	0,48	99,0
13	IN,HS,DS,39	1000	30	6,80	3,60	NA	1700	18	0,54	93,0
14	IN,LS,DS,40	1000	25	6,74	4,15	0,71	220	18	0,00	0,0
15	IN,HS,DS,40	1000	30	6,52	4,05	0,54	180	18	0,00	0,0
16	IN,LS,DS,37.5	1000	5	6,70	NA	NG	0	10	0,00	NA
17	IN,LS,DS,37.5	1000	10	6,70	NA	NA	30	24	0,00	0,0
18	IN,LS,DS,37.5	1000	10	6,80	NA	NA	20	18	0,00	0,0
19	IN,LS,DS,37.5	1000	30	7,00	NA	NA	48	18	NA	NA
20	IN,LS,DS,37.5	1000	20	7,00	4,28	NA	400	20	0,2	85,0
21	IN,LS,DS,39	1000	20	7,00	NA	NA	22	13	0,00	0,0
22	LM,IN,HS,DS,39	1000	25	6,95	NA	NA	25	15	NA	NA
23	LM,IN,LS,DS,39	1000	30	7,00	NA	NA	10	9	NA	NA
24	IN,HS,DS,39	2000	20	6,70	NA	NA	10	7	NA	NA
25	IN,An,LS,DS,39	1000	10	6,70	NA	NG	0	20	0,00	NA
26	IN,An,LS,DS,39	1000	10	7,00	NA	NA	25	30	NA	NA
27	IN,An,LS,DS,39	1000	50	7,00	NA	NA	40	20	0,00	0,0
28	IN,An,HS,DS,39	1000	20	7,00	6,90	NG	0	30	0,00	NA
29	IN,An,HS,DS,37	1000	100	6,60	NA	NA	16	13	NA	NA
30	IN,An,HS,DS,37	1000	50	6,90	4,80	0,55	38	20	0,00	0,0
31	IN,An,HS,DS,38	750	50	6,80	NA	NA	50	25	0,00	0,0
32	IN,An,HS,DS,38	750	50	6,70	NA	NA	50	13	0,00	0,0
33	LM,IN,An,HS,C,38	800	50	6,50	4,50	1,61	4280	12	0,82	98,0
34	IN,An,HS,C,38	800	20	6,80	4,80	NA	4530	13	0,86	98,0
35	IN,An,CT,HS,C,38	800	30	6,75	NA	NA	4270	12	0,83	100,0
36	IN,An,CT,HS,C,36	1000	20	7,00	4,56	NA	1980	13	0,30	100,0
37	IN,An,CT,HS,C,37	1000	20	6,70	NA	NA	10	14	NA	NA
38	IN,An,CT,HS,C,37	1000	25	6,80	NA	NA	200	16	0,00	0,0
39	IN,An,CT,HS,C,38	1000	25	6,75	NA	NA	30	14	0,00	NA
40	H,IN,An,CT,HS,C,37	550	50	6,80	4,50	NA	120	16	0,00	0,0
41	H,LM,IN,An,CT,HS,C,25	750	50	6,9	5,03	NA	170	30	0,00	0,0

(1) IA: Argon is used as inert gas, IN: Nitrogen is used as inert gas, HS: High stirring rate (360 rpm.), LS: Low stirring rate (120 rpm.), AN: Anaerobically grown cells were inoculated, CT: CO_2 trap was used, LM: Liquid membrane was used, C: E. *coli* C600, DS: E. *coli* DS 1576, NA: Not available, NG: No growth, Inoc.:Inoculation.

(2) In $Lgas.Lculture^{-1}.h^{-1}$ (3) Argon free bases

passages. Therefore in runs 33 to 41 *E. coli* C 600 was used. However, after Run 36 *E. coli* C 600 lost its H_2 productivity.

The effect of various parameters such as: temperature, stirring rate, inert gas used, placing liquid membrane above the culture, inserting a CO_2 trap at the gas outlet, and formate addition on the H_2 production in the bioreactor were studied. The results are given in the following sections.

3.2.1. Temperature

H_2 gas production was measured for the experiments held at 37, 39 and 40°C using *E. coli* DS 1576 (Run 12, Run 13 and Run 15 respectively). In all the experiments the stirring rate was 360 rpm. Total H_2 gas production and the rate of H_2 gas evolution with respect to time are shown in Figure 3.4 and Figure 3.5., respectively. Purity of H_2 was 99% at 37°C and 93% at 39°C. The highest rate of H_2 gas production is observed at 39°C. At 40°C, cell growth decreased significantly (Final OD_{660} was 0.535-0.711).

Run 12, 13, and 15 were initiated with 3% inoculum of aerobically grown cells. In all the experiments a long lag period was observed.

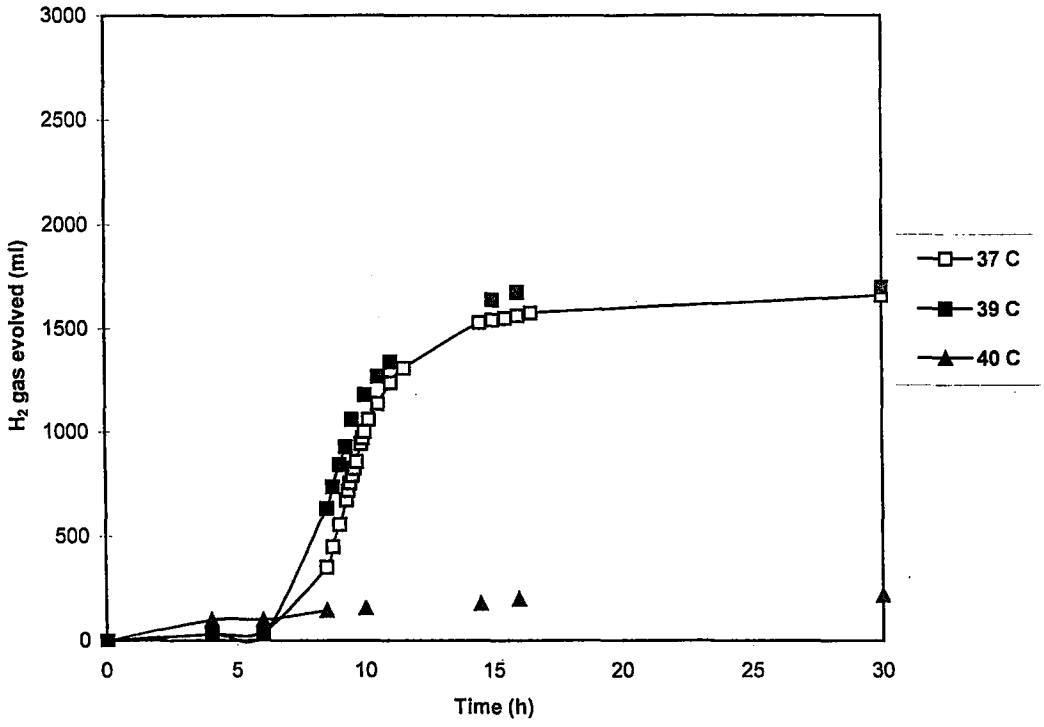

Figure 3.4. H_2 gas production in bioreactor at various temperatures

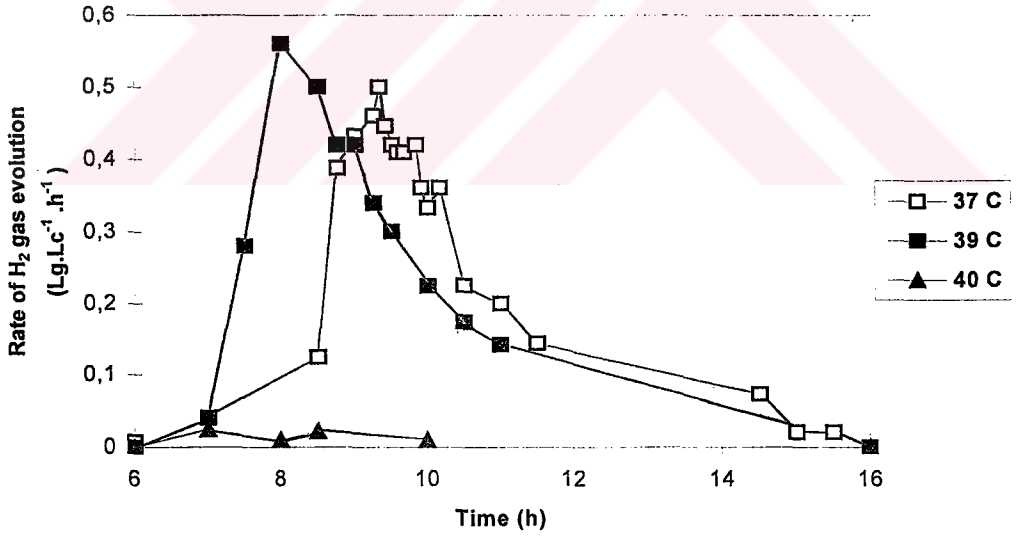

Figure 3.5. Rate of H_2 gas production in bioreactor at various temperatures

3.2.2. Stirring rate

H_2 gas production is measured for the experiments held at 120 and 360 rpm stirring rates (Run 1 and Run 8 respectively). Both experiments were performed at 37°C. Total H_2 gas production and the rate of H_2 evolution are shown in Figure 3.6. and Figure 3.7., respectively.

As it can be seen from Figure 3.6., the lag period of Run 1 was significantly longer than Run 8. This might be due to the low stirring rate in Run 1. However it should be noted that the inoculation volume in Run 1 was 1%, which was the half of the inoculation volume used in Run 8. Although lag phase was shorter in Run 8, total H_2 production was less than Run 1. At high stirring speed, when the rate of gas evolution became its maximum value, it suddenly decreased. Maximum rate of H_2 gas production was lower in Run 1, However, duration of gas production was much higher.

3.2.3 Initial pH

The bioreactor experiments were started at pH values 7.10 to 6.52 where cell growth was observed. It should be indicated that, H_2 production could only be observed for the experiments started between pH 7 to 6.75. Initial pH did not have significant effect on H_2 production within this range.

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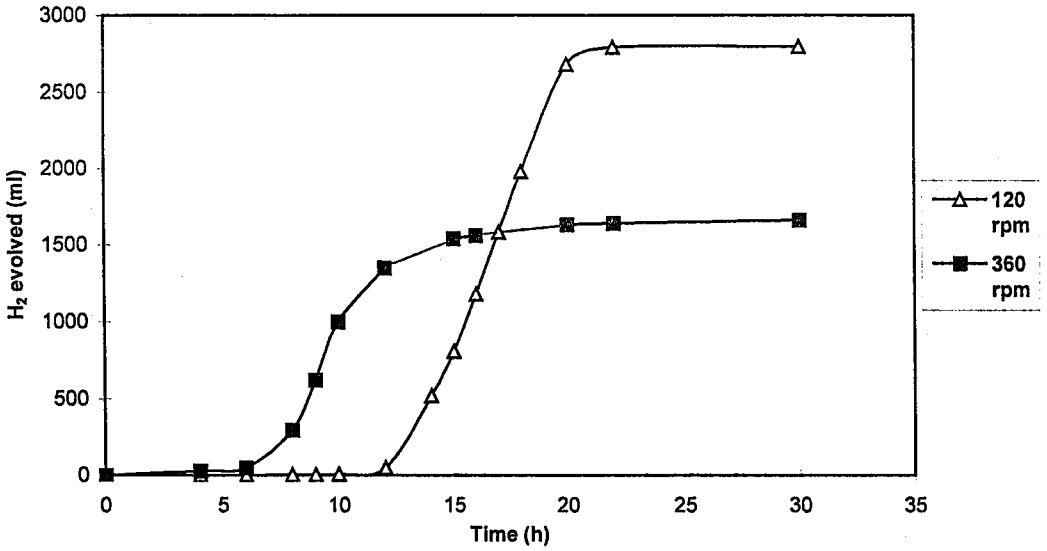

Figure 3.6. H_2 gas production in bioreactor under different stirring conditions at 37°C

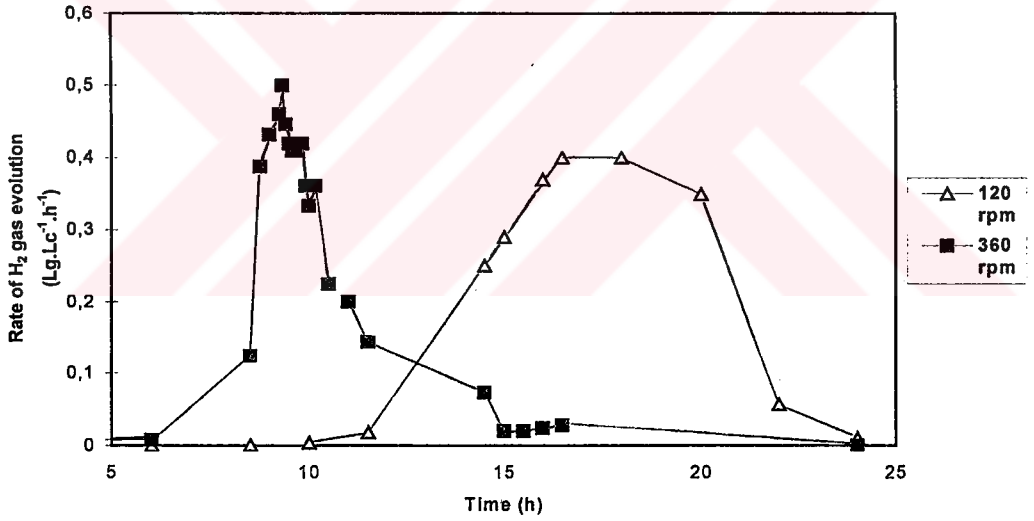

Figure 3.7. Rate of H_2 gas production in bioreactor under two different stirring conditions at 37°C

3.2.4. Inert gas

Argon atmosphere is essential for H_2 producing photosynthetic bacteria (Arık, 1995). Kaya (1995) also worked under argon atmosphere for the H_2 production by *E. coli* DS 1576. In the present study, the experiment carried out in Run 8 under argon atmosphere was repeated with N_2 as the inert gas in Run 12. Figure 3.8. illustrates H_2 gas evolved versus time under Ar or N_2 atmosphere. No difference was observed. This might indicated that, nitrogen does not effect the H_2 productivity. Since nitrogen is cheaper, it was selected as inert gas.

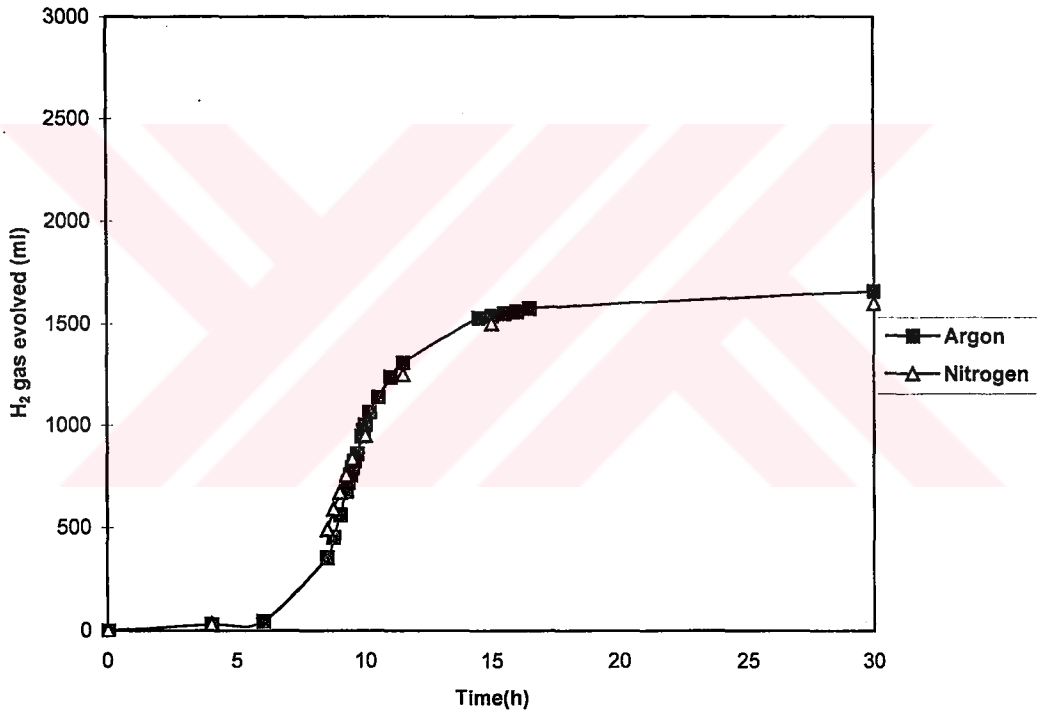

Figure 3.8. Total H_2 gas evolved versus time for nitrogen and argon used as inert gas.

3.2.5. Liquid membrane

Figure 3.9. illustrates the total H_2 gas evolved versus time in Run 33 and Run 34 both carried out under nitrogen atmosphere. In addition to that, liquid vaseline was placed above the culture as a liquid membrane to keep anaerobic conditions during Run 33. H_2 gas production was the same in both runs. In Run 33, 12.5% inoculation of anaerobically grown cells were used. There was no lag phase observed. However, in Run 34, 2.5% inoculation of anaerobically grown cells were used which caused 2 hours lag phase. This result indicates the importance of inoculation ratio.

Since liquid vaseline is to set anaerobic conditions, it can be used in test tubes to set anaerobic conditions, to observe gas evolution.

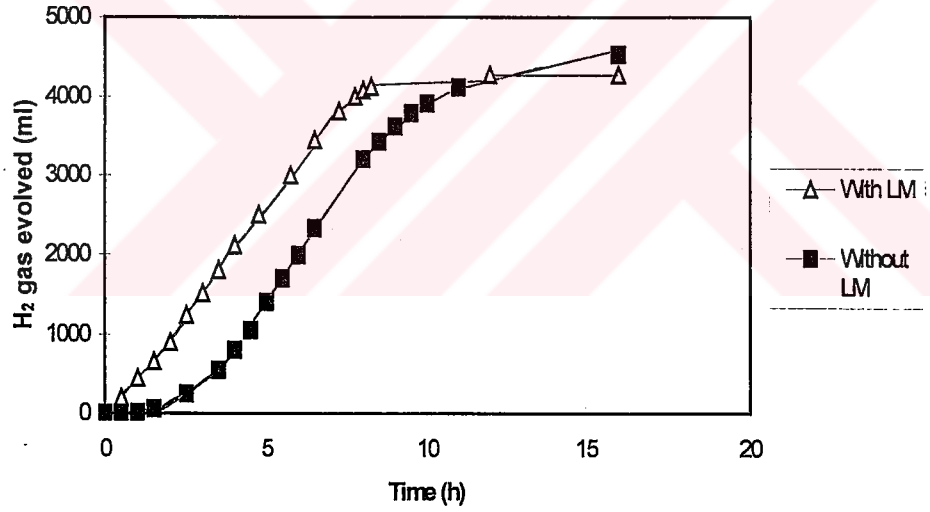

Figure 3.9. Effect of using liquid membrane

3.2.6. CO_2 trap

In order to purify evolved H_2 gas, a CO_2 trap was inserted into the system, in Run 35. Total H_2 gas evolved versus time with and without a CO_2 trap was compared in Figure 3.10., by comparing Run 35 and 34. No change was observed. In Run 35, H_2 purity of the evolved gas was 99% before the CO_2 trap and 100% after the trap.

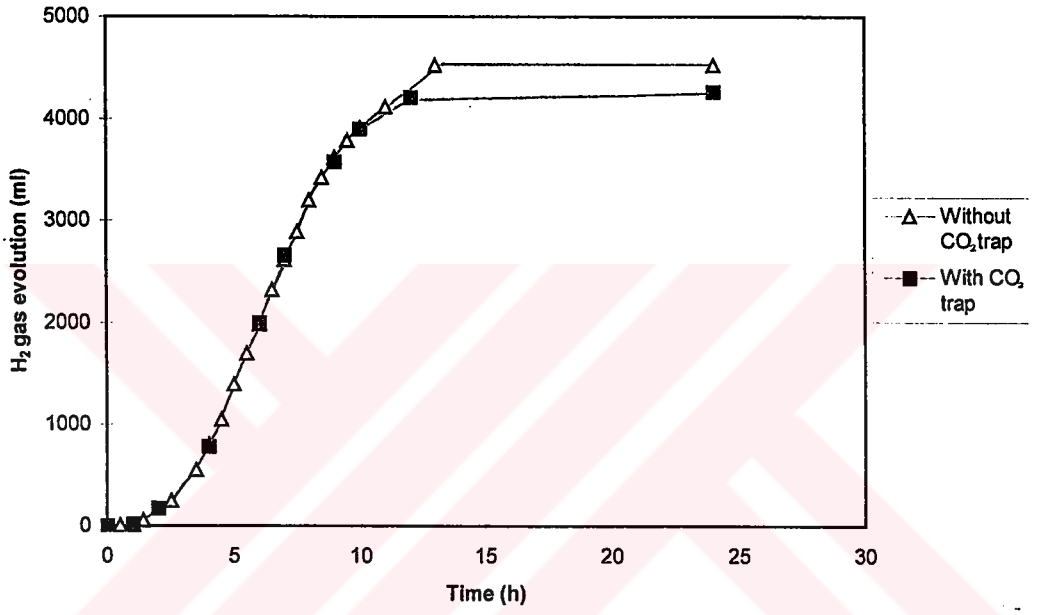

Figure 3.10. Effect of using CO_2 trap

3.2.7. Formate addition

In the experiments Run 32 and Run 34, 0.2% Sodium formate were injected to the reaction medium at the 24th hour after inoculation. In Run 32, formate addition

did not initiate H_2 production. Exogenous addition of formate restarted the gas evolution in Run 34. Figure 3.11. shows the evolved H_2 gas by the exogenous addition of formate considering the injection time in Run 34. When 0.2% (w/v) formate was injected into the bioreactor at the 24th hour of the experiment, 2200 ml of H_2 gas was evolved. This amount corresponds to 95% utilization of formate into H_2 and CO_2. At the 48th hour of the experiment, same amount of formate was injected into bioreactor, but the utilization of formate was lower (69%).

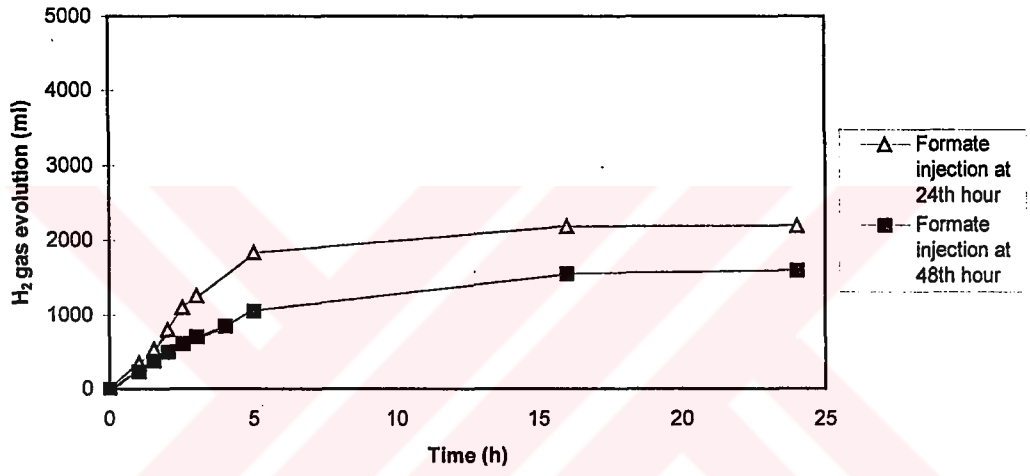

Figure 3.11. Effect of formate injection in Run 34

3.3. Factors affecting hydrogen productivity of *E. coli*

During the study, it was detected that, hydrogen productivity of a strain can be ceased during the passages. Because the stability of the strain is very important for the continuous operation, many methods were investigated to avoid such instability.

When it was discovered that *E. coli* strain DS 1576 have lost its hydrogen productivity totally, two other strains were tested via this purpose; *E. coli* C600 and *E. coli* W677. *E. coli* strain C600 was found to be active in hydrogen productivity. Other two strains grown well in the test tubes, however, no hydrogen evolution was observed. Study was focused on two different parts; how to restore the hydrogen productivity of *E. coli* strain DS 1576 and how to prevent *E. coli* strain C600 to loose its hydrogen productivity.

Precultivation temperature was thought as a possible reason for the decrease in hydrogen productivity of *E. coli* C 600. During the passages, 21°C was examined instead of 37°C as precultivation temperature (Section 2.5.). Hydrogen productivity dissipated in two weeks at 21°C, and in one week at 37°C.

Cultivation in various media was tested to restore H_2 productivity. However, the tests were not successful. Hydrogenase enzymes of *E. coli*, which did not produce H_2 gas, was found to be active according to the rapid screening method.

3.4. *H. halobium* coupled to *E. coli*

Since, *E. coli* has two separate mechanisms as described in Section 1.1.2. to produce molecular hydrogen, immobilized *H. halobium* cells were coupled to *E. coli* strain, which lost its H_2 productivity, in order to find out the lacking mechanism. Hydrogenase 3 takes role in both mechanisms, however formate dehydrogenase only takes place in conversion of formate to H_2 and CO_2.

Since hydrogenase enzymes of *E. coli* strain that did not produce H_2 gas, was found to be active, it was decided to couple this strain to *H. halobium* strain S-9. Firstly, the activity of the *H. halobium* cells was tested. As it can be seen in Figure 3.12., they were highly active, since pH gradient is a sign of conversion of light energy to chemical energy, by means of proton pumping.

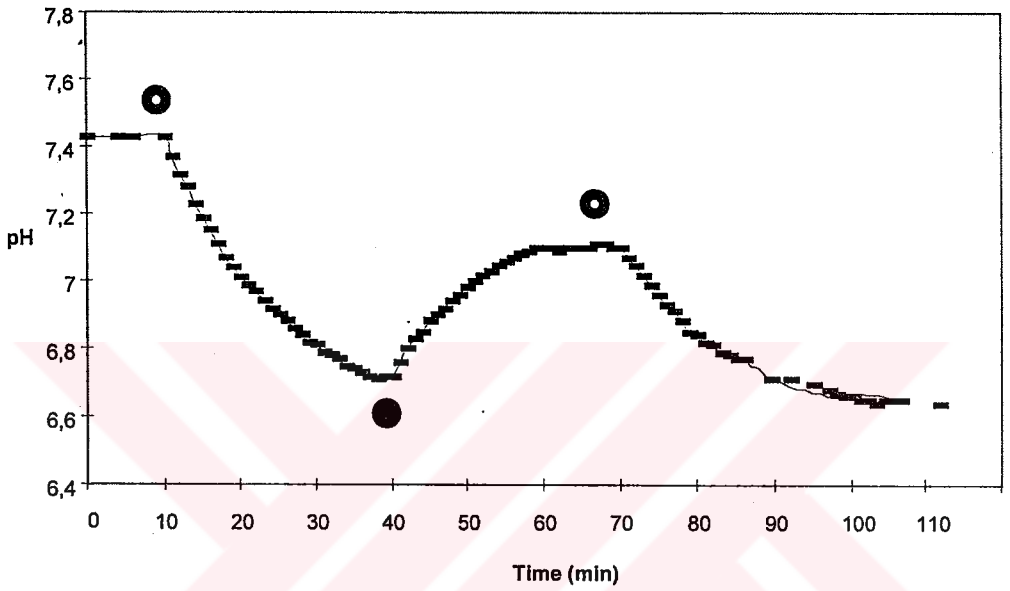

Figure 3.12. Photoactivity of *H. halobium* (: Light on, : Light off)

Photochemically active *H. halobium* strain S-9 was coupled to *E. coli* as it was added to 5-ml test tubes, bioreactor with 550 ml working volume and finally they were immobilized in polyacrylamide gel (PAG) membrane and put into the bioreactor with 750 ml working volume. The bioreactor was illuminated after the 12th hour of the fermentations by a 300 W projector lamp from a distance of 15 cm

apart from the vessel during the periods of 30 min light, 30 min dark. No H_2 gas evolution was observed in all the experiments.

3.5. Discussion

In this study, it was assumed that, except the salt resistance of *E. coli* DS 1576, used *E. coli* strains are genetically same. This assumption was necessary since hydrogen productivity of the *E. coli* strains ceased down by time and the study could not be completed with one strain.

Anaerobically grown *E. coli* produced H_2 gas both in exponential growth phase and in stationary phase. During the study it was discovered that, inoculation conditions affect lag period of the anaerobic growth of *E. coli.* In addition to the anaerobic growth experiments, bioreactor experiments strengthen this idea. When anaerobically grown cells were inoculated, growth and H_2 production ended within 18 to 22 hours (Run 8-Run 12). On the other hand, using anaerobically grown inoculants, growth and H_2 production ended at most in 13 hours (Run 33-Run 36).

Maximum gas production rate was observed at 39°C under 360 rpm stirring. At low stirring rate (120 rpm), maximum rate of H_2 production was lower, but total H_2 production was higher. Final pH of Run 8 is lower than Run 1. At high stirring rate, cells grew earlier and acidic end products may accumulated in the medium causing a sudden decrease in H_2 production. Buffer solutions can be used to test this idea.

When our results (maximum 4.35 μmole H_2/ mg cell. h with *E. coli C 600*, 2.7 μmole H_2/ mg cell. h with *E. coli* DS 1576) are compared with literature (listed at Table 1.5.), highest H_2 productivity was observed using *E. coli C 600*. Using *E. coli* DS 1576, higher rate was observed than Kaya (1995) reported. This might be due to the higher operation temperature (39°C).

Somehow, theoretically produced CO_2 could not be observed in the results of the gas chromotography. As it can be seen in Figure 1.1., evolved CO_2 may be used in the biosynthesis of succinate.

During anaerobic fermentation, *E. coli* uses endogenously produced formate for hydrogen production. When Na-formate was injected into the bioreactor, H_2 evolution was observed at the 24^{th} hour of Run 34. Kaya (1995) reported that gas evolution stopped when the pH of the solution became acidic (4.6-4.9) and when pH was adjusted to initial value by addition of 1 M KOH, gas production restarted. On the other hand, as it can be seen in Figure 3.11., addition of formate even at lower pH values was started gas production. This proves that formate hydrogen lyase system is active at low pH values. However, mixed acid fermentation is halted. Since FHL system does not cause a decrease in pH, higher amounts of formate can be converted to H_2 during the growth of *E. coli.* When sufficient amount of formate added, the rate of H_2 gas evolution does not increase proportionally with formate addition, since the hydrogenase activity of the strain limits the gas evolution.

In Run 32 no H_2 production was observed. Injection of formate at the 24^{th} hr of the experiment did not initiate H_2 production. This might show that, *E. coli* cells

without H_2 productivity, lacks formate hydrogen lyase pathway that consists of formate dehydrogenase and hydrogenase 3.

Nandi and Sengupta (1998) showed production of H_2 from 1.15 M formate over a 96 hr cycle with loss of 25% efficiency per cycle. In Run 34, when formate was injected at the 48^{th} hr of the experiment, loss of 23% efficiency in formate conversion was observed considering the injection at the 24^{th} hr of the experiment.

H. halobium, lacks hydrogenase enzyme system which is essential for the production of molecular hydrogen. Therefore a hydrogenase system has to be supplied to *H. halobium* for the successful production of hydrogen gas (Taqui Khan and Bhatt, 1989; 1990b; 1991a; Taqui Khan, *et al.*, 1992a). On the other hand, under anaerobic growth conditions, *E. coli* induces the over expression of hydrogenase 3, which is responsible from the H_2 evolution.

When *E. coli* cells without H_2 productivity were coupled to *H. halobium*, they did not produce H_2 gas. The lacking enzyme in these cells must be hydogenase 3, since it is the only enzyme used in both H_2 producing mechanisms of *E. coli*. Although these cells were detected to be active in hydrogenase activity by the rapid screening test, the test is not specific for hydrogenase 3. *E. coli* cells with no H_2 productivity were grew well under anaerobic conditions. During the passages, *E. coli* cells might have developed an alternative metabolism to utilize the substrates in the medium without producing H_2 gas. Factors affecting the H_2 productivity of *E. coli* were investigated and the results are presented in Section 3.3. When *E. coli* cells were stored at -20°C even for six months, the H_2 productivity of the cells did not lost. However, all the *E. coli* strains loose their H_2 productivity through bacterial transfers.

Kanayama *et. al.* (1987) reported that recombinant *E. coli* precultured at a temperature (18°C) lower than the optimum temperature for the cell growth (30-37°C) showed a higher H_2 evolution rate from glucose than cells cultured at 30-37°C. Therefore they precultured the cells at 18 °C, but operated the reactor at 30°C. Cells grown during operation, therefore, showed lower H_2 productivity.

When *E. coli* C 600 cells were precultivated one week at 21°C, higher H_2 productivity was observed than the cells precultivated at 37°C. Low precultivation temperature must increase hydrogenase activity of the strain. However, during two weeks of passages, H_2 productivity lost totally. Sode *et. al.* (1990) reported restoration of Hydrogenase lacking *E. coli* cells by cultivation in nickel containing medium. When *E. coli* cells without H_2 productivity were cultivated in nickel containing medium, H_2 productivity of the cells did not increase. This phenomenon could not be explained yet. Cultivation of *E. coli* in vitamin solutions may increase expression of hydrogenase 3.

CHAPTER 4

CONCLUSION

In this study, biological H_2 production was achieved using *E. coli* in an anaerobic bioreactor.

The gas production was observed in the medium containing Luria Broth, glucose, formate and metal ions including Ni, Mo, Se, Mg, Fe, Na, K, S, phosphate and nitrogen. During anaerobic growth of *E. coli,* maximum 0.858 L_{H_2}/Lculture. h (4.35 μmol H_2/mg cell. h) of gas evolution rate was observed. Optimum operating temperature and stirring rate were determined as 39°C and 360 rpm, respectively.

Anaerobic conditions were found to be strictly required. It was found that nitrogen could be used instead of argon as inert gas to achieve the anaerobic conditions. Also, liquid vaseline was selected as a liquid membrane for the same purpose. Using liquid vaseline in test tubes was introduced as an easy method during anaerobic cultivation.

It was detected that, apart from growth conditions, *E. coli* strains do not produce H_2 gas all the time, lacking hydrogenase 3 enzyme. However, they grew well under anaerobic conditions. It was found possible that *E. coli* cells could develop an alternative metabolism to utilize the substrates in the medium during the bacterial transfers.

Stability of the microorganisms is critical for continuous operation. The cause of the instability of *E. coli* cells must be well investigated to achieve continuous H_2 production.

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APPENDIX A

LIST OF CHEMICAL AND SUPPLIERS

Sodium chloride (NaCl)	Merck
Magnesium sulphate ($MgSO_4.7H_2O$)	Merck
Potasssium chloride (KCl)	Merck
Bacteriological Agar	Oxoid
Luria Broth Base	Sigma
α-D+-Glucose	Sigma
Sodium formate (Na(HCOO))	Riedel-Germany
Sodium molybdate ($Na_2MoO_4.2H_2O$)	Merck
Nickel (II) chloride ($NiCl_2.6H_2O$)	Merck
Sodium selenate ($Na_2SeO_4.10H_2O$)	Analar-England
Iron (II) sulphate ($FeSO_4.7H_2O$)	Merck
Ammonium sulphate ($(NH_4)_2\ SO_4$)	Merck
Potassium phosphate (KH_2PO_4)	Merck
Sodium phosphate (Na_2HPO_4)	Merck
Potassium phosphate (K_3PO_4)	Merck

APPENDIX B

GROWTH MEDIUM USED FOR CULTIVATION OF ESCHERICHIA COLI

Component	grams in 1000 ml
LB	25
Glucose	16,5
Sodium formate *	2
$MgSO_4 .7H_2O$	0,2
$(NH_4)_2SO_4$	1
KH_2PO_4 *	0,8
Na_2HPO_4 *	0,5
NaCl	2
$FeCl_3 .6H_2O$	0,1
$NiCl_2$	0,12
Sodium Selenate	0,002
Sodium molybdate	0,0024

* : dissolved separately

APPENDIX C

GROWTH MEDIUM PREPARED TO TEST ESCHERICHIA COLI

Component	grams in 1000 ml
LB	25
Glucose	16,5
Sodium formate *	1
$MgSO_4 .7H_2O$	0,1
$(NH_4)_2SO_4$	0,5
KH_2PO_4 *	0,4
Na_2HPO_4 *	0,25
NaCl	2
$FeCl_3 .6H_2O$	0,05
$NiCl_2$	0,06
Sodium Selenate	0,001
Sodium molybdate	0,0012

* : dissolved separately

APPENDIX D

RATE CALCULATION

Sample calculation of number of moles of H_2 produced and rate of H_2 production is given below. Gas collection units are at room temperature.

$$P.V = n\,R\,T$$

$$(1 atm)(V\ ml) = (n\ mole)\ 82.06 \frac{ml.atm}{mole\ K}(273+25)$$

$$n(mmole) = \frac{V\,(ml) \times 10^3}{24454} \qquad \text{(Equation 1)}$$

Volume of the gas evolved:

Run 34: 4530 ml n= 185 mmole

According to gas chromatography results, 98% of evolved gas was hydrogen,

So;

185 x 0.98 = 181 mmole H_2 was evolved.

Rate calculation:

$R_{H2} = (GP_2 - GP_1)*(H_2\%)/(\text{Volume of culture}*(t_2-t_1))$

Where;

R_{H2} is the rate of H_2 production (L H_2 / L culture. h),

GP_1 and GP_2 are the amounts of the produced gas at time t_1 and t_2, respectively.

Between the 4.5 and 5th hrs of Run 34, 350 ml H_2 was produced.

$GP_2 - GP_1 = 0.35$ L,

$t_2 - t_1 = 0.5$ h => $R_{H_2} = (0.35 \text{ L})*(0.98)/(0.8 \text{ L culture})*(0.5)$,

$R_{H_2} = 0.858$ L H_2 / L culture. h

From equation 1.

0.858 ml corresponds to $n = \frac{858 \times 10^3}{24454} = 35 \text{mmole } H_2$

The calibration curve between the optical density and the dry weight of the samples of *E. coli* culture is shown in Figure D.1. In order to calculate the dry weight of the samples, they were centrifuged at 6300 rpm for 30 min and the liquid part is removed from the samples.

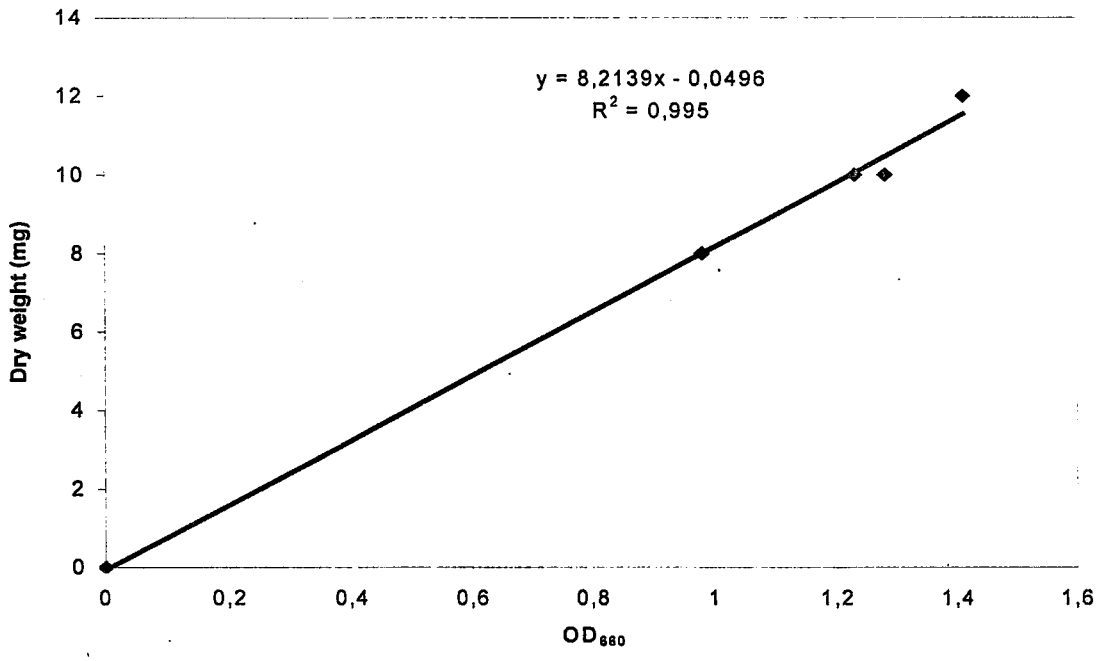

Figure D.1. Calibration curve between the optical density and the dry weight of the samples of *E. coli* culture.

Dry weight of the *E. coli* cells in the reactor was calculated as;

Dry weight=8.214*optical density

(5^{th} h of Run 34) OD_{660}=0.98 => Dry weight=8.05 mg

Since one litre culture contains 8050 mg cell;

R'_{H_2} = 35 mmole H_2 / (8050 mg cell)* 1 h = 4.35 μmole H_2/mg cell. h

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