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Production of biological hydrogen and bacterial carotenoids from sugar beet molasses with *Rhodobacter sphaeroides* in a biorefinery concept

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Abstract: In this study, the goal was to produce biohydrogen and bacterial carotenoids with *Rhodobacter sphaeroides* O.U.001, a purple non-sulfur photosynthetic bacterium, utilizing sugar beet molasses in the context of biorefinery. First, media with different sugar concentrations (10 g/L, 20 g/L, 30 g/L, 40 g/L, 50 g/L) were prepared for bacterial growth. Then, hydrogen production was carried out using these media in anaerobic conditions in 100 ml bioreactors. After hydrogen gas was collected from the bioreactors, carotenoid extraction was performed from the remaining bacteria. As a result of the analyzes, it was found that the amount of biohydrogen and the amount of bacterial carotenoids obtained were inversely proportional to the increased sugar concentrations. The maximum hydrogen formation was detected in the medium containing 10 g/L of sugar (19.18 mL). According to the results of gas chromatography analysis, the quantity of hydrogen in the total gas was found to be around 23.6%. The highest yield of carotenoids was again obtained from bacteria reproduced in a medium containing 10 g/L of sugar (3.12 mg/g, carotenoid/dry biomass). As a conclusion, this study provides an example for the successful realization of two high value-added products within a biorefinery approach by using molasses obtained at an affordable cost.

Keywords: Carotenoid, Hydrogen, Molasses, *Rhodobacter sphaeroides*

Biyorafineri konseptiyle şeker pancarı pekmezinden Rhodobacter sphaeroides ile biyolojik hidrojen ve bakteriyel karotenoid üretimi

Özet: Bu çalışmada biyorafineri konsepti ile şeker pancarı melasından mor kükürtsüz fotosentetik bir bakteri olan *Rhodobacter sphaeroides* O.U.001 ile biyohidrojen ve bakteriyel karotenoidin üretilmesi amaçlanmıştır. İlk önce, bakteri büyütmek için farklı şeker konsantrasyonlarında (10 g/L, 20 g/L, 30 g/L, 40 g/L, 50 g/L) besiyerleri hazırlanmıştır. Daha sonra, hidrojen üretimi bu besiyerlerini kullanarak 100 mL'lik biyoreaktörlerde anaerobik koşullarda çalışılmıştır. Biyoreaktörden hidrojen gazı toplandıktan sonra kalan bakterilerden karotenoid ekstraksiyonu gerçekleştirilmiştir. Analizler sonucunda, artan şeker konsantrasyonu ile elde edilen biyohidrojen miktarı ve bakteriyel karotenoid miktarının ters orantılı olduğu bulunmuştur. En yüksek hidrojen üretimine 10 g/L şeker içeren besiyerinde görülmüştür (19,18 mL). Gaz kromatografisi analiz sonuçlarına göre toplam gaz içerisinde hidrojen miktarı %23,6 civarında tespit edilmiştir. En yüksek karotenoid verimi yine 10 g/L şeker içeren besiyerinde çoğaltılan bakterilerden elde edilmiştir (3,12 mg/g, karotenoid/kuru biyokütle). Sonuç olarak, bu çalışma uygun maliyetle elde edilen melasın kullanılmasıyla yüksek katma değerli iki ürünün biyorafineri yaklaşımı ile başarıyla gerçekleştirilmesi adına bir örnek sunmaktadır.

Anahtar Kelimeler: Karotenoid, Hidrojen, Melas, *Rhodobacter sphaeroides*

1. Introduction

The vast majority of the world's energy needs are provided from non-renewable energy sources such as fossil-based energy sources. But the use of these energy sources seriously harms nature. The global population growth also increases the need for energy. For this reason, it will become very important from a strategic point of view for countries to develop their own alternative energy sources. Examples to alternative renewable energy sources are solar energy, wind energy, wave energy, hydrogen energy, hydro power and geothermal energy. Hydrogen is a renewable and clean energy source among the energy sources of the future because it is seen as one of the alternative energy sources of

the future (Sagir et al. 2018). Hydrogen can be used as a fuel, and the combustion reaction of hydrogen is called clean combustion. In the clean combustion reaction, hydrogen reacts with oxygen and water is released and it does not harm the nature in any way. Furthermore, even though hydrogen is the lightest element in the universe, it was said to have 2.75 times more energy content (123 kJ/g) when compared to the hydrocarbon-based sources (Dursun and Gülşen 2019). Hence, it is recognized as the energy carrier of the future.

Today, hydrogen is usually produced by thermochemical means. However, biological hydrogen production is also possible through dark fermentation and photobiologically with the help of various microorganisms using various waste streams like sugar beet molasses (Kars and Alparslan 2020) and waste black cumin (Dursun and Gülşen, 2021). And, since biological hydrogen production takes place at lower temperatures and pressures, it requires less energy input than thermochemical methods and production is more advantageous in terms of side products. Among others, photobiological hydrogen production can be carried out by microorganisms in a photoautotrophic or photoheterotrophic way. Various purple non-sulfur bacteria were used in photoheterotrophic hydrogen production (Özsoy Demiriz et al. 2019; Kars and Gündüz, 2010). *Rhodobacter sphaeroides* (*R. sphaeroides*), a purple non-sulfur (PNS) photosynthetic bacteria, can produce hydrogen in photosynthetic conditions (under light and under anaerobic conditions) using various carbon sources as substrate (Kars and Gündüz 2010). The enzyme responsible for the production of hydrogen is nitrogenase, and this enzyme catalyzes the production of hydrogen using ATP during the conversion of nitrogen to ammonia. So, biological hydrogen production occurs as a result of an enzyme driven reaction and it is affected from physiological conditions such as vitamins and minerals (Kars and Alparslan 2020). It is also possible to produce high value-added products such as bacterial carotenoids, coenzyme Q10, vitamin B12, 5-ALA by *R. sphaeroides*. Although hydrogen production can be done both thermochemically and biologically, the amount of hydrogen obtained from these methods has not yet reached a commercial scale. For this reason, in recent years biorefinery approach has been applied to increase the efficiency of hydrogen production processes. In this approach, in addition to an energy source, the production of some marketable products (carotenoids, and valuable chemicals such as polymer) were also aimed and this route was defined as the process of biorefining (Cherubini and Jungmeier 2010).

Carotenoids are fat-soluble, antioxidant pigments found in photosynthetic organisms (such as bacteria, plants and algae (Liu et al. 2015). The task of carotenoids is to absorb light for use in photosynthesis and also to protect chlorophylls from the harmful effect of light. Studies have shown that carotenoids prevent various types of cancer, cardiovascular diseases, and aging-related ailments with their antioxidant activities and increase antibody production (Young and Lowe 2001; Kiokias and Oreopoulou 2006; Liu et al. 2015). Additionally, previous studies have reported that a carotenoid called capsanthin obtained from pepper can be used to prevent multidrug resistance in cancer cells (Motohashi et al. 2003). Therefore, these pigments are very promising to be used for medical purposes.

It is very important that the substrate to be used in the biorefinery concept should be sustainable and renewable. For this reason, sustainable, affordable and easily obtainable sugar beet molasses was used as a substrate in this study. Then, several sets of media with varying sugar beet molasses concentrations were formulated to disclose the optimal molasses concentrations for concurrent production of biohydrogen and bacterial carotenoid with *R. sphaeroides* in a cost-effective manner within the context of the biorefinery concept. Sugar beet molasses contains about 50% sucrose and therefore can be a rich source of nutrients for bacteria. Thus, optimal conditions for the production of hydrogen and carotenoids will be determined and a significant contribution will be made to the development of a cost-effective bioprocess within a frame of biorefinery approach.

2. Materials and Method

2.1. General cultivation circumstances

In this study, *R. sphaeroides* was used in the production of biological hydrogen and bacterial carotenoids. Bacteria can use various carbon sources as substrates. In the study, sugar beet molasses (Konya Şeker, Turkey) was used as a carbon source and L-glutamate (15 mM/2 mM) was used as a nitrogen source. The sugar concentration in the medium was increased gradually (10 g/L, 20 g/L, 30 g/L, 40 g/L, 50 g/L) from 10g/l to 50g/L. In addition, trace element, iron sulfate and vitamin solutions were put into the cultures as documented earlier (Kars and Ceylan 2019). According to the previous findings, the amount of Na was found to be very high after elemental investigation of molasses by ICP-MS (Kars and Alparslan 2013). For this reason, extra NaCl was not added to the medium. KH_2PO4 (0.5 g/L), $MgSO_4.7H_2O$ (0.2 g/L) and $CaCl_2.2H_2O$ (0.025 g/L) were added to the medium. 50 µL of iron solution (13.2 mM $FeSO_4$), 1 mL of molybdenum containing trace element solution (0.83 mM $Na_2MoO_4.2H_2O$) and 2.5 µL of vitamin solution (Niacin 500 mg/L, Biotin 15 mg/L and Thiamine 500 mg/L) were added to the prepared media. The initial pH of the media was fixed to 6.9.

After adding the components, the media were sterilized by autoclaving at 121°C for 15 minutes. Since the structure of the vitamin will deteriorate due to temperature, the vitamin solution was added to the media after autoclaving. The gas phases of the bioreactors were passed through argon gas for 3 minutes in order to form anaerobic environs. 100-watt tungsten filament incandescent bulbs were used as the light source for the photosynthetic condition. The cultures were incubated at approximately 29 °C for growth.

2.2. Biomass and pretreatment

Molasses is a by-product that occurs as a result of the refining of sugar beet. Sugar beet molasses possesses a

sucrose (w/w) content of about 50% (Sagir et al. 2018). In addition to sucrose, molasses is rich in many types of carbons like organic acids (Kars and Alparslan 2013). This feature makes sugar beet molasses a prosperous nutrient for many bacterial cells. Sugar beet molasses obtained from Konya Sugar Factory was utilized as a substrate for both biohydrogen and bacterial carotenoid production. However, molasses was not used directly due to its dark color and high sugar content. Before preparing molasses and different sugar concentrations media, they were centrifuged for 20 minutes at 10.000 rpm due to the particles in their contents. After centrifugation, the solutions were filtered via 0.22 µm filters and diluted with water and media were prepared in 100 ml bioreactors.

2.3. Hydrogen production with R. sphaeroides using molasses

Initially, hydrogen productions were carried out in the study. Hydrogen production setup was established after the cultures (5 different media with different sugar contents) were incubated for about 3 days. Hydrogen production setup was based on the principle of collecting the hydrogen gas produced by *R. sphaeroides* in bioreactors in glass tubes with 0.5 cm markings. Changes in the water levels in tubes were observed at 24-hour intervals. After collecting hydrogen gas in tubes, gas chromatography (GC) analysis was performed. An Rt-Msieve 5A column and a thermal conductivity detector were used to examine the constituents of the accumulated gas using GC (Shimadzu GC-2010 Plus, Japan). The detector and column were heated to 250 and 50 degrees Celsius, respectively. Argon gas ran inside the column with a flow rate of 4 mL per minute to carry the gas samples.

2.4. Carotenoid production from R. sphaeroides

After the completion of hydrogen production, bacterial cultures were centrifuged for 20 minutes at 10 000 rpm to obtain the cells. Then, the upper phase was discarded and the precipitate was dissolved in 2 mL of distilled water to wash each cell pellet. Cell suspension was re-centrifuged at 10 000 rpm for 20 minutes and the washing process was repeated. After final centrifugation, the remaining liquid was thrown away and the entire precipitate was transferred to a beaker and dried in an oven at 65°C for one night. Bacterial cells were lysed by adding 4 mL (3 M) HCl onto the dry bacterial mass obtained as a result of drying. The cell lysate was mixed at 28°C, 100 rpm for 30 minutes. The solution was spinned at 10 000 rpm for 20 minutes. Extraction of the carotenoid was done by adding 5 mL of acetone onto the precipitate and vortexing briefly. To this extract, another 35 mL of acetone was added to reach up to 40 mL of acetone. At 30°C, 40 minutes of extraction of the carotenoids at 150 rpm was performed, and then whole extract was spinned at 10 000 rpm for 20 minutes. The carotenoids were dissolved in the supernatant which was then was transferred to a clean 50 ml tube. The optical density of the carotenoid solution was measured at 480 nm and the amount of carotenoid was calculated using the Equation 1 (Kars et al. 2020; Gu et al. 2008).

$$Carotenoid\ yield\left(\frac{\mu g\ carotenoid}{g\ dry\ cell\ weight}\right) = \frac{A \times D \times V}{0{,}16 \times W} \qquad \text{(Equation 1)}$$

A: Absorbance (480 nm)

D: Dilution coefficient

V: Volume of solvent (acetone)

W: Mass of dry bacteria (g)

3. Results

3.1. Hydrogen production with R. sphaeroides using molasses

Biological hydrogen production was tested at five different sugar concentrations. The tubes where hydrogen gas was collected were observed at 24-hour intervals and graphs were created using these data. During the study, the experiments were repeated two times and error bars were added to the graph. The results of the experiments were figured out in a single graph. The gas chromatography (GC) analysis was carried out to reveal the total amount of pure hydrogen collected in these tubes. According to the results of GC analysis, the amount of hydrogen in the total gas was assigned to be circa 23.6%. Taking into account the results of hydrogen production (Figure 1), it was found that hydrogen production decreases as the concentration of sugar (molasses amount) increases. The highest hydrogen production occurred in the medium containing 10 g/L sugar (19.18 mL gas accumulation). It was noticed that increasing sugar concentration did not lead to an increase in hydrogen production and hydrogen production was the highest at the lowest sugar concentration. In order to raise the sugar concentration in the medium, the amount of molasses was also augmented. And, it was realized that increasing the amount of molasses darkened the color of the medium and increased its density. This, in turn, can reduce the penetration of light through the medium and prevent bacterial cells from reaching the light they need. In the end, it was anticipated that this adversely affected the amount of hydrogen produced.

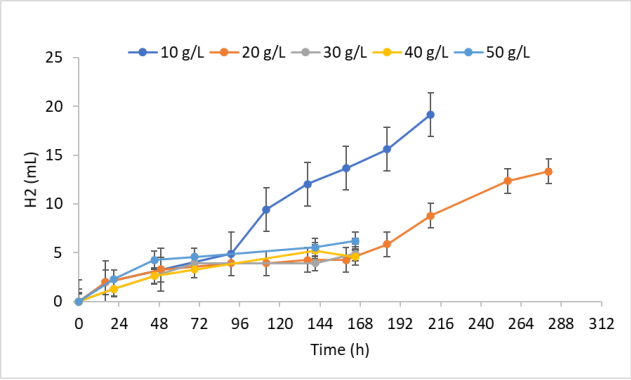

Figure 1. Hydrogen production at different molasses concentrations.

Molasses has long been used as substrate by our group and others and studies have been done for years to find the most efficient hydrogen production process. Examples of these

studies were gathered and shown in Table 1. It was observed that different hydrogen production yields were obtained using different bacterial strains in various hydrogen production processes. We basically thought that these variations arise from different physiological parameters (bacterial strains, media composition and processs type) implemented in these processes. The amount of hydrogen obtained from a concentration of 10 g/L (0.27 mol H_2/mol sucrose) was also included in the table. As can be observed from the table, the greatest amount of hydrogen production was achieved by successive dark and photo fermentation with the mutant progeny of *R. capsulatus* (13.7 mol H_2/mol sucrose) (Özgür et al. 2010). Among photofermentative hydrogen generation processes, the greatest amount was attained with *R. sphaeroides* O.U.001 (0.5 mol H_2/mol sucrose) (Kars and Alparslan 2013).

Table 1. Examples of biohydrogen production using molasses as substrate.

Microorganism	Process	Yield (mol H_2/mol sucrose)	Reference
Rhodobacter sphaeroides O.U.001	Photofermantative	0.27	This study
Rhodobacter sphaeroides O.U.001	Photofermantative	0.5	(Kars and Alparslan 2013)
Rhodobacter capsulatus JP91	Photofermantative	10.5	(Keskin and Hallenbeck 2012)
Mixed anaerobic culture	Dark fermentation	2.8	(Chang et al. 2011)
Caldicellulosiruptor saccharolyticus and *Rhodobacter capsulatus hup*-YO3	Dark and Photofermantation	13.7	(Özgür et al. 2010)

3.2. Carotenoid production from R. sphaeroides

In this study, it was aimed to produce bacterial carotenoids besides hydrogen generation in a biorefinery frame. The source of carotenoids is *R. sphaeroides*. The lack of immunity and pathogenicity of *R. sphaeroides* makes it a valuable source of carotenoids. There are two types of carotenoids in the *R. sphaeroides*; spheroidene (SE) and spheroidenone (SO) (Kars et al. 2020). These carotenoids have the potential to be used as antioxidants by effectively neutralizing the surrounding singlet oxygen species. There are two possible ways to increase the current bacterial carotenoid production. These are either the application of recombinant DNA technology or the optimization of culture conditions. Genetic methods for *R. sphaeroides* are well established, but gene manipulations require significant effort and there are many targets to improve. Therefore, in this study, five distinct sugar concentrations (molasses amount) were tested to reveal the best substrate concentration for the highest carotenoid production. Based on previous experience and literature findings, it is known that sugar beet molasses is a rich medium that supports bacterial growth very well (Kars and Alparslan 2013).

In the current work, sugar beet molasses harvested in Konya was used as the substrate for the cultivation of bacteria for the production of carotenoids. In this way, locally produced molasses was utilized for the production of value-added products contributing to the circular economy. *R. sphaeroides* was cultured in media with various sugar concentrations (10 g/L, 20 g/L, 30 g/L, 40 g/L and 50 g/L). The bioprocess was run in intermittent mode using a 100 mL bioreactor. After hydrogen production was ceased, the remaining cells were collected and bacteria were dried to obtain cell dry weights (Table 2). Carotenoids were extracted from dry cells using acetone as a solvent. Optical densities were measured after 8 times dilution at 480 nm. The maximum carotenoid yield was calculated as 3.12 mg/g (carotenoid/dry biomass), taking into account the dilution factor and solvent volume (40 mL). Similar to the hydrogen generation results, it was noted that the amount of carotenoids obtained decreased as the sugar ratio (molasses amount) increased.

Table 2. The amounts of carotenoids obtained from cultures after hydrogen production

Sugar (g/L)	OD (480 nm)	Dry weight (g)	Carotenoid yield (µg/g)
10	5,0296	0,4029	3120,87
20	6,1904	0,5927	2611,10
30	2,4392	0,5702	1069,45
40	0,6584	0,4132	398,35
50	0,3184	0,369	215,72

4. Discussion

Sugar beet molasses used in the preparation of growth media is a waste product of the sugar factory and contains organic acids, phenolic substances and ammonium in addition to sucrose (Kars and Alparslan 2013). The organic and inorganic composition of sugar beet molasses is very diverse and rich; therefore, it supports the growth of bacteria very well. In the present study, as a common trend, as the amount of molasses in the media increased, the amounts of hydrogen and carotenoid decreased. There are many factors which might have led to this result. For instance, it is a well-known fact that phenolic substances and ammonium negatively affect hydrogen production (Kars and Alparslan 2013). This is basically due to the fact that ammonia reversibly inactivates the nitrogenase enzyme that is the enzyme functioning in hydrogen evolution. Moreover, phenolics substances also thought to poison the hydrogen production metabolism. Taking into account the previous findings, the results (decrease in hydrogen production as a result of elevated levels of molasses) obtained here is consistent with previous studies. Another common factor is the light whose sufficient amounts are needed for an efficient hydrogen generation (Uyar et al. 2007; Kars and Alparslan 2013). Because of the darkish color and density of molasses, the delivery of light through the medium and reaching the cells is blocked. As the amount of molasses

increases, the culture darkens and the light penetration efficiency decreases. This causes the cells to absorb less light and therefore less hydrogen production occurs.

Like hydrogen generation results, the biggest carotenoid yield was achieved at the lowest sugar concentration in the study. This shows that bacteria proliferate best at this concentration. So, considering both hydrogen and carotenoid production results, it seems that the most suitable sugar (sugar beet molasses) concentration for *R. sphaeroides* is 10 g/L. The amount of hydrogen production by using molasses might be less when compared to minimal media, but molasses may be preferred over synthetic media in biotechnological processes because it can be obtained at much cheaper cost compared to synthetic carbon sources. The processes carried out using molasses are described as sustainable and cost-effective. In the current work, considerable quantities of both hydrogen and carotenoids were obtained in a single process. Thus, an exemplary study was implemented for the purpose of developing a sustainable and cost-effective processes within a biorefinery concept.

Considering previous findings and present results, it was noticed that the physical features (viscosity, color etc.) of the sugar beet molasses used so far differ from each other. It is also suspected that the composition of molasses used in different times may also change. Therefore, the differences in physical and chemical properties of molasses may result in deviations in the results. Moreover, the use of inorganic iron sources like $FeSO_4$ instead of organic iron sources like ferric citrate probably led to variations in both hydrogen and carotenoid productions. These experiences demonstrated that design and composition of growth media strongly influence the hydrogen production and bacterial reproduction. So, it is of great importance to find out best medium formulations for better yields.

5. Conclusion

R. sphaeroides is a versatile microorganism able to grow in different growth modes and capable of producing many marketable products such as biopolymers, vitamins and carotenoids. It proliferates very well in media prepared by using sugar beet molasses which is an affordable, renewable and sustainable substrate. Molasses was shown to be a very efficient substrate in the production of biohydrogen as a fuel and carotenoid as a marketable product within a biorefinery concept. *R. sphaeroides'* being a non-immunogenic and non-pathogenic bacterium makes it a unique cell factory for the production of many marketable products in addition to biohydrogen as shown in the present work.

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Authors' contributions

Planning, funding applications, experimental work and manuscript preparation were done by GK. KD contributed to experimental work and manuscript preparation. BNB contributed to the experimental work.

Conflict of interest disclosure

There is no conflict of interest.

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