Cumhuriyet Science Journal
CSJ

e-ISSN: 2587-246X
ISSN: 2587-2680

Cumhuriyet Sci. J., Vol.40-2 (2019) 414-423

Hydrogen generation by *Rhodobacter sphaeroides* O.U.001 using pretreated waste barley

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Received: 08.02.2019; Accepted: 24.05.2019 *http://dx.doi.org/10.17776/csj.524612*

Abstract. In the present study, valorization of waste barley by producing hydrogen (H_2) and 5-aminolevulinic acid (5-ALA) using *Rhodobacter sphaeroides* O.U.001 was aimed. Firstly, 3 % (w/v) waste barley hydrolysate was prepared by treating 3 g of powdered waste barley with H_2SO_4 in a total volume of 100 mL mixture and then autoclaving this mixture at 121 ºC for 30 min. Upon generation of fermentable simple sugars by pretreatment and analytical examination of the hydrolysate in terms of ammonium content, element composition and light transmittance, various types of growth media containing various concentrations of sugar (5 - 6 - 7 - 8 g/L) were prepared. The cells were cultivated in these media under photo-heterotrophic conditions which favor H_2 and 5-ALA generations. pH changes, growth, H_2 production and 5-ALA generation were monitored in the media. The results showed that all the media prepared from 3 % (w/v) waste barley hydrolysate sustained the cell growth appreciably. The highest OD value (OD_{660}: 1.71) was attained when using 8 g/L sugar. Furthermore, biological H_2 evolution was seen in each bioreactor. In particular, the highest hydrogen accumulation (0.29 L H_2/L) was achieved in 6 g/L sugar-containing medium. However, 5-ALA was not detected in any of the media. To conclude, considerable cell growth and biological hydrogen production was achieved using 3 % (w/v) waste barley hydrolysate under the conditions tested but there was no detectable 5-ALA generation.

***Keywords*:** Biological hydrogen, 5-aminolevulinic acid, *Rhodobacter sphaeroides*, waste barley.

Ön işlemden geçirilmiş atık arpa kullanarak *Rhodobacter sphaeroides* O.U.001 ile hidrojen üretimi

Özet. Bu çalışmada, *Rhodobacter sphaeroides* O.U.001 kullanılarak hidrojen (H_2) ve 5-aminolevulinik asitin (5-ALA) üretilmesi ile atık arpa'nın değerlendirilmesi hedeflendi. Öncelikle, 3 g toz halindeki atık arpa H_2SO_4 ile karıştırılarak 100 mL toplam hacimde karışım elde edildi ve sonrasında bu karışım 121 °C' de 30 dakika boyunca otoklavlanarak % 3'lük (a/h) atık arpa hidrolizatı hazırlandı. Fermente edilebilir basit şekerlerin önişlem ile ortaya çıkarılması ve hidrolizatın amonyum muhtevası, element bileşimi ve ışık geçirgenliği bakımından analitik olarak incelenmesinin ardından, farklı şeker konsantrasyonlarına sahip çeşitli büyüme ortamları (5 - 6 - 7 - 8 g/L) hazırlandı. Hücreler bu ortamlarda H_2 ve 5-ALA yapımlarını destekleyen foto-heterotrofik koşullar altında çoğaltıldı. Ortamlardaki pH değişimleri, büyüme, hidrojen üretimi ve 5-ALA üretimi izlendi. Sonuçlar, % 3'lük (a/h) atık arpa hidrolizatından hazırlanan tüm ortamların hücre büyümesini önemli ölçüde desteklediğini gösterdi. En yüksek OD değeri (OD660: 1.71) 8 g/L şeker kullanılarak elde edildi. Ayrıca, her bir biyoreaktörde biyolojik H_2 üretimi gözlemlendi. Özellikle, en yüksek hidrojen birikimi

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(0.29 L H_2/L), 6 g/L şeker içeren ortamda elde edildi. Ancak, hiçbir ortamda 5-ALA tespit edilmedi. Sonuç olarak, test edilen koşullar altında % 3'lük (a/h) atık arpa hidrolizatı kullanılarak önemli miktarda hücre büyümesi ve biyolojik hidrojen üretimi sağlandı, ancak saptanabilir miktarda 5-ALA üretimi yoktu.

***Anahtar Kelimeler*:** Biyolojik hidrojen, 5-aminolevulinik asit, *Rhodobacter sphaeroides*, atık arpa.

1. INTRODUCTION

Currently, there is increasing energy demand in the world and it is primarily supplied from the fossil-based fuels. However, it is generally recognized that the use of fossil-based fuels has negative impacts on environment. Sulfur oxides (SO_x), carbon monoxide (CO), nitrogen oxides (NO_x), and carbon dioxide (CO_2) are the main pollutant gasses emitted as a consequence of the use of fossil-based fuels [1, 2]. For instance, while CO_x emissions cause greenhouse effect, SO_x and NO_x emissions lead to acid rains. Moreover, these emissions might also cause serious health problems. Therefore, alternative and clean energy sources need to be developed to alleviate these environmental and health problems.

There are several alternative renewable and sustainable sources like geothermal energy, hydropower, solar energy, wind energy and biomass energy. Hydrogen can be considered as an energy carrier since there is no hydrogen source in nature but rather it is produced through biological and non-biological means [3, 4]. If hydrogen could be produced in a renewable and sustainable way, it can partially meet the world's energy demand. In our country, there are also sustainable and renewable sources for the generation of hydrogen and considerable amount of electricity could potentially be generated from the hydrogen produced [5]. Moreover, when used as a fuel, it does not produce any toxic chemicals. Biological hydrogen production occurs mainly by dark fermentation and photo-biological processes. In some processes, a combination of two was realized and called as two-stage hydrogen production method [6]. In biological hydrogen processes, the microorganisms take role and the responsible enzymes are hydrogenases and nitrogenases [7]. There are various types of these enzymes in microorganism [7, 8]. Photo fermentative hydrogen production is mainly performed by purple non-sulfur (PNS) bacteria. The representatives of them could be listed as *Rhodopseudomonas palustris* (*Rps. palustris*), *Rhodospirullum rubrum* (*Rsp. rubrum*), *Rhodobacter capsulatus* (*R. capsulatus*) and *Rhodobacter sphaeroides* (*R. sphaeroides*). The responsible enzyme in PNS bacteria is nitrogenase and hydrogen production is an inherent character of the enzyme [7]. Under anoxic conditions and nitrogen limitations, the hydrogen is evolved at the expense of ATP using light energy [7, 8]. PNS bacteria are versatile in that they can grow under different physiological conditions and they can produce various valuable chemicals like 5-aminolevulinic acid (5-ALA), poly-hydroxy butyrate (PHB) and vitamin B_{12} [9-13]. In a recently developed concept called biorefinery, more than one product (more than one fuel or fuel and value-added chemicals) is produced in the same bioprocess [12-16]. The logic behind this approach is to maximize the benefit from the biomass. In this way, cost-efficient bioprocesses will be developed. Kars et al. [12] reported the production of 5-ALA (23 mM) and hydrogen (1.01 L H_2/L) in the same bioprocess from sugar beet molasses.

In biological hydrogen production processes, different types of carbon sources could be used [17]. Industrial waste streams, agricultural and forestry lignocellulosic materials could effectively be used as substrate in the biological hydrogen production processes [12, 13, 17]. The selected carbon source should be cheap, found in large amount, easily fermentable by microbe and non-toxic to the cells. The case of using lignocellulosic biomass, the pretreatment of biomass is of great importance to produce useable simple sugars and organic acid. The basic pretreatment methods may be listed as mechanical, biological, chemical and physical techniques [18]. The motivation behind

this study is to find cheap and accessible feedstock for biological hydrogen and 5-ALA productions. For this purpose, waste barley was chosen, pretreated and used as carbon source. In the present study, 3 % (w/v) waste barley hydrolysate was obtained. The growth media containing various quantities of sugar (5, 6, 7 and 8 g/L) were prepared using this hydrolysate. And, the formation of hydrogen and 5-ALA by *R. sphaeroides* O.U.001 in the bioreactors was monitored.

2. MATERIAL AND METHOD

2.1. The microbial strain and its cultivation conditions

In this study, *R. sphaeroides* O.U. 001 whose DSM number is 5864 was chosen for both photo-biological hydrogen and 5-aminolevulinic acid productions. The medium used for the general maintenance of this bacterium was Biebl and Pfennig minimal medium [19]. One liter of minimal medium includes K_2HPO_4 (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), DL-Malic acid (2 g), $Na \cdot Glutamate \cdot H_2O$ (0.37 g), NaCl (0.4 g), $CaCl_2 \cdot 2H_2O$ (0.05 g), trace element solution (0.05 mL from 20X stock solution), $FeSO_4$ (1 mL from 2X stock solution) and vitamin solution (0.05 mL from 20X stock solution). One liter of 20X concentrated stock trace element solution includes $Na_2MoO_4 \cdot 2H_2O$ (40 mg), $NiCl_2 \cdot 6H_2O$ (20 mg), $CuCl_2 \cdot 2H_2O$ (20 mg), $CoCl_2 \cdot 6H_2O$ (200 mg), H_3BO_3 (60 mg), $MnCl_2 \cdot 4H_2O$ (100 mg), $ZnCl_2$ (70 mg) and HCl (0.675 mL from % 37). 2X concentrated $FeSO_4$ solution was prepared by adding 0.02 g of $FeSO_4$ into 10 mL of dH_2O. One liter of 20X concentrated vitamin stock solution contains Thiamin (500 mg), Nicotinic acid or Niacin (500 mg) and Biotin (15 mg). The trace elements solution and $FeSO_4$ solution were sterilized by autoclave while vitamin element solution was sterilized through micro filtration since vitamins were heat-labile. The trace elements solution and $FeSO_4$ solution were separately autoclaved and added to the basal media to prevent possible precipitations such as calcium phosphate precipitate. When solid media were needed, 1.5 % agar was used in minimal media. 55 ml glass bottles were used as bioreactors. The incubation temperature for the cultures was 29 ºC and the light energy was provided by incandescent lamps (100 W). The intensity of the light bioreactor surface was circa 775 ± 25 lux. For general purposes, either aerobic or anaerobic condition was used.

2.2. Pretreatment of waste barley

R. sphaeroides is a versatile bacterium since it has the capability to utilize a wide variety of substrate as a source carbon [12, 20, 21]. However, the waste materials and lignocellulosic biomass should be pretreated to decompose the complex biomaterials into its monomers so that it could be utilized by the PNS bacteria. In the present study, waste barley was utilized as carbon source so that a cost-efficient hydrogen production process could be achieved. Firstly, 3 g of powdered waste barley was dissolved in certain amount of dH_2O after which the pH of the concoction was set to 3 by adding H_2SO_4 before completion of the final volume to 100 mL. The acid hydrolysis of waste barley (3 % w/v) was performed by autoclaving this mixture at 121 ºC for 30 min. To remove the insoluble components, the suspension was spun at high speed (9418 x g) for about 10 min and then the liquid part above the pellet was filtered (Whatman Grade No: 41) to eliminate remaining insoluble particles. After completion of pretreatment process, the neutralization of the hydrolysate to pH: 7 was done by NaOH addition. Upon neutralization, color of the hydrolysate became darker and some precipitates were formed. These were removed by re-centrifugation of the hydrolysate at 9418 x g for 10 min and then the amounts of total sugar, organic acids, total phenol and ammonium were analyzed.

2.3. Analytical methods

Many factors like a type of carbon source [20], presence of ammonia [22] and existence of necessary elements such as molybdenum and iron [23] influence biological hydrogen production. These factors exert their effect either directly on the nitrogenase enzyme or on the selected PNS bacterium. For this reason, waste barley

hydrolysate was analyzed in terms of these aspects in order to prepare a suitable medium for biological hydrogen production. Acid-phenol spectrophotometric method applied for the determination of total simple sugar concentration of the waste barley hydrolysate [24]. First of all, a series of samples with pre-determined sugar concentrations were prepared and then their spectrophotometric measurements were done. After the graph was drawn, the unknown sample concentration was calculated by interpolation. After knowing the sugar content of the hydrolysate, 4 different culture media were prepared so as to contain 5, 6, 7 and 8 g/L sugar concentration from % 3 waste barley hydrolysate. The total ammonium ion content of the hydrolysate was found out with the use of an ammonium test kit (Norateks, Turkey). For this, 1 mL of barley hydrolysate was put into a glass tube and mixed with 4 mL of dH_2O. Then, 2 drops from the NH_4-1 solution and 4 drops from the NH_4-2 solution were put onto the diluted hydrolysate in order. The suspension was held at room temperature (RT) for 5 min. for the formation of color. Finally, the color of the suspension was matched with the identical reference color on the manual to find out the ammonium concentration. The elements found in the 3 % barley hydrolysate were identified by inductively coupled plasma mass spectrometry (ICP-MS, Perkin Elmer elanDRC-e, USA).

The produced hydrogen was collected in graduated glass tubes by water displacement method and purity of collected gas was measured by gas chromatography (GC, Agilent, 6890N). Supelco carboxen 1010 column and thermal conductivity detector were used in GC. The argon gas was utilized as carrier gas and the flow rate was set to 22.3 mL/min. The temperature of detector, injector and oven was adjusted to 170 ºC, 160 ºC and 140 ºC, respectively. The amount of 5-ALA in media was quantified by a spectrophotometric technique [25] and explained in detail in [26]. Similar to other methods mentioned above, 8 samples having pre-determined amounts of 5-ALA were first made and then their spectrophotometric measurements were performed. Afterward, the quantities of 5-ALA in the studied samples were calculated from the graph.

2.4. Media preparation and culture conditions

For the hydrogen and 5-ALA productions, four kinds of medium with various sugar concentrations (5, 6, 7 and 8 g/L) were made from % 3 barley hydrolysate. The contents of the 5-ALA and hydrogen production media were same as the contents of the Biebl and Pfennig minimal medium except that glutamate, malate and NaCl were not added since barley hydrolysate contains sufficient amount of carbon, nitrogen and NaCl sources. After putting all the ingredients of the medium into the bioreactors (55 mL glass bottles), the pH of the bioreactors was adjusted to 7 and sterilized by autoclaving. The sterile vitamin solution was then added into the bioreactors aseptically. The cultures were made anoxic by flushing with argon gas for 3 min. The photoheterotrophic growth mode was provided by incubating the cultures under an illumination of 100 W incandescent lamps providing circa 775 ± 25 lux on the surface of the bioreactors.

2.5. Statistical analysis

In this study, pH, growth and hydrogen production experiments were performed in duplicate. Standard deviations from the mean values were calculated. The error bars were inserted into the graphs.

3. RESULTS AND DISCUSSION

3.1. The analytical features of barley hydrolysate

The quality of barley used in this study is low and it cannot be used as human food. The percentages of fat, protein, and starch in barley were determined previously and found to be 2.45 ± 0.035, 11.19 ± 0.46 and 37.5 ± 0.49, respectively [26]. Especially, the starch content of the waste barley was almost half of the normal quality barley. In the present study, hydrolysate was prepared as 3 % (w/v) as opposed to our previous study where 9 % (w/v) hydrolysate was used [13]. Therefore, the content of 3 % (w/v) hydrolysate

was different from 9 % (w/v) hydrolysate. After acid hydrolysis, the sugar content of the 3 % (w/v) barley hydrolysate was found to be 11.58 g/L. Then, 4 different media with various concentrations of sugars (5, 6, 7 and 8 g/L) were made from this barley hydrolysate. PNS bacteria could efficiently use glucose [27] and sucrose [12] for the generation of hydrogen. Moreover, type and composition of substrate were told to strongly influence both the hydrogen production and biomass accumulation [28]. In the present study, considerable amount of simple sugars was obtained after acid hydrolysis of waste barley. Therefore, it was shown that 3 % (w/v) waste barley hydrolysate could be used as carbon source for *R. sphaeroides* O.U.001.

The ammonium content of the medium utilized for the generation of hydrogen influence the hydrogen generation ability of microorganism significantly since above 2 mM concentration, the ammonium suppresses the nitrogenase enzyme resulting in the cessation of hydrogen evolution [22]. The ammonium content of the 3 % (w/v) hydrolysate was found to be 0.139 mM which is below suppressive concentrations. After preparation of the media with different concentrations of sugar, the ammonium concentrations turned into 0.060 mM, 0.072 mM, 0.084 mM, 0.096 mM in the media having initial sugar quantities of 5, 6, 7 and 8 g/L culture, respectively. These ammonium concentrations are far below the suppressive concentration; therefore, these media are suitable for the production of biological hydrogen.

It is known that elements such as iron and molybdenum are needed for the proper functioning of Mo-nitrogenase enzyme and hydrogen generation is mediated by this enzyme in *R. sphaeroides* O.U.001 [23]. In this context, the element composition of the 3 % (w/v) barley hydrolysate was found out by ICP-MS. In the hydrolysate, the analyzed elements and their quantities were (mg/L); B (0.449), Cr (0.052), Mn (0.4), Fe (0.153), Co (-), Ni (-), Cu (0.05), Zn (0.227), Mo (0.003), Al (0.041), Ca (13.405), Mg (33.7), Na (>800), K (>400). Since sufficient amount of Na is present in the hydrolysate, NaCl was not added to the media. The other significant factor which also affects the hydrogen generation capability of microorganisms is the presence of high amount of phenols in the waste materials which are going to be used as substrate. For instance, the quantity of whole phenol (1.9 g/L) was found to be at the toxic level in olive oil mill wastewater (OOMW) [29]. Therefore, OOMW could only be used as substrate after dilution with water for hydrogen production [30]. Total amount of phenolic substances in barley was found as 10.13 mg GAE/g barley in our previous study [13]. The overall amount of phenol in the hydrolysate is significantly lower than that in OOMW. After preparation of the media by diluting with dH_2O, the quantity of phenols becomes much lower in the culture media. Therefore, waste barley hydrolysate is also suitable for hydrogen production in terms of total phenol content.

Another important parameter which should be considered for bio-hydrogen generation processes is the availability of sufficient energy in the form of light to the cells in the bioreactor. According to Uyar et al. [31], invisible light (infrared) at which the bacteriochlorophyll a shows the maximal absorption is more effective than the visible light where the carotenoid has absorption maxima. In this context, in order to see the transmittance of the barley hydrolysate, the % transmittance of barley hydrolysate at infrared region (800 and 860 nm) was measured and it was found that the % transmittance of each media was above % 90. The suitability of the barley hydrolysate in terms of light transmittance was also demonstrated.

3.2. pH changes and bacterial growth

Several media with various sugar concentrations (5, 6, 7, and 8 g/L) were made from % 3 barley hydrolysate. After 10 % inoculation into 45 mL fresh media, 50 mL of cultures were obtained and incubated under light. pH and the turbidity of the cultures were followed at certain time intervals (Figure 1 and Figure 2). pH of the medium is mainly dependent on the type carbon source and it

is crucial for biological hydrogen production [32]. Even though the preliminary pH values of the cultures were adjusted to 7, they dropped to 6.6 after inoculation of bacteria and incubation for about 32 h (Figure 1). Then, the pH of the cultures rose back to 7 after 23 h incubation. The pH of the media remained almost the same at around 7 until 175. h. Afterward, a slight increase (pH: 7.23) occurred in especially 5 g/L sugar-containing medium. There were no significant changes in the pH of the other media. As a general tendency, the pH of the media followed a similar pattern and did not change significantly during the bioprocess. This result shows that the buffering capacity of phosphate buffer used in this study is high enough to resist the pH changes due to the metabolism of microorganisms. This uniform pH provides the bacteria with a suitable environment for hydrogen production.

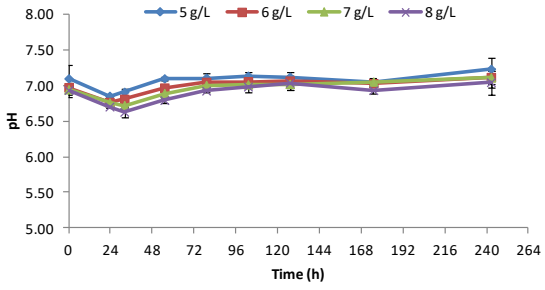

Figure 1. The pH changes during the growth of *R. sphaeroides* O.U.001. The experiment was done duplicate and standard deviations were added.

One of the aims is to find the optimal amount while using biomass as carbon source for bacterial growth. The use of high amount of hydrolysate might be toxic to the cells due to the ingredients of hydrolysate such as phenols or it might prevent light transmission in the bioreactor. Furthermore, the lower concentrations might be inadequate to sustain cell growth. For this reason, the growth of *R. sphaeroides* O.U.001 in the cultures having various concentrations of sugar was followed by taking spectrophotometric measurements periodically. In this way, the suitability of selected concentrations (5, 6, 7 and 8 g/L) in order to sustain bacterial growth was assessed. In the present study, relatively lower quantities of waste barley hydrolysate were used when compared to our previous study [13]. As shown in Figure 2, comparable OD values were attained when compared to our previous studies [13, 23]. A rapid rise in turbidity continued until 79. h. and then the rate of increase slowed down. The maximal OD values were 1.25, 1.44, 1.50 and 1.71 in the media having initial sugar concentrations of 5, 6, 7 and 8 g/L cultures, respectively. The higher the sugar concentration, the higher the OD value (Figure 2). Demiriz et al. [33] used acetate as carbon source to produce hydrogen and poly-β-hydroxybutyric acid by *R. capsulatus* DSM 1710 and they obtained similar results such that total biomass of bacteria in the bioreactor increased in parallel to raising the amount of acetate from 10 mM to 65 mM. In our previous study which was done with 9 % waste barley hydrolysate, the highest OD value (OD_{660}: 1.78) was attained when using the sugar concentration at the highest concentration (11 g/L sugar) [13]. When compared to previous findings, it can be asserted that the media prepared with 3 % waste barley hydrolysate is also sufficient to sustain *R. sphaeroides* O.U.001 and the growth pattern showed the similar tendency when the literature findings were taken into account.

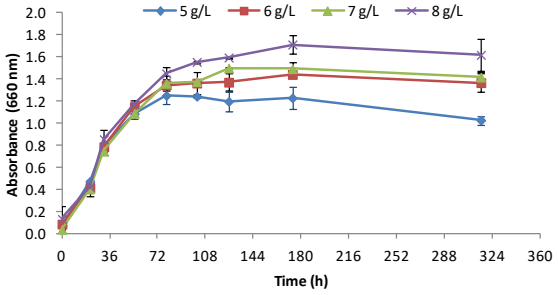

Figure 2. Growth of *R. sphaeroides* O.U.001 in media prepared by using % 3 waste barley hydrolysate. The experiment was done duplicate and standard deviations were added.

3.3. Hydrogen and 5-ALA formation by *R. sphaeroides* O.U.001

The color and content of the hydrolysate might also affect the hydrogen generation capability of *R. sphaeroides* O.U.001. For this reason, four different growth media (5, 6, 7 and 8 g/L) were prepared from 3 % (w/v) waste barley hydrolysate

and tested for hydrogen production. The total culture volume was 50 mL and the gas evolved from the culture was accumulated in the graduated glass tubes. Then, analysis of the collected gas was performed by GC. The percentage (%) of H_2 in collected gas was 81.5, 87.3, 89.2 and 80.4 in 5, 6, 7 and 8 g/L sugar-containing cultures, respectively. Remaining gas was identified as CO_2 and air. In Figure 3, total hydrogen accumulation of hydrogen was illustrated. In the preparation of graph, the pure hydrogen amounts were used not the total gas accumulated. Almost the same amount of hydrogen was accumulated in the bioreactors. The highest hydrogen accumulation (0.29 L H_2/L) was achieved in the bioreactor having initial sugar quantity of 6 g/L culture. 0.27 L H_2/L, 0.27 L H_2/L and 0.24 L H_2/L were produced in the bioreactors having initial sugar quantity of 5, 7 and 8 g/L culture, respectively. These results are comparable to our previous findings where 0.4 L H_2/L was produced in medium having an initial sugar quantity of 11 g/L culture (Table 1). The interesting point was that the hydrogen accumulation in higher amount of sugar-containing culture (8 g/L) was always lower than that in lower amount of sugar-containing media as opposed to the case observed in growth of bacteria in Figure 2. That is, increase in sugar concentration did not result in an increase in hydrogen production. This may be owing to the darker color of this medium which can prevent the access of light into the bioreactor because 34.48 mL of hydrolysate was used for the preparation of 8 g/L sugar-containing medium while 30.17, 25.86 and 21.55 mL of hydrolysate were used to make the media having sugar quantities of 7, 6 and 5 g/L culture, respectively. Moreover, the ammonium and other potentially toxic chemicals like phenols are also found in higher amount in the medium having the highest initial sugar amount of 8 g/L culture and this might negatively affect the hydrogen generation capability of the cells in the bioreactor. Light limitation and presence of ammonium seem to be harmless to the cells in terms of their growth but they are thought to be highly effective and restrictive for hydrogen production capability of bacterium. Similar findings were reported by Feng et al. [34] in their study where hydrogen was produced using corn straw (CS) hydrolysate by *R. capsulatus* JL1. They observed that hydrogen yield increased from 1402 to 2966 mL/L when the amount of CS hydrolysate was increased from 7.0 g CS/L culture to 16.4 g CS/L culture. However, further increase in substrate concentration resulted in a decrease in hydrogen yield due to the inhibition effect of high amount of hydrolysate. In the current study, 5-ALA production was also investigated; however, 5-ALA was not detected in any of the media. Several possible explanations could be done for situation. Firstly, this may be due to the low sugar concentrations in the media. Probably, the substrate sustained the cells for hydrogen generation and growth but it was not sufficient to produce excess reducing equivalents for the generation of 5-ALA. In our earlier work where higher amounts of substrates were used, 67.4 µM of 5-ALA was obtained in the bioreactor having an initial sugar concentration of 9 g/L culture [13]. In previous study, levulinic acid and vitamin B_{12} additions were done to promote 5-ALA synthesis but in the present study such additions were not done. This might be reason why 5-ALA was not produced. Finally, the previous study was performed using 9 % (w/v) waste barley hydrolysate but the present study was done using 3 % (w/v) waste barley hydrolysate. The two hydrolysates were different in terms of sugar content, ammonium content, element composition, phenol content and transparency. In example, color of the media prepared from 3 % (w/v) waste barley hydrolysate was much darker than the color of the media prepared from 9 % (w/v) waste barley hydrolysate. These differences might also be the reason why 5-ALA could not be produced in the present study.

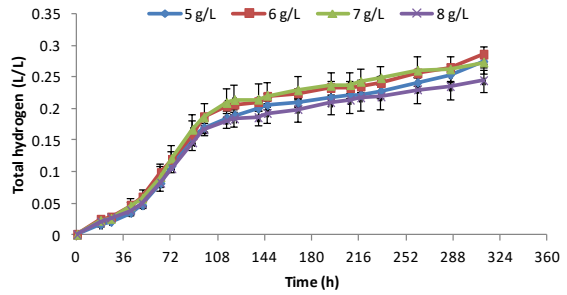

Figure 3. Total accumulation of hydrogen in media prepared by using 3 % waste barley hydrolysate. The experiment was done duplicate and standard deviations were added.

Table 1. Hydrogen generation by *R. sphaeroides* O.U.001 from different carbon sources.

Initial carbon source	H_2 production (L H_2/L culture)	Ref.
Simple sugars, 6 g/L	0.29	This work
Simple sugars, 11 g/L	0.40	[13]
Acetate, 30 mM	0.21	[21]
Malate, 15 mM	1.97	[21]
Sucrose, 28 g/L	1.01	[12]

4. CONSCLUSION

Different type of feedstock has been used as carbon source for biological hydrogen production for many years. The important point in these studies is to find the optimum concentration of the substrate. While inadequacy of carbon source leads to lower hydrogen yield, the excess of it leads to inhibition of hydrogen production and cell growth. In this context, in the current study, pH variations, growth of *R. sphaeroides* O.U.001, biological hydrogen production and 5-aminolevulinic acid production were investigated using waste barley as carbon source. Relatively low concentrations of sugar-containing media (5, 6, 7 and 8 g/L) were prepared from 3 % (w/v) waste barley hydrolysate. The pH of the cultures did not change significantly during the bioprocess. The initial amounts of sugars in the bioreactors sufficiently sustained cell growth. Similarly, comparable amounts of hydrogen were obtained. However, the 5-ALA was not detected in any of the media. To conclude, 3 % waste barley hydrolysate could also be used for the cultivation and hydrogen generation by *R. sphaeroides* O.U.001.

ACKNOWLEDGEMENTS

This research was supported by Selçuk University (BAP-12201030). Prof. İnci Eroğlu and Dominic Deo Androga are acknowledged gratefully for their technical support.

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