Novel approach of ethanol waste utilization: Biohydrogen production by mixed cultures of dark- and photo-fermentative bacteria using distillers grains

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Abstract
The combination of dark- and photo-fermentative bacteria is quite a new approach in hydrogen (H2) production biotechnology, which offers potential efficient production of H2 from renewable resources. This study is focused on the attempt to obtain high H2 yield by pure and mixed cultures of dark-fermentative (Escherichia coli) and photo-fermentative (Rhodobacter sphaeroides) bacteria using distillers grains (DG), the ethanol fermentation waste. During the growth in diluted DG media H2 production by pure and mixed cultures started at 24 h growth. Moreover, the mixed culture produced significantly more H2 from DG: 2-folds diluted media provided the ~1.5–3-folds higher H2 yield in comparison with pure culture. These effects were obtained for continuous H2 production at 72–96 h growth. DG dilution and neutralization to pH 7.0 were required. The results indicated that DG can be used as effective and valuable source of organic substances in H2 production by bacteria. This study can provide novel approach for an inexpensive energy generation, as well as to resolve the problem of waste utilization.

Introduction

Dark- or photo-fermentation are the main pathways for biological hydrogen (H2) production. H2 generation through dark- or photo-fermentation of organic compounds and wastes conversion is of great interest, thus acting as H2 production promising choice [1–3].

Constantly increasing demand for energy needs novel and cheaper substrates. Disposal of organic wastes, originating from food, sugar, wine and ethanol industry, makes a new promising approach of H2 production [1,4–6]. Distillers grains (DG) are industrial wastes generated during wine and ethanol fermentation. DG of wine fermentation has been used to produce H2 by mixed microflora [6]. However, undefined mixed cultures, heating (up to 95 °C) treatment and acidic pH
Escherichia coli pathways of higher H2 yield. The changes of the external pH and redox such as waste dilution and pH were optimized to reach a potential (Eh) during the bacterial anaerobic growth were measured by optical density (OD) changes OD at 600 nm for E. coli and at 660 nm for Rh. sphaeroides using Spectro UV—Vis Auto spectrophotometer (Labomed, USA). Specific growth rate was calculated as described [13,14].

Wet DG from common (bread) wheat Triticum aestivum L. was obtained from “Alex Grig” Alcohol Plant Co. LTD (Yerevan, Armenia). DG is a yellow-brown color polydisperse system with yeast scent, in which compounds are dissolved and suspended (with visible milled caropsis). DG was obtained during ethanol fermentation, which was performed by yeast Saccharomyces cerevisiae. In DG were found various sugars (glucose, xylose, arabinose and other), glycerol, fatty acids (linoleic, palmitic, oleic, and linolenic acids), different proteins (peptides) and 14 amino acids with a predominance of glutamate, including 8 essential amino acids: arginine, lysine, valine, histidine, threonine, phenylalanine, leucine, isoleucine, which can be used by bacteria as carbon and nitrogen source for H2 production [7–9]. The chemical composition of DG depends on the cereals fermentation conditions and the type of ethanol produced. The untreated DG was filtered through cotton wool, then paper filter, next sterilized by autoclaving at 120 °C for 20 min. As pH of DG were ~3.5, before autoclaving the pH of the waste was adjusted to 7.0 by means of 0.1 M NaOH. DG were diluted 2-, 5-, and 10-folds using distilled water. In this study diluted DG was used without any medium supplements, because DG contained various carbon-containing compounds.

**Determination of medium pH, E_h and H_2 yield**

The pH of the growth medium was determined by a pH-meter (HI 122-02, HANNA Instruments, Portugal) with selective pH electrode, as described [13,14].

The medium E_h was measured during bacterial pure and mixed cultures anaerobic growth using a pair of redox electrodes: platinum (Pt) and titanium-silicate (Ti–Si) electrodes, as described [13,14]. In spite of Ti–Si electrode, Pt one was sensitive to H2. Therefore, the changes of E_h and the differences between these electrode readings provided information about not only redox processes, but also H2 yield, which was determined by the decrease of E_h to low negative values during bacterial growth [13,14,22]. This determination of H2 is close to the method with Clark-type electrode employed by the other authors [23]. The correlation between E_h and H2 production was shown; the supplementation of H2 didn’t affect medium pH [13,14,23]. H2 production in gas phase was confirmed chemically by the method based on the bleaching of KMnO4 solution in H2SO4 with H2 as described [24].
Reagents, data processing and others

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

Results and discussion

Effects of DG dilution on H2 yield in E. coli and Rh. sphaeroides pure and mixed cultures

E. coli BW 25113 and Rh. sphaeroides MDC6521 were grown separately in the appropriate culture media with carbon sources – succinate and glucose. The H2 production ability of E. coli and Rh. sphaeroides pure cultures during DG dark- and photo-fermentation was determined. H2 production was detected during 96 h anaerobic growth. In diluted (2–10-folds) DG media E. coli produced H2 only during 24 h growth, and H2 production by Rh. sphaeroides was started at 24 h and continued till 96 h growth (Fig. 1a). It is interesting, that H2 production by Rh. sphaeroides control cells, grown in Ormerod medium, was detected at 48 h growth, whereas H2 production by E. coli cells, grown in peptone medium, was only observed in 24 h culture (Fig. 1a).

The H2 yield in E. coli during growth in 2–5-folds diluted DG media was ~2-fold higher in comparison with culture, grown in peptone medium with glucose; the H2 yield was decreased at 10-fold dilution (Fig. 1a). H2 production was not observed, when undiluted DG were used (not shown). This is possible due to high organic compounds content in DG of ethanol fermentation [7–9]. It is known, that high content of sugars has inhibitory effect on bacteria growth and H2 production ability [25–27]. Thus, dilution of DG is necessary to optimize the organic compounds concentration for the growth and H2 production by bacteria.

The highest H2 yields of ~5.2–6.3 mmol L\(^{-1}\) were obtained in Rh. sphaeroides in the 2–5-folds diluted media after 48 h growth, whereas the highest H2 production by Rh. sphaeroides control cells was observed in 72 h culture (Fig. 1a). The H2 yields in 2- and 5-folds diluted media after 48 h anaerobic growth were ~4.0- and 4.8-folds higher in comparison with control cells, grown in Ormerod medium (Fig. 1a). DG contain various organic acids and amino acids, particularly glutamate, which can be used as the carbon and nitrogen sources for H2 production, and can affect the activity of the key H2-producing enzyme of purple bacteria – nitrogenase [12,28]. Thus, diluted DG have an evident effect on H2 yield in E. coli and Rh. sphaeroides pure cultures and can be efficiently used in H2 production.

The H2 yield in E. coli and Rh. sphaeroides mixed culture using DG was also determined during 96 h growth. Mixed culture was cultivated in anaerobic conditions upon illumination. H2 production by mixed culture was also started at 24 h and continued during the growth up to 96 h (Fig. 1b). The H2 yield in co-culture during the first 24 h growth in 2–5-folds diluted DG media was lower ~1.1- and ~2.7-folds, in comparison with E. coli and Rh. sphaeroides pure cultures, respectively. Then, H2 production by co-culture was increased. The H2 yield by mixed culture was higher than that of pure cultures: H2 yield in mixed culture during 72–96 h growth in 2-fold diluted DG media was increased ~1.5–3-folds in comparison with Rh. sphaeroides, grown in 2-fold diluted DG medium (Fig. 1b). The maximal rate of H2 production by mixed culture calculated was 5.16 mmol H2 per L and day. It should be noted that the highest H2 production rate was 7.9 mmol L\(^{-1}\) per day which has been reported with unidentified mixed culture and from DG of wine industry for heating treatment conditions and acidic pH [6]. The H2 yields in mixed culture of E. coli and Rh. sphaeroides in 5-fold diluted DG media were 1.1–1.7-folds higher than that of Rh. sphaeroides pure culture (Fig. 1b). E. coli can convert the DG to various organic acids and other products. Then, the DG fermentation end-products can be utilized by Rh. sphaeroides for continued H2 generation. The increase of H2 yield can be also related with formation of reductive power and ATP synthesis [13,14]. Thus, the combination of dark- and photo-fermentative bacteria can enhance H2 yield.

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Fig. 1 – The H2 yields of pure (a) and mixed (b) cultures of E. coli BW 25113 and Rh. sphaeroides MDC6521 during anaerobic growth in 2-, 5-, and 10-folds diluted DG.
**Effect of DG dilution on medium \( E_h \) during pure and mixed cultures growth**

\( E_h \) of growth medium is considered as important environmental parameter, which can be defined as the biological system ability to reduce or oxidize various compounds \[13,22\].

\( E_h \) of \( E. \) coli control cells has decreased only up to \(-405 \text{ mV}\) during growth till 24 h, whereas \( E_h \) of \( R. \) sphaeroides has gradually decreased up to \(-580 \text{ mV}\) during the growth up to 72 h (Fig. 2). This negative value of \( E_h \) is coupled with \( \text{H}_2 \) generation, because for the \( 2\text{H}^+ + 2e^- \rightarrow \text{H}_2 \) reaction \( E_h \) equals to \(-414 \text{ mV}\) \[13,22,29\].

\( E_h \) of \( E. \) coli, grown in 2-fold diluted DG media, has decreased up to \(-505 \text{ mV}\) during the growth up to 24 h, and then increased up to \(-60 \text{ mV}\) (Fig. 2b). The same kinetics of \( E_h \) was obtained in 5- and 10-folds diluted DG media (Fig. 2a).

The dilution of DG affected the \( E_h \) of pure cultures. \( E_h \) of \( R. \) sphaeroides cells, grown in 2-5-folds diluted DG media, have gradually decreased during the 48 h growth up to \(-610 \text{ to } -680 \text{ mV}\), and then increased during the growth up to 96 h (Fig. 2b). Such changes of \( E_h \) can be connected with the reducing equivalents (NADH or FADH\(_2\)) production. These compounds can have a pronounced effect on the bacterial metabolism, because greater availability of NADH considerably alters the nature of end-products. \( E_h \) of \( R. \) sphaeroides, grown in 10-fold diluted DG medium, has decreased during the 48 h growth up to \(-535 \text{ mV}\), and then increased up to \(-225 \text{ mV}\) (Fig. 2b).

The \( E_h \) in mixed culture using DG was also determined during anaerobic growth. \( E_h \) of mixed culture, grown on 2-5-folds diluted DG media, have gradually decreased during the growth till 72 h up to \(-570 \text{ to } -610 \text{ mV}\) (Fig. 2c). Such change of \( E_h \) also indicates \( \text{H}_2 \) generation by mixed culture.

**Effect of DG dilution on pure and mixed cultures growth properties and medium pH**

The growth properties and pH changes were monitored during bacteria cultivation on diluted DG media. \( E. \) coli and \( R. \) sphaeroides were unable to grow on undiluted medium (not shown). However, the growth yields of cells, grown in 2-10-folds diluted DG media, were considerably lower than those of control cells (not shown). Specific growth rates of pure and mixed cultures have decreased during growth on DG media in dilution-dependent manner (not shown).

Medium pH is another significant parameter, which affects the \( \text{H}_2 \) production by bacteria, because it can change the activity of \( \text{H}_2 \)-producing enzymes, as well as the metabolic pathways \[1-3,11\]. In our previous studies we have shown the correlation between the decrease of \( E_h \) and the increase of pH in \( R. \) sphaeroides, which points out not only the fermentation end-products formation, but also the redox reactions on the bacterial membrane surface \[13,22\].

\( \text{pH} \) of \( E. \) coli, grown in peptone medium, up to 24 h has decreased from pH 7.0 to 6.0 (Fig. 3a). This decrease can be coupled with end products of glucose or glycerol fermentation, such as formic and other organic acids and \( \text{H}_2 \) \[10,11,22,25,27\]. Value of pH after 24–96 h \( E. \) coli growth in DG media was higher in comparison with control (Fig. 3a). During the \( R. \) sphaeroides control cells growth till 72 h in Ormerod medium the pH of medium increased from 7.0 (initial pH) to ~8.75 (Fig. 3b). In 2-10-folds diluted DG media pH of \( R. \) sphaeroides pure culture was increased to ~9.0–9.5 (Fig. 3b).

Such changes of pH can be connected with the available substrates uptake and fermentation products formation, as
polyhydroxybutyrate shown before [13,28]. The mixed culture pH (2–5-folds DG media) has decreased to 6.1–6.2 during the first 24 h, then gradually increased up to 8.3–8.75 (Fig. 3c). Besides, the disproportion between rates of substrates uptake and fermentation end products formation in E. coli and Rh. sphaeroides in mixed culture can change pH. This might change activity of hydrogenases and formate hydrogen lyase in E. coli [2,10,11,27,29] and possibly nitrogenase and hydrogenase in Rh. sphaeroides [1-3,5] and improve H₂ production by mixed culture, as suggested [21].

Concluding remarks

In the present study H₂ production by pure and mixed cultures of dark- (E. coli) and photo-fermentative bacteria (Rh. sphaeroides) using DG of ethanol fermentation has been investigated during batch fermentation at pH 7.0. The H₂ yields in pure cultures during growth up to 48 h in 2–5-folds diluted DG media were higher in comparison with cultures, grown in appropriate media. The mixed culture produced significantly more H₂ from DG: 2-folds diluted media provided the ~1.5–3-folds higher H₂ yield in comparison with pure culture. Moreover, H₂ production by mixed culture was continued during the growth up to 96 h. DG dilution and neutralization are necessary to adjust the organic acids concentration and the pH 7.0 for the optimal growth of dark- and photo-fermentative bacteria.

The results have shown the possibility of using DG of ethanol industry (in optimal dilution), as an effective substrate for H₂ production.

This study can provide novel approach for an inexpensive energy generation using identified mixed cultures of dark- and photo-fermentative bacteria as well as it can resolve the problem of waste utilization. During this study potentially new experimental data have been obtained, which can develop the understanding of bacterial metabolism mechanisms through the optimization of the conditions for dark- and photo-fermentative cooperation for efficient H₂ production.

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