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Research paper

# The distillers grains with solubles as a perspective substrate for obtaining biomass and producing bio-hydrogen by *Rhodobacter sphaeroides*

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ABSTRACT

The photosynthetic purple non-sulfur bacterium *Rhodobacter sphaeroides* MDC6521, isolated from Armenian mineral spring, and distillers grains with solubles (DGS) (by-product of bio-ethanol fermentation) were applied for obtaining biomass and producing bio-hydrogen ($H_2$) upon illumination. During growth on diluted DGS media $H_2$ production was started at 24 h growth, whereas $H_2$ photoproduction by *R. sphaeroides* cells, grown on Ormerod medium, was detected after 48 h. Moreover, the *R. sphaeroides* produced considerably more $H_2$ during DGS photo-fermentation: the $H_2$ yields from 2- fold and 5-fold diluted media were ~4.6–5.5-folds higher in comparison with control. $H_2$ yield has decreased at higher dilution. The growth yields of *R. sphaeroides* cells, grown in the 2–5-folds diluted DGS media, were considerably higher than those of control cells, grown in Ormerod medium. The results can provide with new cheaper and more effective source of biomass and bio-hydrogen and as well as solve the problem of ethanol by-product utilization.



## 1. Introduction

Molecular hydrogen ($H_2$) is an energy carrier with high energy content (−122 kJ $g^{-1}$). Among the developing alternative energy resources, $H_2$ is recognized as the most promising alternative to fossil fuels and is expected to play a major role in future energy supply as it is clean, renewable, and efficient [1,2]. Biological $H_2$ production with microorganisms is considered as one of the perspective field of biotechnology, which suggests the generation of renewable and ecologically clean energy from a variety of substrates and organic wastes [3–7].

Photosynthetic purple non-sulfur bacteria such as *Rhodobacter* species are perspective candidates for the $H_2$ production due to their high substrates conversion rate [8–10]. Under anaerobic conditions *Rhodobacter sphaeroides*, isolated from Armenian mineral springs, has been shown to perform a photo-fermentation of various carbon- and nitrogen-containing organic substrates with $H_2$ production [11–14].

The selection of the source for $H_2$ becomes a serious problem, because it strongly affects the $H_2$ yield by photosynthetic bacteria. It is important to choose such sources, which can be effectively utilized by bacteria and would be less expensive providing the enhanced $H_2$ yield. Various organic substrates, generally used in laboratory for research on $H_2$ production, permit a fast and large amount of $H_2$ production [8–10]. But one of the key problems in $H_2$ production technology is the high cost of various organic carbon and nitrogen sources. The use of different organic wastes and new substrates, which are cheaper and more effective, for $H_2$ production can provide inexpensive energy generation and simultaneous waste utilization.

Practically unlimited source of natural nutrients are the food industry wastes [4–7,15–19]. These sources can include cereal distillers grains (DG) as a by-product of ethanol industry. This source is widely available and very cheap. During ethanol fermentation the conversion yield of one tone wheat or corn to wet DG is ~460–480 kg.

The detailed principles of DG generation are reported in the literature [20–22]. The starch in cereals is transformed into ethanol. The rest of the cereals components such as proteins, lipids,

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minerals, and other remain unchanged chemically. These residual stocks are known as distillers dried grains with solubles (DDGS). The process includes various steps such as fermentation, distillation, by-product recovery and other [21,22]. During by-product recovery the nonvolatile components (obtained during distillation process) are centrifuged to produce a liquid fraction (thin stillage) and a solid fraction (distillers wet grains, DWG). The thin stillage is concentrated into condensed distiller solubles (CDS) via evaporation, and then CDS are mixed with DWG to become distillers wet grains with solubles (DWGS) and then dried into DDGS [21].

Distillers grains with solubles (DGS) contain carbohydrates and essential fatty acids as well as trace elements such as iron, magnesium, manganese, and others, which are necessary for growth of purple bacteria [18–22]. In DGS were also present various amino acids with a predominance of glutamate, including 8 essential amino acids: arginine, lysine, valine, histidine, threonine, phenylalanine, leucine, isoleucine as shown [20–22].

Thus, DGS are a valuable source of natural biological compounds and can be used for $H_2$ production. However, this aspect of DGS has not been considered yet. Some authors were shown that DGS proved to be a suitable feedstock for bioenergy production [16,17]. Given the possibility of change in the ethanol fermentation, it must be assumed that as a result of deep chemical research can justify the use of DGS as a new source of bio-hydrogen. The solution of this problem will provide a new cheap source of $H_2$. Increasing energy requirement needs a new substrates and methods of energy generation. Disposal of various by-products makes the $H_2$ production a new perspective approach towards satisfaction of energy demand.

DGS are a very cheap substrate: their cost in Armenia is 1.5 cent per liter. The use of DGS for $H_2$ production can be better than the sole carbon and nitrogen sources. Application of DGS can alter the modes of bacterial metabolic pathways and improve the $H_2$ production.

In this study the photosynthetic purple bacterium *R. sphaeroides*, isolated from Armenian mineral spring, was employed to produce $H_2$ during the photo-fermentation of new substrates such as DGS. It is very important to choose such source, which can be effectively utilized by *R. sphaeroides* for obtaining biomass and provide the enhanced $H_2$ yield technology.

## 2. Materials and methods

### 2.1. Bacterial strains, cultivation conditions and determination of growth

Phototrophic bacterium *R. sphaeroides* strain MDC6521 (Microbial Depository Center, National Academy of Sciences of Armenia, Yerevan, Armenia, WDCM803), isolated from Arzni mineral spring in Armenian mountains, was cultivated in glass vessels of 150 ml capacities with plastic press caps in anaerobic conditions on Ormerod medium with succinate as carbon source upon illumination (~36 W $m^{-2}$) as described previously [12–14]. The growth of batch culture was monitored by changes in optical density (OD) by Spectro UV–Vis Auto spectrophotometer (Labomed, USA), and by determining bacterial biomass dry weight (DW), which was correlated with OD at 660 nm: DW (g $L^{-1}$) = $OD_{660} \times 0.50$. Specific growth rate was calculated as ln2/doubling time of OD within an interval, where the logarithm of culture OD increased with time in a linear manner (logarithmic growth phase), and it was expressed as $h^{-1}$ [12,13].

DGS from common (bread) wheat *Triticum aestivum* L. were obtained from "Alex Grig" Alcohol Plant Co. LTD (Yerevan, Armenia). DGS are a yellow-brown color polydisperse system with yeast scent, in which compounds are dissolved and suspended (with visible milled caryopsis). DGS were obtained during ethanol fermentation, which was performed by yeast *Saccharomyces cerevisiae*. The untreated DGS were filtered through cotton wool, and then paper filter, next filtrate was sterilized at 120 °C by autoclaving for 20 min, and then used during experiments. As pH of DGS was ~3.5, before autoclaving the pH of the DGS was adjusted to 7.0 ± 0.1 by means of $10^2$ mol $m^{-3}$ NaOH. DGS were diluted at 2-fold, 5-fold, 10-fold, and 25-fold using distilled water. Diluted DGS were used without any medium supplements, because DGS contained various carbon-containing compounds. DW of undiluted DGS was 20 g $L^{-1}$.

### 2.2. Determination of medium pH, redox potential ($E_h$) and $H_2$ yield

The initial pH of the culture growth medium was measured at time intervals 0 h–96 h by a pH-potentiometer (HI 122-02, HANNA Instruments, Portugal) with pH electrode (HJ1131B) as described [13,14].

The medium $E_h$ was determined during *R. sphaeroides* anaerobic growth by potentiometric method using a pair of redox electrodes: platinum (Pt) and titanium-silicate (Ti–Si) electrodes as described [11–14]. The kinetics of $E_h$ measured simultaneously by both electrodes during bacterial growth provides information about general redox processes and also $H_2$ production [14,23–25]. The electrochemical approach, although indirect, is a useful means of determining $H_2$ production and gives accurate and reproducible data [24,26].

The $H_2$ yield was calculated by the decrease of $E_h$ to low negative values during bacterial growth and expressed in mmol $g^{-1}$ DW as described [12–14]. This determination of $H_2$ is close to the method with Clark-type electrode used by the other authors [27]. The correlation between $E_h$ and $H_2$ production was shown; the supplementation of $H_2$ didn't affect medium pH [14,27]. $H_2$ production in gas phase was also confirmed by the chemical method based on the bleaching of $KMnO_4$ solution in $H_2SO_4$ with $H_2$ as described [11,12,28]: $2KMnO_4 + 3H_2SO_4 + H_2 \rightarrow K_2SO_4 + 2MnSO_4 + 4H_2O$.

The pH and redox electrodes were immersed into the glass vessels, and the electrodes readings were registered each 24 h as shown in Figures.

### 2.3. Reagents, data processing and others

Various reagents of analytical grade were used in this study. Each experiment was repeated three times to determine deviation, which is presented as error bars on the figures. Standard errors were calculated using Microsoft Excel 2013. The standard errors and Student criteria (p) were employed to validate the difference in average data between various series of experiments as described previously [13,14].

## 3. Results and discussion

### 3.1. $H_2$ yield in R. sphaeroides during DGS photo-fermentation

The bio-hydrogen production ability of *R. sphaeroides* during DGS photo-fermentation was tested. $H_2$ production was measured till 96 h growth (Table 1). The results showed that $H_2$ production by *R. sphaeroides* control cells, grown on Ormerod medium, was detected at 48 h growth.

No $H_2$ production was observed, when undiluted DGS were used (not shown). This is possible due to high organic compounds content in DGS [20–22]. It is known, that high content of sugars has inhibitory effect on bacteria growth and generation of $H_2$ [29]. Thus, dilution of DGS is necessary to optimize the organic compounds concentration for the growth and $H_2$ production by purple bacteria.

The results obtained have shown that in diluted 2-fold, 5-fold, and 10-fold DGS media $H_2$ production was started at 24 h and

**Table 1**
The $H_2$ yield of *R. sphaeroides* MDC6521 during DGS photo-fermentation.

| | $H_2$ yield,[a] mmol $g^{-1}$ DW | | | |
|---|---|---|---|---|
| | 24 h | 48 h | 72 h | 96 h |
| Control (Ormerod medium) | – | 1.57 | 3.86 | 1.72 |
| 2-fold diluted DGS | 9.71 | 7.18 | 4.74 | 1.03 |
| 5-fold diluted DGS | 9.43 | 8.70 | 3.20 | 1.14 |
| 10-fold diluted DGS | 2.40 | 3.11 | 1.48 | – |
| 25-fold diluted DGS | – | – | – | – |

[a] The mean values calculated by decrease in $E_h$ (see Materials and methods) are represented. Minus (–) sign represented absence of $H_2$ production.

continued during the growth up to 96 h (see Table 1). The results showed that the $H_2$ yield in *R. sphaeroides* from DGS was higher than that of control. The highest $H_2$ yields were obtained for *R. sphaeroides* in the 2-fold and 5-fold diluted media after 24 h growth (see Table 1). The $H_2$ yields in 2-fold and 5-fold diluted media after 48 h anaerobic growth were ~4.6-fold and 5.5-fold higher in comparison with the control, grown on Ormerod medium (see Table 1). Utilization of available substrates makes it possible. It can be connected with formation of reductive power and ATP synthesis as suggested [13,14]. DGS contain significant amount of amino acids, which are the nitrogen and carbon sources for $H_2$ production, and can affect the nitrogenase activity, the key $H_2$ producing enzyme of purple bacteria, involving in $H_2$ production process [2,7,8]. $H_2$ yield was decreased at higher dilution (10-fold and 25-fold) (see Table 1).

Thus, diluted DGS have a pronounced effect on $H_2$ yield in *R. sphaeroides* during the anaerobic growth upon illumination.

### 3.2. *R. sphaeroides* growth properties and medium pH changes during DGS photo-fermentation

The growth properties and medium pH changes were monitored during *R. sphaeroides* cultivation on various diluted DGS media. As for $H_2$ yield, dilution of DGS resulted in growth properties changes. *R. sphaeroides* was unable to grow on undiluted medium (not shown), which is probably connected with the high organic compounds concentration in DG.

During the anaerobic growth of *R. sphaeroides* dry weight of the bacterial suspension was increased; this testified growth of the culture (Fig. 1). The growth yields of cells, grown in the 2–5-folds diluted DGS media, were higher than those in Ormerod medium (see Fig. 1). *R. sphaeroides* MDC6521 was unable to grow well on more diluted DGS medium (see Fig. 1).

However, specific growth rate of *R. sphaeroides* has decreased during growth on DGS media in dilution-dependent manner, in compare to control (Fig. 2). 5–25-folds dilutions gave ~1.3–1.4-folds suppressed growth rate.

The results have shown that DGS in optimal dilution (2–5-folds) can be used as effective substrates, because they contain various amino acids with a predominance of glutamate, which is served the best nitrogen source for $H_2$ production by photosynthetic bacteria [20–22].

Medium pH is an essential parameter, which affects the photo-fermentative $H_2$ production by bacteria, because it can affect the activity of enzymes such nitrogenase and hydrogenase, involved in $H_2$ production, as well as the metabolic pathways [3,26,30]. During the anaerobic growth of *R. sphaeroides* MDC6521 control cells up to 72 h pH of medium increased from 7.0 ± 0.1 (initial pH) to ~8.75 (Fig. 3A). Thus, pH of growth medium was changed during growth on DGS media (see Fig. 3A). During growth (72 h) on 2–10-folds diluted DGS media pH rose from 7.0 ± 0.1 to ~9.0–9.5, then decreased 8.8–9.0 (see Fig. 3A).

ΔpH (the difference between initial pH of growth medium and value of pH after 24–96 h bacterial growth) during growth on DGS media was higher in comparison with the control (Fig. 3B). Such variation of pH can be connected with the uptake of available substrates and formation of some products of photo-fermentation such as bio-hydrogen [12,30].

### 3.3. Redox potential changes during *R. sphaeroides* growth on DGS media

Bacterial growth medium $E_h$, like pH, is considered as another important factor of the environment, which can be defined as biological system ability to reduce or oxidize various compounds [24,25]. In our previous studies we have shown the correlation between the increase of pH and the decrease of $E_h$ by *R. sphaeroides* [12,14]. Such relationship indicates not only the formation of fermentation end-products but also the redox processes on the surface of bacterial membrane [24,25].

During growth (0 h–72 h) on Ormerod medium the $E_h$ of *R. sphaeroides* gradually decreased up to −580 mV ± 10 mV (Fig. 4).

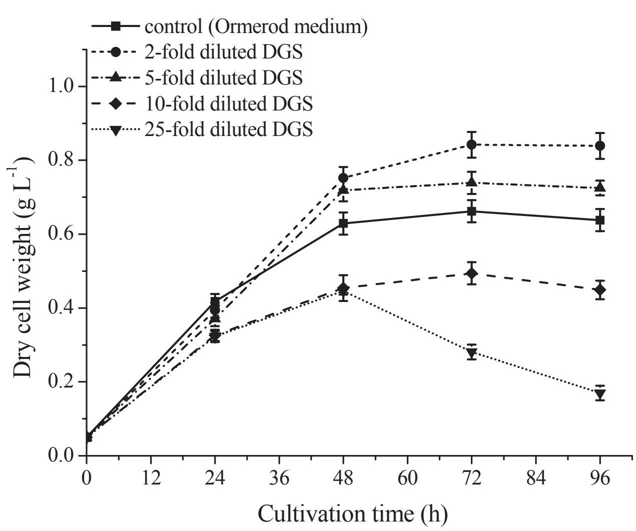


**Fig. 1.** *R. sphaeroides* MDC6521 growth in batch culture during DGS photo-fermentation.

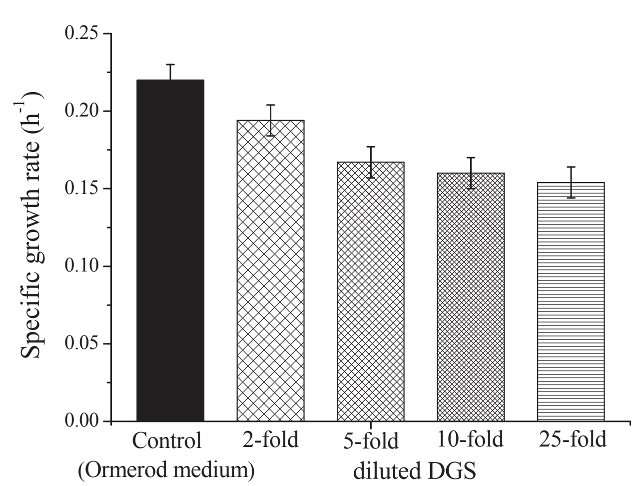


**Fig. 2.** Specific growth rates of *R. sphaeroides* MDC6521 in batch culture during DGS photo-fermentation.

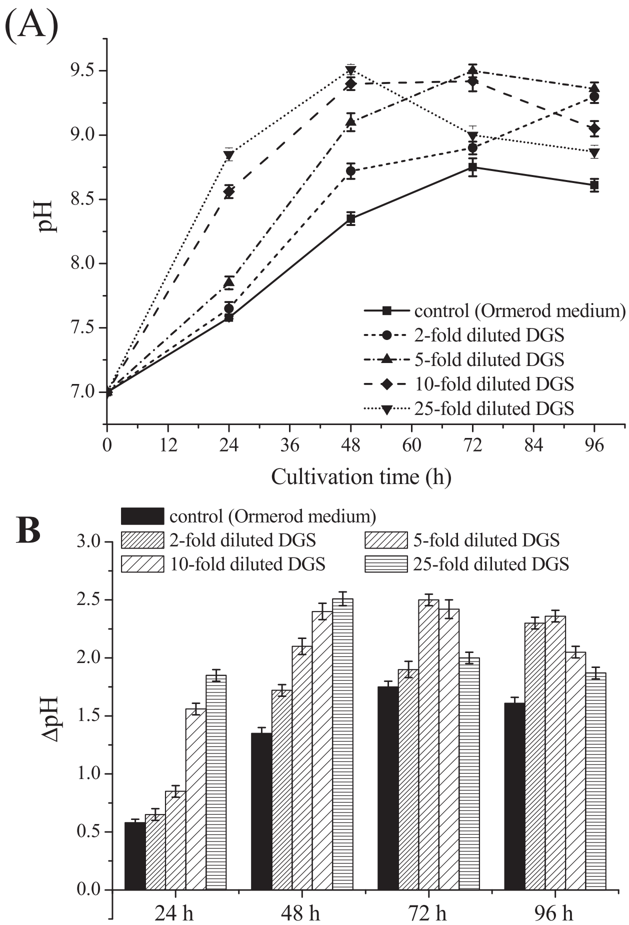


**Fig. 3.** Change of *R. sphaeroides* strain MDC6521 medium pH (A) during DGS photo-fermentation. ΔpH (B) is the difference between initial pH and pH value after 24 h–96 h bacterial growth.

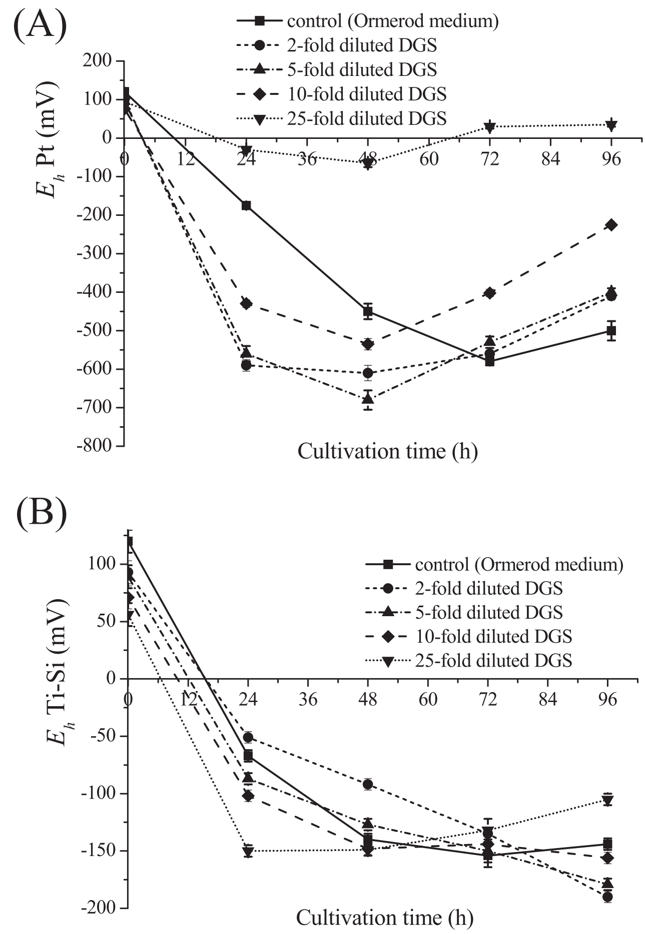


**Fig. 4.** Change of *R. sphaeroides* MDC6521 medium $E_h$, measured by Pt (A) and Ti–Si (B) electrodes, during DGS photo-fermentation.

This negative value of $E_h$ is connected with $H_2$ production, because for the $2H^+ + 2e^- \rightarrow H_2$ reaction $E_h$ equals to −414 mV [31,32].

The dilution of DGS affected the $E_h$ of *R. sphaeroides* suspension (see Fig. 4). $E_h$ of cells, grown on 2–5-folds diluted DGS media, measured by a Pt electrode, were gradually decreased during the growth (0 h–48 h) up to −610 mV to −680 mV, then increased up to −410 mV ± 10 mV (Fig. 4A). Such changes of $E_h$ can be connected with the production of reducing equivalents such as NADH or $FADH_2$, which can have a pronounced effect on the bacterial metabolism, because greater availability of NADH considerably alters the nature of end-products. $E_h$ of cells, grown on 10-fold diluted DGS medium, was decreased during the growth (0 h–48 h) up to −535 mV ± 15 mV, and then increased up to −225 mV ± 5 mV. But $E_h$ of *R. sphaeroides*, grown on 25-fold diluted DGS medium, was not changed much during the growth up to 96 h (see Fig. 4A). $E_h$ of *R. sphaeroides* control cells, measured by Ti–Si electrode, was gradually decreased up to −144 mV ± 5 mV, whereas $E_h$ of bacterium, grown on 2–10-folds diluted DGS media decreased up to −156 mV to −190 mV during the growth up to 96 h (Fig. 4B).

## 4. Conclusion

The present study has shown that DGS can serve as an effective source for *R. sphaeroides* growth and $H_2$ photoproduction. During growth on diluted distillers grains media $H_2$ production started at 24 h growth, whereas $H_2$ production by *R. sphaeroides* control cells, grown on Ormerod medium, was detected after 48 h. Moreover, the *R. sphaeroides* produced significantly more $H_2$ from DGS: 2-fold and 5-fold diluted media provided the ~4.6–5.5-folds higher $H_2$ yield in comparison with the control. DGS dilution and neutralization are necessary to adjust the organic acids concentration and the pH 7.0 for the optimal growth and $H_2$ production by *R. sphaeroides*.

The results have shown the possibility of using DGS (in optimal dilution) as an effective substrate for obtaining biomass and producing bio-hydrogen, because it contains various organic acids, amino acids with a predominance of glutamate, trace elements, and other compounds, which can be easily utilized by purple bacteria. This study can provide not only inexpensive energy generation but also solve the problem of by-product utilization.

## Conflict of interest

The authors declare no financial or commercial conflict of interest.

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