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RESEARCH ARTICLE / ARAŞTIRMA MAKALESİ

Evaluation of High Concentrations of Sugar Beet Molasses as Substrate for Hydrogen and 5-Aminolevulinic Acid Productions

Yüksek Miktarlarda Şeker Pancarı Melasının Hidrojen ve 5-Aminolevulinik Asit Üretimi için Substrat Olarak Değerlendirilmesi

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Abstract
Sugar beet molasses is a valuable raw material and it contains high amount of sugar especially sucrose. Therefore, it could be used as substrate for the generation of highly valuable chemicals by microorganisms. Here, considerably high concentrations of molasses were tested for the first time to investigate if they could enhance the growth of *Rhodobacter sphaeroides* O.U.001 and generations of hydrogen and 5-aminolevulinic acid (5-ALA). Firstly, five distinct growth cultures having sugar contents of 34 g/L, 41 g/L, 48 g/L, 55 g/L and 61 g/L were made ready using molasses. Then, in batch processes, bacterial growth and generations of hydrogen and 5-ALA were investigated in these media. As a result, the highest cell growth (OD_{660}: 9.26, 4.54 g cdw/L) to date was achieved in 34 g/L sugar containing medium. Similarly, the highest quantity of 5-ALA (37.44 mM) to date was attained in the same growth culture. In addition to these significant improvements, at maximum 21.02 mL (0.42 L H_2/L) of hydrogen was collected from 34 g/L sugar containing medium. To conclude, using a sugar concentration of 34 g/L yielded the highest bacterial growth and 5-ALA generation so far. And, it also supported the generation of considerable amount of hydrogen.
Keywords: 5-aminolevulinic acid, hydrogen, molasses, *Rhodobacter sphaeroides*

Öz
Şeker pancarı melası değerli bir hammaddedir ve sükroz başta olmak üzere yüksek miktarda şeker içerir. Bu nedenle, mikroorganizmalar tarafından son derece değerli kimyasalların üretimi için substrat olarak kullanılabilir. Bu çalışmada, *Rhodobacter sphaeroides* O.U.001'in çoğalması ile hidrojen ve 5-aminolevulinik asit (5-ALA) üretimlerini arttırıp arttırmadıklarını araştırmak için ilk kez oldukça yüksek melas konsantrasyonları test edilmiştir. İlk önce, şeker içeriği 34 g/L, 41 g/L, 48 g/L, 55 g/L ve 61 g/L olan beş farklı ortam, melas kullanılarak hazırlandı. Daha sonra, kesikli süreçlerle, bu ortamlarda bakteri çoğalması ile hidrojen ve 5-ALA üretimleri incelenmiştir. Sonuç olarak, bugüne kadarki en yüksek hücre çoğalması (OD660: 9.26, 4.54 g cdw/L), 34 g/L şeker içeren ortamda elde edildi. Benzer şekilde, aynı çoğalma kültüründe bugüne kadarki en yüksek miktarda 5-ALA (37.44 mM) elde edildi. Bu önemli gelişmelere ek olarak, 34 g/L şeker içeren ortamdan maksimum 21.02 mL (0.42 L H_2/L) hidrojen toplanmıştır. Sonuç olarak, 34 g/L'lik bir şeker konsantrasyonunun kullanılması, şimdiye kadarki en yüksek bakteri üremesini ve 5-ALA oluşumunu sağladı. Ayrıca, önemli miktarda hidrojen üretimini de destekledi.
Anahtar Kelimeler: 5-aminolevulinik asit, hidrojen, melas, *Rhodobacter sphaeroides*

I. INTRODUCTION

The generation of highly valuable chemicals together with biofuels through valorization of biomass and waste products is among the trending topics in the scientific fields of study. This approach is termed as "biorefinery concept" [1]. One of the driving forces behind this approach is the transition from petroleum derived fuel and chemical generation to renewable biomass-based fuel and chemical production. In this way, cost-efficient and sustainable processes will be developed. These processes will also help to develop healthier and environmentally friendly fuels and chemicals. The use of lignocellulosic residues and waste streams ensures the settlement of bio-based and circular economy in each country. Therefore, for the countries to be self-sufficient, sustainable processes through which value-added chemicals and fuels are generated from local feedstocks need to be developed. The three pillars of the biorefinery approach could be counted as feedstock, products and conversion process. Regarding the feedstock, a broad spectrum of raw materials can be utilized for the generation of high value-added chemicals and fuels [1-3]. In a broader sense, the starting raw material could be divided into two main categories as being lignocellulosic and non-lignocellulosic feedstocks. Lignocellulosic raw materials come from mainly the

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forest and agricultural plants and their residues while non-lignocellulosic feedstocks come out as a result of industrial processes. The lignocellulosic raw materials and waste streams could be converted into chemicals and fuels either by thermo-chemical or biological ways [1]. Thermo-chemical conversion of biomass into high value-added products and fuels generally requires stringent conditions; however, biological conversion processes operate at moderate conditions [4-6]. In case of biological generation of chemicals and bio-fuels, the raw material should be first pretreated to make it usable for the microorganisms. And, the media prepared with pre-treated feedstock should be free from any toxic chemicals which might inhibit growth and biochemical generation of chemicals and fuels.

Hydrogen, butanol, biodiesel, ethanol and methane could be generated as fuels in the context of biorefinery approach [6,7]. The production of these fuels contributes to the energy diversification and it also partially meets the increased energy demand of the country. Therefore, these potential benefits promote the alternative and sustainable energy research. Among them, hydrogen could be considered as both sustainable energy source and valuable chemical. Molecular hydrogen cannot be found in nature freely but rather it is produced through thermo-chemical and biological ways [4]. Thermo-chemical hydrogen production processes include biomass pyrolysis and biomass gasification and occur at very high temperatures (400-1000 °C) [8]. However, the biological hydrogen production processes operate at much lower temperatures (28-70 °C). The amount of hydrogen that could be generated by biological systems is still below practical applications; therefore, hydrogen production studies carry on to enhance amount and cost efficiency through biological and process optimization studies. Nevertheless, purple non-sulphur (PNS) bacteria such as *Rhodobacter sphaeroides* are promising candidates for hydrogen generations and substantial amounts of hydrogen were achieved so far [9].

The most powerful aspect of the biorefinery approach is the capability of producing several high value-added products like polyhydroxyalkanoates (PHAs), furans (5-hydroxymethylfurfural, HMF, and furfural), succinic acid and levulinic acid (LA) [1,6,10]. Among others, hydroxymethylfurfural (HMF) and levulinic acid (LA) could be used as the monomer for the fabrication of many industrially significant chemicals like pharmaceuticals. The generation of such valuable chemicals from forest and agro-industrial resources in processes with low environmental impact will certainly promote biorefiney studies. 5-aminolevulinic acid (5-ALA) is one of the target chemicals to be produced in biorefinery approach [11] though chemical synthesis is also available [12]. It is a versatile chemical that can be used in several medical and biotechnological applications and it takes special attention due to its use as a photosensitizer substance in photodynamic therapy [13]. The biological formation of 5-ALA occurs by either C-4 pathway (Shemin pathway) using succinyl-CoA and glycine or C-5 pathway using glutamate [13]. In anaerobic photosynthetic bacteria such as *R. sphaeroides*, 5-ALA biosynthesis occurs through C4 pathway in which glycine and succinyl CoA are converted into 5-ALA by ALA synthase enzyme and as much as 27.5 mM of 5-ALA was obtained [13]. The capability of *R. sphaeroides* to produce 5-ALA and hydrogen from various biomass resources could make it a robust and sustainable cell factory in biorefinery studies.

Aforementioned discussions imply that there is a trend for shifting from petroleum-based fuels and chemicals to more green production technologies. The biomass resources play a key role in setting of such processes. Therefore, significant efforts are being made to find out sustainable feedstocks to be used in the productions of fuels and chemicals. As a part of these efforts, sugar beet molasses has been utilized as feedstock for several years in our studies to produce high value-added products [14]. Molasses is the product of sugar refining process and contains up to 50 % (w/w) sucrose in addition to many organic acids and elements [14]. In Turkey, 996000 tons of sugar beet molasses were produced in 2018 [15]. Therefore, it stands as a sustainable substrate in bioprocesses. In the current work, molasses obtained from sugar beet was utilized as feedstock for *Rhodobacter sphaeroides* O.U.001 to generate hydrogen and 5-ALA. In our previous study [14], the highest tested sugar quantity was just 28 g/L; however, in the present study, much higher concentrations (up to 61 g/L) were tested. As a result, the maximum amount of evolved hydrogen (0.62 L H_2/L culture) was declined but significantly higher amounts of cell growth (OD_{660}: 9.26) and 5-ALA (37.44 mM) were achieved. In the current studies, saturation points above which no further improvements in bacterial growth, hydrogen and 5-ALA productions were observed upon addition of molasses were determined. In other words, the upper limits of sugar beet molasses concentration that could efficiently be used by *R. sphaeroides* for growth and generations of hydrogen and 5-ALA were revealed.

II. MATERIALS AND METHODS

2.1. Culture Compositions

Rhodobacter sphaeroides O.U. 001 (DSM number: 5864) which is a photosynthetic purple non-sulfur bacterium was used in the present work. It is a versatile bacterium which could act as a robust cell factory for the formation of hydrogen and 5-ALA. As a raw material, sugar beet molasses was used. It is the byproduct of sugar beet processing plants and it has high sucrose content (50 % w/w) in addition to various organic acids (malic, succinic, fumaric, lactic, acetic, propionic and formic acid) and elements (K, Na, Mg, Ca, Al, Zn, Cu, Ni, Co, Mn, Cr and B) [14]. Due to its

rich content, it is expected to support both cell proliferation and generation of valuable metabolites. In the present work, the aim was to test the higher concentrations of molasses and to observe the upper limits of molasses concentrations. In this context, the sugar concentrations in the growth media were adjusted as 34 g/L, 41 g/L, 48 g/L, 55 g/L and 61 g/L knowing that the sucrose content of beet molasses was circa 50 % (w/w). The media were named according to their sugar contents and used as such throughout the study. Before preparation of these media, the molasses was diluted and centrifuged at 8500 rpm for 10 min. to get rid of insoluble materials. *R. sphaeroides* O.U. 001 is ordinarily maintained in Biebl and Pfennig minimal medium [16] where glutamate and malate were utilized as sources of nitrogen and carbon, respectively. Here, instead of malate, the sucrose in molasses was utilized as a source of carbon but 2 mM of glutamate was again included in the cultures as a nitrogen source. $CaCl_2.2H_2O$ (0.025 g/L) and $MgSO_4.7H_2O$ (0.2 g/L) were included in the media as major elements while KH_2PO_4 (0.5 g/L) was used as buffer. $FeSO_4$ (2 g/L), $Na_2MoO_4.2H_2O$ (0.2 g/L) and vitamins which were Niacin (0.5 g/L), Thiamine (0.5 g/L) and Biotin (0.015 g/L) were added into the cultures at given concentrations. It was stated that supplementation of levulinic acid (LA) enhanced the extracellular accumulation of 5-ALA [13,17]; therefore, 15 mM of levulinic acid (LA) was also included in the growth culture as suggested. To prevent the LA addition from interfering with the generation of hydrogen and bacterial growth, limited quantities of LA was supplemented towards the termination of exponential growth stage. Additional trace elements were not added since molasses already contained sufficient amount of these elements.

2.2. Culture Conditions

Hydrogen generation by *R. sphaeroides* O.U. 001 occurs only in anoxic environments in the presence of light [18]. 5-ALA production takes place under same conditions as well. Therefore, the experimental setup established here was suitable for the generation of both products. In this way, a cost-effective process was obtained. The anoxic environment in 55 mL glass bioreactors was ensured by flushing argon gas for 3 min. while the light energy (3 klux) was supplied from incandescent bulbs (100 W). Furthermore, the back of the photobioreactors were coated with aluminum folio so that the light beams were benefited more. The cultures were maintained at 29 °C without shaking because *R. sphaeroides* O.U. 001 can move by the help of its flagella. Having no need for agitation is another property of the process that increases the cost-effectiveness of the process.

2.3. Analytical Techniques and Measurements

In the case of using biomass and waste streams as substrate, it is of great importance for them to be pretreated so that they become suitable for the growth of bacteria. Therefore, the feed should be analyzed in terms of several parameters which influence the hydrogen and 5-ALA generations by bacteria. The first consideration is the presence and amount of ammonia excess of which inhibits nitrogenase enzyme and thus preventing hydrogen generation [19]. Sugar beet molasses was previously shown to contain 32.57 mM of ammonium [14] and the quantity of ammonium in the media (34 g/L, 41 g/L, 48 g/L, 55 g/L and 61 g/L) was calculated considering this information. The second consideration is the total amount of phenolic substances in the molasses since excess of it might have toxic effects on cells. The phenolics were already quantified in terms of gallic acid equivalent (GAE) and found as 13.36 mg GAE/g molasses in a previous study [14]. And, its amount in each medium was calculated accordingly. Another consideration is light transmittance of the media which were prepared using dark colored molasses. *R. sphaeroides* O.U. 001 is photosynthetic bacterium and generations of hydrogen and 5-ALA are light dependent events. Therefore, the light beams should successfully penetrate the culture and reach to the cells effectively. Taking into account these issues, the light transmittance of each medium was measured with a spectrophotometer (Biochrom Libra S22, UK) at different wavelengths (860, 800 and 522 nm). The wavelengths were opted on the basis of a fact that the light is absorbed by bacteriochloropylls and carotenoids in *R. sphaeroides* O.U.001 and they have maximum absorptions at around these wavelengths [20].

Five different growth cultures possessing varying quantities of sugar (34, 41, 48, 55 and 61 g/L) were prepared in a total volume of 45 mL in bioreactors. After inoculating each of these media by 10 % fresh culture, they were incubated under the conditions mentioned above. Several measurements (pH, OD, hydrogen) were done throughout the process whereas the amount of 5-ALA was quantified at the end of the process. The optical density (OD) measurements of the cultures was performed using a spectrophotometer (Biochrom Libra S22, UK). At certain time intervals, samples were taken from the cultures and OD values were measured at 660 nm after making appropriate dilutions. The exact OD values of the cultures were calculated by multiplying the measurements by the dilution factors. Then, the growth curves were constructed using these data. Depending on the type of substrate, various metabolites might come out and these byproducts could significantly alter the pH of the medium. As a result, these drastic pH changes could negatively affect the hydrogen production performance of the bacteria. For this reason, though the pH of the cultures was buffered to 6.8 at the beginning, pH variations in the media were followed throughout the process and pH graph for each culture was drawn.

The emerged hydrogen gas was gathered in water-filled graduated tubes using pipes and quantity of hydrogen

was recorded throughout the process until cessation of hydrogen generation. The purity analysis of agglomerated gas was performed by a gas chromatography (Shimadzu GC-2010 Plus, Japan) which was equipped with an Rt®-Msieve 5A column and a thermal conductivity detector. The specific conditions for GC were explained in detail in a previous study [14]. After termination of the process, the extracellular quantity of 5-ALA was measured with a colorimetric method which rely on the formation of reddish-purple color upon addition of Ehrlich's reagent and measurement of the resulting color intensity with a spectrophotometer at 533nm [21]. In the protocol, first of all, each of the five culture liquids were centrifuged and filtered to eliminate cells and cell debris. Then, clear filtrate was freezed at -80°C overnight and then freeze-dried in a lyophilizator (ScanVac CoolSafe 110-4 Pro) at –111 °C under 0.009 mbar pressures for 2 days. The freeze-dried samples were re-dissolved and used in the measurements. At first, a calibration curve was drawn with known quantities of 5-ALA and later certain amount of samples was taken from the unknown samples and processed like the standard samples. After formation of colors, the spectrophotometric measurements at 533 nm were done and the concentrations were determined through interpolation on the standard curve.

2.4. Statistical Analysis

pH of the media, turbidity of the cultures and amount of evolved hydrogen were followed and recorded at certain time intervals. In order to calculate standard deviations, the experiments were done in duplicate. The error bars were depicted in the graphs.

III. RESULTS AND DISCUSSION

3.1. Bacterial Growth and pH Changes in Cultures

Although, various concentrations of sugar beet molasses were used in a previous study [14], such high concentrations were tested for the first time for *R. sphaeroides* O.U.001. Specifically, upper limits of the molasses' concentrations were determined in this study. In this context, five distinct media with varying amount of sucrose (34, 41, 48, 55 and 61 g/L) were prepared for growth and formations of hydrogen and 5-ALA. Since each medium was prepared using different amount of molasses, they are unique in terms of their sugar, ammonium and total phenol contents in addition to light transmitting property. In the present study, the quantities ammonium and total phenol were calculated based on the previous findings [14] and depicted in Table1. The light transmission of each medium at given wavelengths was also measured as percent transmission (% T) with a spectrophotometer and illustrated in the Table 1.

Table 1. Properties of growth cultures prepared by using sugar beet molasses

Medium	Ammonium content (mM)	Phenol content (mg GAE)	% T_{522}	% T_{800}	% T_{860}
34 g/L	1.73	47.84	8	81.1	88
41 g/L	2.01	57.42	4.2	79.1	86.9
48 g/L	2.42	67.00	2.4	76.9	84.2
55 g/L	2.77	76.57	1.6	76.1	84
61 g/L	3.11	86.14	0.9	72.1	82.1

As a measure of cell growth, the OD values of the cultures were measured at certain time intervals and illustrated in Figure 1A. Each value in the graph represents the instant optical density measurement of the culture. The instant measurements continued until observing stationary phase of growth. As seen from the graph, the best growth was observed in 34 g/L medium where maximum absorbance value (OD660: 9.26) was obtained. This absorbance value can be interpreted in terms of cell dry weight (cdw) as 4.54 g cdw/L culture knowing the fact that 1 OD value at 660 nm corresponds to 0.49 g cdw/L culture for *R. sphaeroides* O.U.001 [22]. In a previous study, the maximum absorbance was 8.3 (4.07 g cdw/L culture) which was attained in medium possessing 28 g/L sugar. Thus, the highest cell density to date was achieved in the current work utilizing sugar beet molasses as substrate. A further increase in sugar concentration above 34 g/L had a negative impact on cell growth. Specifically, as the concentration of sugar was raised, the cell density decreased. In order to prepare media with higher sugar concentrations, more sugar beet molasses was included in the media which in turn resulted in higher accumulation of other accompanying substances like ammonium, phenol, elements and other impurities. In this context, these issues should be taken into account to elucidate the negative impact of high molasses concentration. Firstly, high osmolarity due to high sugar concentrations might affect the cells adversely. Secondly, the quantity of phenolic substances in these media might reach to toxic levels so that cells might be influenced negatively. In other words, high phenol content might interfere with the overall cellular metabolism. Finally, high amounts of elements and other accompanying chemicals in sugar beet molasses might have negative impact on growth of the bacteria.

Although the pH of the growth cultures was fixed to 7 initially, considerable variations occurred during the process as demonstrated in Figure 1B. All of the cultures showed similar trend in that the pH first climbed up to 9 and then declined back to 7. However, it is obvious from the growth curve that this pH alteration did not interfere with the growth. A very similar pH profile was also observed in case of using relatively lower sugar concentrations [14]. It can be

deduced from these results that upon utilization of sugar beet molasses, basic molecules or by-products were produced and they caused an increase in pH of the culture. In the case of using waste barley as feedstock, a different pH profile was attained [11,23] such that the pH of the cultures first dropped below 7 and then climbed back to 7. From these results, it is also confirmed that the type of carbon source and thus the type of metabolism has a determinative effect on the pH. Further metabolomic studies might help to reveal the types of metabolites which results in such pH changes.

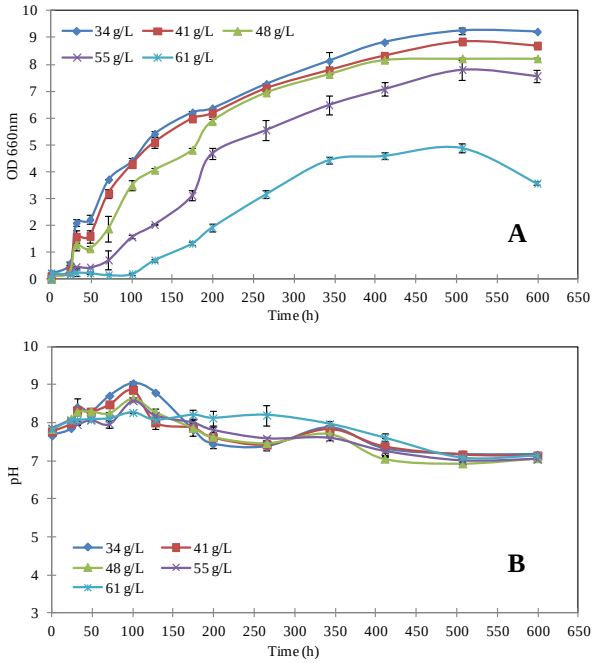

Figure 1. The growth (A) and pH (B) changes in cultures with distinct sugar concentrations

3.2. Hydrogen Accumulations in Cultures

Hydrogen evolution was recorded in five distinct growth media (34, 41, 48, 55 and 61 g/L) throughout process and illustrated in Figure 2. After GC analyses, it was realized that the percentage of pure hydrogen was circa 70 % in the collected gas samples. And, the amounts of pure hydrogen were used in the course of plotting hydrogen accumulation curves. The hydrogen production yields were also calculated and expressed as mg H_2/g sugar. In calculations, only the sugar (sucrose) was taken into account and contribution of glutamate was ignored. The highest amount of hydrogen (21.02 mL, 0.42 L H_2/L culture, 0.99 mg H_2/g sugar) was recorded in case of using 34 g/L sugar concentration. In addition, 18.87 mL (0.38 L H_2/L culture, 0.74 mg H_2/g sugar), 12.92 mL (0.26 L H_2/L culture, 0.44 mg H_2/g sugar), 9.84 mL (0.20 L H_2/L culture, 0.29 mg H_2/g sugar) and 9.23 mL (0.19 L H_2/L culture, 0.24 mg H_2/g sugar) of hydrogen was obtained in 41, 48, 55 and 61 g/L sugar-containing cultures, respectively. In a previous study where 28 g/L sugar concentration was used, 1.01 L H_2/L culture was achieved [14]. When these two studies were compared, it can be concluded that 28 g/L is the most suitable concentration for hydrogen production. And, it was realized that above 28 g/L, increasing sugar (molasses) concentration led to decreased hydrogen accumulation. Putting more sugar (molasses) above 28 g/L obviously exerted a negative effect on hydrogen generation. This result might be explained in several ways by taking into account the culture compositions. First consideration is related with the ammonium concentration. It is of special importance since ammonium suppresses the nitrogenase enzyme especially above 2 mM and thus leading to decreased hydrogen evolution [19]. In some of the cultures (48, 55 and 61 g/L), ammonium content was found to be considerably higher than this critical level so this might be the reason for reduction in the hydrogen generation. Second issue is related with the total phenol content of the media. As demonstrated in Table 1, substantial amounts of phenolic substances are present in the media; therefore, these phenolics might exert toxic effect on nitrogenase enzyme. Or, in an indirect way, the phenolic substances might interfere with the reducing power of the cell thus leading to lessened hydrogen generation. The third consideration is the light transmission capacity of the media. The percent transmission values of the media were measured and shown in Table 1. A drastic decrement in the light transmittance of the media was especially noticed at visible wavelength (522 nm). Moreover, considerable amounts of decline in the transmittance of infrared light (800 and 860 nm) were noticed as well. It is a fact that light as an energy source possesses a significant impact on hydrogen production [20]. Since the access of cells to the light was substantially diminished, hydrogen evolution capacity of the cells was lessened accordingly. Although several intrinsic factors resulted in lowered hydrogen accumulations, the quantity of hydrogen (0.42 L H_2/L culture) achieved in this study is still higher than the amount (0.29 L H_2/L) obtained when sugar (6 g/L) obtained from waste barley was used as substrate. Moreover, beet molasses has a rich content such that it contains several elements (K, Na, Mg, Ca, Al, Zn, Cu, Ni, Co, Mn, Cr and B) and organic acids (malic, succinic, fumaric, lactic, acetic, propionic and formic acid) which might have positive influence on growth and hydrogen production [14]. Therefore, sugar beet molasses definitely has potential and value to be used as substrate for the generation of hydrogen and other valuable products.

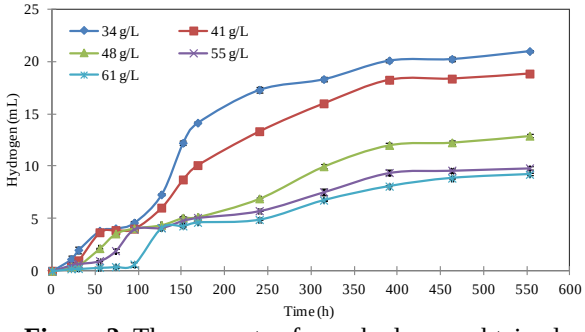

Figure 2. The amounts of pure hydrogen obtained from the cultures with various sugar contents

3.3. 5-Aminolevulinic Acid Productions

The conditions for hydrogen and 5-ALA generations are almost the same. The only distinct attempt to enhance extracellular 5-ALA formation was LA addition into the cultures toward the end of exponential stage of growth. With this approach, a cost-effective bioprocess could be achieved by generating both hydrogen and 5-ALA in the same bioprocess. After the end of the bioprocess, the cultures were first clarified from cells and any suspended solids by centrifugation and filtration. Then, 5-ALA in each purified culture was quantified. The yields for 5-ALA productions were also calculated and expressed as g 5-ALA/g sugar. Sucrose was considered as sole carbon source and contributions of glutamate and LA were ignored during calculations. The maximum amount of 5-ALA (37.44 mM, 0.143 g 5-ALA/g sugar) was observed in the culture with a sugar concentration of 34 g/L. In addition, 34.45 mM (0.109 g 5-ALA/g sugar), 12.04 mM (0.033 g 5-ALA/g sugar), 11.29 mM (0.027 g 5-ALA/g sugar) and 11.29 mM (0.024 g 5-ALA/g sugar) of 5-ALA was attained in 41, 48, 55 and 61 g/L sugar-containing cultures, respectively. It was noticed that the augmentation of sugar concentration (molasses) above 34 g/L influenced the 5-ALA generation negatively. The above-mentioned arguments for the inhibitory effect of the high molasses concentration (above 34 g/L) can also be counted for the case of 5-ALA production. Especially, high phenol content and low light transmittance of the media might have substantial impact on 5-ALA generation. In order to compare these results with the previous findings, various examples for 5-ALA production were itemized in Table 2. As seen from the Table 2, the highest amount of 5-ALA generation (37.44 mM) to date was achieved in the present study using molasses sugar at a concentration of 34 g/L. Taking into account the results in Table, it can be declared that beet molasses is a promising carbon source for *R. sphaeroides* O.U.001 for 5-ALA production.

Table 2. 5-ALA generation by various strains of *R. sphaeroides* at distinct culture conditions

Strain	Substrate	5-ALA (mM)	Reference
O.U.001	Molasses sugar	37.44	This study
O.U.001	Molasses sugar	23.34	[14]
O.U.001	Waste barley	0.067	[11]
CR-720	Glucose and glycine	27.50	[24]
IFO 12203	Volatile fatty acids	16.00	[25]

IV. CONCLUSIONS

In the present study, substantially high amounts of sugar beet molasses were utilized as carbon source to support bacterial growth and generate hydrogen and 5-ALA. In this way, upper limits of sugar concentrations were investigated for the first time. Several conclusions could be drawn from the present study. First, when used as a substrate, sugar beet molasses significantly supported the growth of the bacteria, thereby yielding the highest amount of cell mass (4.54 g cdw/L culture) to date. Even, the molasses could be used for the purpose of obtaining high bacterial cell mass only. On the other hand, compared to previous studies where lower quantities of sugar beet molasses were used, less amount of hydrogen (0.42 L H_2/L culture) was achieved. So, it can be concluded that sugar concentrations equal to or higher than 34 g/L have negative impact on hydrogen production. As a final conclusion, it could be claimed that higher quantities of molasses sugar up to 34 g/L enhance the generation of 5-ALA, remarkably. Specifically, in the current study, the highest amount of 5-ALA to date (37.44 mM) was achieved in 34 g/L sugar containing culture. To sum up, a molasses derived sugar concentration of 34 g/L is the best for the highest bacterial growth and 5-ALA generation. In addition to these, considerable amounts of hydrogen were also achieved at the same time thereby increasing the overall efficiency of the bioprocess.

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