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Hydrogen production by *Escherichia coli* using brewery waste: Optimal pretreatment of waste and role of different hydrogenases

Anna Poladyan [a, b], Karen Trchounian [b], Anait Vassilian [c], Armen Trchounian [a, b, *]

[a] Department of Biochemistry, Microbiology and Biotechnology, 1 A. Manoogian Str, 0025 Yerevan, Armenia
[b] Research Institute of Biology, 1 A. Manoogian Str, 0025 Yerevan, Armenia
[c] Department of Ecology and Nature Protection, Biology Faculty, Yerevan State University, 1 A. Manoukian Str., 0025 Yerevan, Armenia

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ABSTRACT

Brewery spent grains (BSG), one of the by-products of brewery production, were used for *Escherichia coli* growth and hydrogen (H_2) production. The dilute acid and alkali pretreatment methods were used to hydrolyze the rough lignocellulose structure, and optimal conditions for the BSG hydrolysate (BSGH) preparation were developed. *E. coli* BW25113 wild type strain and hydrogenase (Hyd)-negative mutants with deletions of genes encoding key subunits of Hyd 1–4 (Δ*hyaB*, Δ*hybC*, Δ*hycE*, Δ*hyfG*), as well as for a Δ*hyaB* Δ*hybC* double mutant were investigated with regards to growth, acidification of the medium, redox potential kinetics and H_2 production when using BSGH. Readings of redox Pt electrode dropped to −400 ± 10 mV, with H_2 yield of ~0.75 mmol H_2 L^{-1} at the 3rd h wild type strain growth. Changes in redox Ti-Si electrode readings were negligible. H_2 production was not observed with defective Hyd-3 and Hyd-4; therefore, Hyd-3 and Hyd-4 are responsible for H_2 production using BSGH, whereas defective Hyd-1 and Hyd-2 led to a ~2-fold stimulation of H_2 yield. The data were confirmed by determining cumulative H_2 yield. These findings are useful for development of renewable energy, especially H_2 production biotechnology, using different organic wastes as sustainable energy feedstocks.

1. Introduction

There is an urgent need to find alternative sources of ecologically clean, renewable and cheap energy to satisfy the demands of modern industrialized civilization and sustainable development [1]. Another problem that is worth addressing is generation of different wastes, which are produced in huge amount after many industrial and agricultural activities, utilization of which could lead both to waste treatment and cost-effective energy production. In this respect, molecular hydrogen (H_2) is being recognized as an alternative energy source of the future, as it is renewable, ecologically advantageous (only water is formed upon H_2 oxidation) and, moreover, the energy released upon it's combustion (~142 kJ g^{-1}) is ~3.5 fold greater than for oil [2].

Nowadays it is of interest to produce H_2 by using bacteria. *Escherichia coli,* performing dark fermentation resulting in H_2 evolution, is a good candidate to use for these biotechnological proposes, because it is well characterized, genetically and metabolically easy to manipulate bacterium [3]. Moreover, local agricultural and food industry wastes rich in carbohydrate and protein can be used for H_2 formation by implementing proper bio-process technologies [4–6]. In this context, consumption of the mentioned wastes for H_2 production provides a low-priced energy generation and, which is also important, accompanying waste treatment [7]. Transition of H_2 production by dark fermentation from laboratory to pilot-scale systems is emerging [8,9]. This will lead to the strategy in which local energy supply systems are developed; without the need for long-distance transportation of resources, products and associated expenses. The resources can include cereal distiller's grains wastes which are as by-product of ethanol fermentation [5], and also brewery wastes [6,7,10–13].

A major part of brewery wastes is used in agriculture, particularly as an animal feed, but also for production of specialized valuable products such as yeast extract and other compounds. Reductions in dairy farming, limits on farm activities due to disease and other factors have restricted these opportunities [14]. Thus, brewery waste utilization can be used to release energy,

* Corresponding author. Department of Biochemistry, Microbiology and Biotechnology, Yerevan State University, 1 A. Manoogian Str, 0025 Yerevan, Armenia.
E-mail address: Trchounian@ysu.am (A. Trchounian).

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particularly H_2 [15].

Beer is one of the most highly consumed beverages throughout the world. During brewing, various by-products are generated, the most common of which are spent grains (BSG), spent hops and surplus yeast; the latter are generated from the main raw materials [15–17]. BSG is the by-product of the mashing process; which is one of the initial steps of brewery production necessary to solubilize the malt and cereal grains and to extract of the wort (water with extracted biomass) [15–17]. The worldwide annual production of BSG has been estimated to be approximately 38.6×10^6 tons [16].

According to literature, BSG contains cellulose, hemicelluloses and lignin, and it has a high protein content; the most abundant monosaccharides found in BSG are xylose, glucose and arabinose [15,17,18], as well as minerals, many vitamins and amino acids. It is very important to pretreat BSG correctly. Various pretreatment methods are now available to fractionate, solubilize, hydrolyze and separate cellulose, hemicellulose, and lignin components [12,19–21]. The first step of pretreatment starts with mechanical curing that can be followed by chemical, physicochemical, thermal, biological and other procedures to further destroy the tough structure [21]. The next step is conversion of monomeric carbohydrates into H_2 by using appropriate microorganisms. There are some studies reporting the possibility of producing biogas from different brewery wastes [4,6,17]. It was shown that brewery waste can be used for H_2 production via dark- and photo-fermentation by bacteria [4,13,18,22] including *Bacillus* sp., *Enterobacter* sp. or *Rhodobacter sphaeroides* which could be considered as efficient bacteria towards industrially relevant H_2 production. However, mechanisms and optimal conditions for H_2 production using brewery and other food industry wastes should be studied, making possible genetic and metabolic engineering of bacteria to enhance worldwide H_2 production.

It is well known that *E. coli* during utilization of different carbon sources (glucose or glycerol) also efficiently produces H_2 [2,3]. H_2 production from distiller's grains wastes was shown for *E. coli* and *R. sphaeroides* and the optimal pretreatment conditions were investigated [5].

In *E. coli* H_2 is produced from formate decomposition, the end product of both glycerol and glucose fermentation via action of membrane-associated [Ni-Fe]-hydrogenase (Hyd) enzymes [23,24]. In *E. coli* four Hyd enzymes (Hyd 1–4) encoded by the *hya*, *hyb, hyc* and *hyf* operons, respectively, are involved in H_2 metabolism: they are responsible for both H_2 production and oxidation [23,24]. Moreover, the reaction directions and activity of the Hyd enzymes are determined by the substrate of fermentation (glucose or glycerol), pH of the medium, oxidation-reduction potential (ORP) and other factors [2,3,23,25–27]. Consequently, optimal activity of Hyd-1 was shown under anaerobic conditions, at low, acidic pH or upon formate supplementation while Hyd-2 was active under more reducing conditions (less ORP) or at alkaline pH. Formate dehydrogenase H (FDH-H) together with Hyd-3 or Hyd-4 compose the formate hydrogenlyase (FHL) 1 and 2 pathways during glucose anaerobic utilization at acidic and alkaline pHs, respectively [23,28]. *E. coli* might be also considered as a good candidate for industrial H_2 production using brewery wastes. For development of H_2 production technology by bacteria it is essential to investigate and control the conditions of Hyd enzymes activities using appropriate mutant strains during the utilization of different organic wastes, including those from breweries.

The present study aimed to develop optimal pretreatment methods of BSG; to design optimal conditions for growth and H_2 production of *E. coli* upon utilization of BSG hydrolysate (BSGH); to reveal the responsible Hyd enzymes for H_2 production under certain optimized conditions with the help of *E. coli* different Hyd mutants; as well as to enhance H_2 production. Thus, BSG pretreatment conditions were designed to be effective for *E. coli* bacterial growth and H_2 production and conditions; utilization of BSGH were also optimized. The role of Hyd 3 and Hyd 4 in H_2 production was determined; and improved H_2 production was observed in *E. coli* single and double mutants lacking Hyd 1 and Hyd 2.

These findings are novel and might have application with benefits for producing applied and renewable energy when brewery waste is used. Utilizing different wastes for energy production are of significance to supply renewable and sustainable energy and to maintain clean environment.

2. Materials and methods

2.1. Bacteria, the growth conditions for bacteria; growth yield and rate determinations

The *E. coli wild type* parental strain (WT) and different Hyd-negative mutant strains have been kindly provided by Prof. T. Wood (Pennsylvania State University, University Park, PA, USA) [29] and are described in Table 1.

Bacteria were cultivated for 72 h in anaerobic conditions, at 37 °C. The batch culture was performed in 150 mL glass vessels covered by plastic press-caps and composed of growth peptone medium (PM) or BSGH or minimal salt medium (MSM). Bacterial subculture inoculums were grown in PM under the same anaerobic conditions. PM was composed of 20 g L^{-1} peptone, 2 g L^{-1} K_2HPO_4, 5 g L^{-1} NaCl, pH 6.5; 10 g L^{-1}, whereas, MSM contained 8.004 g L^{-1} K_2HPO_4, 3.128 g L^{-1} KH_2PO_4, 1.056 g L^{-1} NH_4SO_4, 0.048 g L^{-1} $MgSO_4$, 0.009 g L^{-1} $FeSO_4$ [26,27]. A pH-selective electrode was used to measure the pH by HJ1131B pH-meter (Hanna Instruments, Portugal). The pH was settled using 0.1 M NaOH or KOH or 0.1 N HCl.

BSG were supplied by "Kilikia" beer factory (Yerevan, Armenia). BSG was treated by using dilute acid and alkali hydrolysis methods in steam sterilizer for 1 h, 121 °C [19,20], and BSGH was obtained.

Bacterial biomass formation in the conditions above was investigated with the help of Spectro UV–vis Auto spectrophotometer (Labomed, Los Angeles CA, USA), by determining the optical density (OD) values of the bacterial culture absorbance at 600 nm. The specific growth rate (μ) was stated as lg2/doubling time, which was counted when the logarithm of OD was increased linearly with time, as described [26]. Finally, bacterial growth yield was estimated by determining bacterial culture dry weight (CDW) and expressing in g L^{-1}, as done before [26].

2.2. H_2 production determination

The pair of ORP platinum (Pt), (EPB-1, GSEEE; or PT42BNC, HANNA Instruments, Portugal) and titanium-silicate (Ti-Si), (EO-02, GSEEE, Gomel, Belarus) electrodes were used to determine the ORP of bacterial culture [26,30,31]. Compared to the Ti-Si electrode, the Pt electrode is sensitive to H_2 or O_2 and its readings drop down to negative values (~400 mV) in the presence of medium H_2. The difference between the readings of the two mentioned electrodes permits to estimate the H_2 production under particular conditions, as earlier reported [26,30,31]. The H_2 production yield was calculated and expressed in mmol H_2 L^{-1}, as described [26,30,31]. H_2 determination using the electrochemical method described [2,3,23] gives more accurate results for cumulative H_2 yield in liquids.

Cumulative H_2 yield measurements were performed in 500 mL glass vessels under permanently stirring conditions; the gases bubbled (H_2 and CO_2) were treated by 1 M NaOH solution (to eliminate CO_2 from gas mixture); and H_2 gas was collected and estimated by the water displacement volume [5,23].

Table 1
E. coli strains characteristics used in the study.

Strains	Genotype	Protein's absent subunit	Reference
BW 25113	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{W116}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rha BAD*$_{LD78}$	WT parental strain	[29]
JW 0955[a]	BW 25113 Δ*hyaB*::Kan	Large subunit of Hyd-1	[29]
JW 2962[a]	BW 25113 Δ*hybC*::Kan	Large subunit of Hyd-2	[29]
JW 2917[a]	BW 25113 Δ*hycE*::Kan	Large subunit of Hyd-3	[29]
JW 2472[a]	BW 25113 Δ*hyfG*::Kan	Large subunit of Hyd-4	[29]
MW1000[a]	BW25113 Δ*hyaB* Δ*hybC*::Kan	Large subunits of Hyd-1 and Hyd-2	[29]

[a] Resistant to kanamycin.

H_2 production upon *E. coli* growth was visualized by the presence of gas bubbles in the test Durham tubes and also was validated by the chemical assay based on the decolorizing of $KMnO_4$ solution in H_2SO_4 with H_2 [26,30,31].

2.3. Reagents used in the study and data processing

Glycerol and peptone (Carl Roths GmbH, Germany) were used, and other reagents were of analytical grade.

The average data are expressed from three experiments done; the standard errors were considered and Student criteria (P) were used to confirm the difference in average data between different series of experiments, as defined before [25–27]; the difference was considered valid when $P < 0.05$.

3. Results and discussion

3.1. Pretreatment of BSG and its optimization for E. coli growth and H_2 production

It is very important for bacterial growth and H_2 production to pretreat BSG correctly prior to use. During the few past years a large number of pretreatment methods have been developed, including dilute acid or alkali treatment, ammonia exposure, ionic liquid pretreatment and others [12,19–21]. So, the initial step of our investigation was directed to hydrolyze the lignocelluloses containing material. For this proposes different acids and alkalis were applied: 1–10% BSG were dissolved in 0.5–3% acid (HCl, H_2SO_4) or alkali (NaOH, KOH) and treated, as described (see Materials and methods). After filtration the next step was adjustment of pH of the BSG hydrolysate (BSGH) from 1.5 to 7.5. The latter was done by using various alkalis (NaOH, KOH) and with potassium salt (K_2HPO_4); the pH value was reached to pH 7.5 by using HCl. Finally, appropriate dilutions of BSGH in a broad range, from 1 to 20, were applied.

Given these observations, the optimal conditions for bacterial growth and H_2 production were found to be 4% (w/v) of BSG treated with 0.7% (v/v) sulfuric acid in a steam sterilizer, at 121 °C, the pH was adjusted to pH 7.5, and 2.5-fold dilutions of BSGH were more optimal for *E. coli* WT strain growth and H_2 production (see Fig. 1).

Next, *E. coli* BW25113 WT strain growth, ORP kinetics and H_2 production were investigated. The results were compared with the data of *E. coli* grown on PM, with 10 g L^{-1} glycerol supplementation (see Materials and methods). It should be noted that glycerol being by-product of various industries, including bio-diesel production, is currently very cheap; and it was shown that it can be fermented by *E. coli* [3]. Medium acidification was observed upon *E. coli* WT strain growth on both BSGH and PM with glycerol: after 6 h growth the pH dropped from 7.5 to 5.5 and to 6.9, respectively (Fig. 2b).

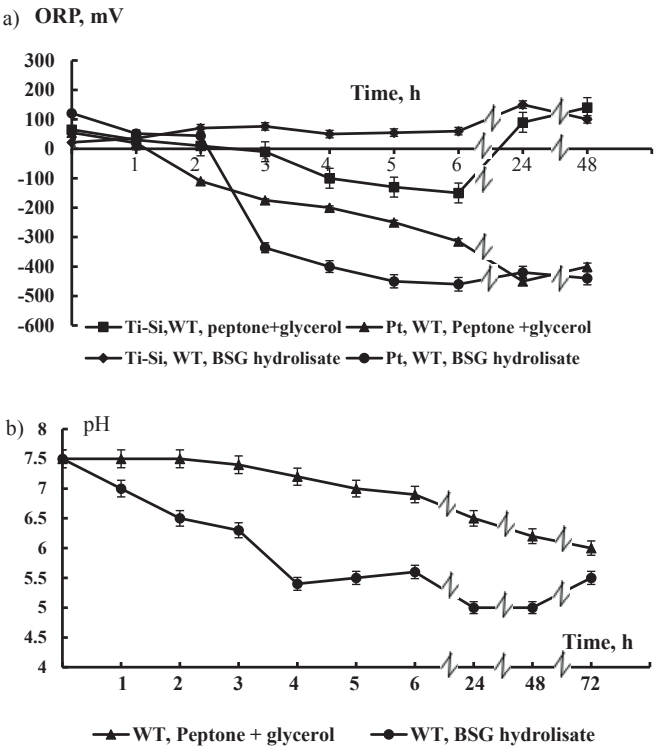

Fig. 2. ORP (a) and pH (b) changes during growth of *E. coli* BW25113 on peptone medium (PM) with glycerol and BSGH. Bacteria were grown anaerobically, at pH 7.5, with 10 g L^{-1} glycerol added when necessary. WT is wild type parental strain. ORP was determined by platinum (Pt) and titanium-silicate (Ti-Si) electrodes and expressed in mV (vs Ag/AgCl (saturated by KCl)).

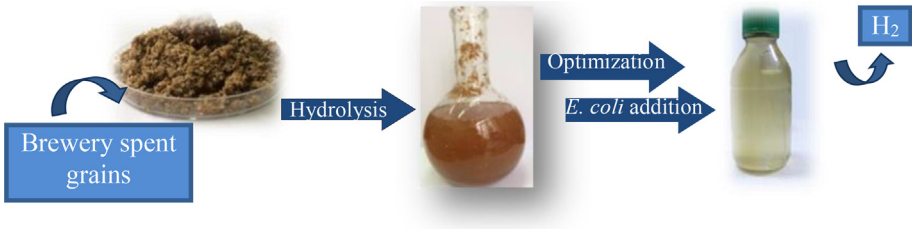

Fig. 1. Main steps of H_2 production by *E. coli* bacteria using BSGH. See the text.

Bacterial biomass yield was ~1.8 fold less after 24 h growth on BGSH compared with that on PM under glycerol fermentation (Fig. 3a). There was no difference between bacterial specific growth rates (μ) values (0.63 ± 0.02 h^{-1}) in both cases (Fig. 3b). It was reported that pretreatment of lignocellulosic biomass may produce degradation products with an inhibitory effect on the fermentation process which might affect bacterial growth [12,19–21]. On the other hand, lower biomass yield might be regarded due to medium rapid acidification (Fig. 2b) or limitations of fermentation substrates.

ORP is one of the important physicochemical parameters determining bacterial growth; moreover, ORP is parameter linked to the activity of Hyd enzymes and H_2 production [32]. Readings of the Pt electrode dropped from positive values down to negative ones, −400 ± 10 mV value, with a H_2 production yield of 0.74 ± 0.02 mmol H_2 L^{-1} at the 3rd h of growth, corresponding to the beginning of the log growth phase of *E. coli* BW25113 fermenting BSGH (see Fig. 2a). Upon bacterial glycerol fermentation in PMH_2 production was observed at the end of the log growth phase with the H_2 production yield of 0.75 ± 0.03 mmol H_2 L^{-1}. In contrast, the drop in Ti-Si electrode readings was negligible and remained positive when compared to glycerol fermentation in PM (see Fig. 2a). H_2 was not produced after 72 h growth (data not shown).

BSGH used in the study is a typical hemicellulosic hydrolysate which contains a mixture of xylose, arabinose, glucose, formic acid, acetic acid, etc. [15,17,18], all of which can be fermented by *E. coli* [2,3,28,33,34]. Thus utilization of these substrates might lead to bacterial biomass formation, medium acidification and H_2 production. Future studies are required to enhance bacterial biomass and to prevent rapid drop of medium pH upon *E. coli* growth on BSGH.

3.2. Production of H_2 by the E. coli wild type strain BW25113 and Hyd-negative mutants upon fermentation of BSGH

As was mentioned above, H_2 is produced from formate decomposition upon glucose or glycerol fermentation [2,3,28], and Hyd enzymes are involved in this process [23,24]. To understand the role and participation of individual Hyd enzymes both in bacterial growth and H_2 production upon utilization of BSGH, the growth and H_2 production of *E. coli* Δ*hyaB,* Δ*hybC,* Δ*hycE,* Δ*hyfG* mutants with deletions of genes for different key subunits of Hyd-1 to 4, respectively, and a Δ*hyaB* Δ*hybC* double mutant with deletions of genes for key subunits of both Hyd1 and Hyd 2 were studied (Table 1).

All of the mutants were able to grow on BSGH: after 24 h of growth the biomass yield values were around 0.3 (g CDW) L^{-1}, close to the BW25113 value, and again, ~1.8-fold less compared with the data of the WT strain grown on PM with glycerol (see Fig. 3a). μ was more or less similar each strain, even ~1.1-fold stimulated in the Δ*hyaB* Δ*hybC* double mutant (see Fig. 3b). Medium pH dropped from 7.5 to 5.5 upon bacterial all strains at 6 h growth on BSGH (data not shown).

From the beginning of the lag growth phase of the WT strain and all mutants a drop of the Pt electrode readings from positive to negative values (down to ~ −100 to −500 mV) was detected (Fig. 4): the H_2 production yield was stimulated ~2 fold (~1.45 mmol H_2 L^{-1}) in the Δ*hyaB*, Δ*hybC* single and the Δ*hyaB* Δ*hybC* double mutants (see Table 1) compared with the WT strain. In contract, H_2 production was absent in the Δ*hycE* and Δ*hyfG* mutants (see Table 1) during log growth phase (see Fig. 4): the Pt electrode readings dropped to ~ −200 mV and −100 mV, respectively.

As was mentioned above, activity of the Hyd enzymes and their

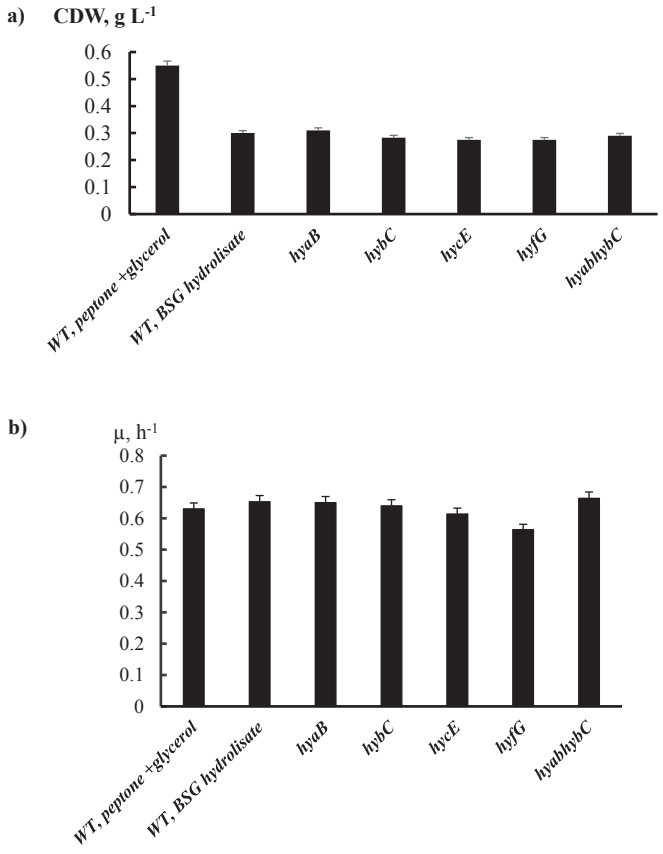

Fig. 3. Growth characteristics of *E. coli* BW25113 and the Hyd-negative mutants. Bacteria were grown anaerobically, at pH 7.5. a) Cell dry weight (CDW) (g L^{-1}); b) specific growth rate (μ) during growth on BSGH and PM with 10 g L^{-1} glycerol addition. WT is wild type parental strain; BSGH is brewery spent grains hydrolysate; PM is peptone medium. For strains used, see Table 1.

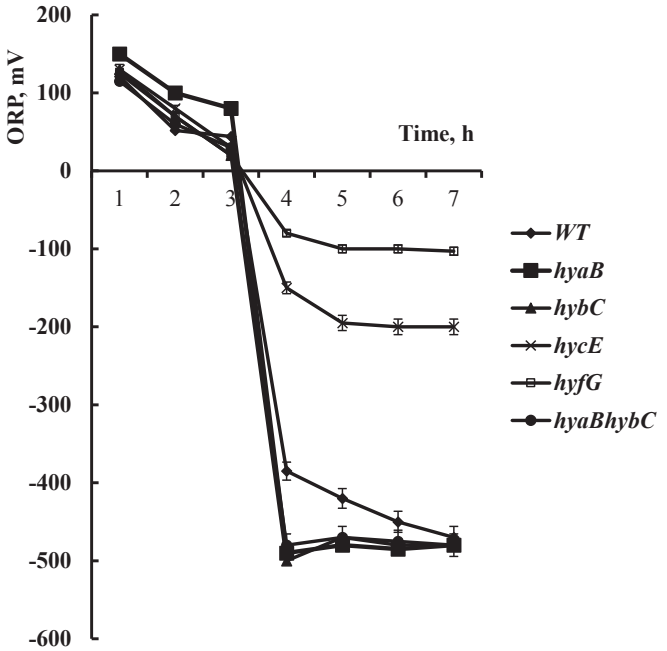

Fig. 4. The ORP kinetics of by *E. coli* BW25113 and the Hyd-negative mutants during utilization of BSGH. BSGH is brewery spent grains hydrolysate. For the others, see the legends to Figs. 2 and 3.

operation direction (H_2 producing or oxidizing mode) depended on the substrate of fermentation, medium pH, etc. [23,24]. So, our results point out the key roles of Hyd-3 and Hyd-4 in H_2 production and verify the role of the Hyd-1 and Hyd-2 in reverse, H_2 oxidizing mode upon BSGH utilization by *E. coli*. The results for Hyd-3 are in accordance with the previous findings obtained, when it was shown that Hyd-3 is the major in H_2 production by *E. coli* in the presence of formate, however Hyd-4 activity is inhibited by high concentration of glucose mainly at pH 7.5 [23,28,35].

3.3. Cumulative H_2 production measurements during growth of the *E. coli* BW 25113 and ΔhyaB ΔhybC double mutant under different conditions

Cumulative H_2 production was measured upon bacterial anaerobic growth in 500 mL medium upon permanent stirring conditions (to remove H_2 gas from the growth medium) (Fig. 5, Table 2). As was mentioned above, H_2 and CO_2 are produced during growth of *E. coli* on different fermentation substrates [23]. Thus, CO_2 was eliminated from the gas mixture by 1 M NaOH solution; and H_2 gas was collected and estimated by water displacement volume (see Materials and methods).

For standard comparison, glucose was used as a reference for comparing the yield of H_2 obtained from BSGH to the yield of glucose. Moreover, the influence of the medium composition on the yield of H_2 was also tested where the bacteria were grown in PM and minimal salt medium (MSM) with 0.2% glucose as fermentation substrate. Thus, cumulative H_2 yield reached up to 170 ± 5 mL and 39 ± 3 mL for growth of the WT strain on PM and MSM, respectively (Fig. 5) at the 11th h of growth on 0.2% glucose (1 g glucose was present in 500 mL PM). It should be noted, that upon bacterial growth on MSM the lag phase of growth was prolonged and noticeable H_2 production was observed after 7 h of growth. With growth of the WT strain in 500 mL of BSGH (with 8 g BSG) cumulative H_2 yield reached up to 26 ± 3 mL, but stimulated ~ 1.7-fold (46 ± 2 mL) in the Δ*hyaB* Δ*hybC* double mutant (Fig. 5). Data of cumulative H_2 production per bacterial CDW are presented in Table 2: thus, growth of the WT strain on rich (PM) medium with glucose led to ~3 and ~1.7-fold higher amount of H_2 yield compared to growth of the WT strain and mutant on BSGH, respectively.

Although when compared with BSGH PM medium with glucose

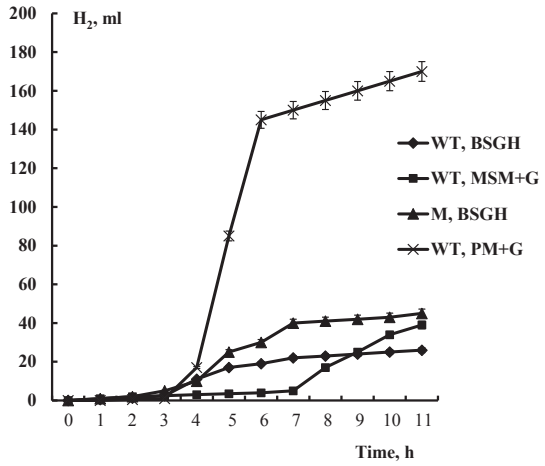

Fig. 5. Kinetics of cumulative H_2 yield during growth of *E. coli* BW25113 and the Δ*hyaB* Δ*hybC* double mutant under different conditions. In all cases, bacteria were grown in 500 mL growth medium: MSM is minimal salt medium; PM is peptone medium; BSGH is brewery spent grains hydrolysate. WT is wild type parental strain and M is the Δ*hyaB* Δ*hybC* double mutant. G is glucose. For strains used, see Table 1.

Table 2
Cumulative H_2 yield of *E. coli* wild type parental strain BW25113 (WT) and the Δ*hyaB* Δ*hybC* mutant MW1000 upon growth in different conditions.

Growth peculiarities[a]	H_2, mL (g CDW)$^{-1}$	Estimated price (USD)[b]
WT grown on BSGH	100 ± 3	1.28
Mutant strain grown on BSGH	175 ± 5	0.7
WT grown on PM with glucose	290 ± 5	2.20
WT grown on MSM with glucose	160 ± 5	4

[a] Data on the 11th h of bacterial growth are presented; PM is peptone medium; MSM is minimal salt medium (see Materials and methods); for *E. coli* strains, see Table 1.

[b] Prices are calculated for 1000 L of H_2 yield considering only the prices for glucose [37] and BSG [16,36].

is better for achieving higher H_2 production levels, nevertheless, glucose and peptone are much more expensive substrates for sustainable, long-term H_2 production than BSG [37]. Moreover, the results demonstrate that upon utilization of BSGH by the bacterium MW1000, with abolished H_2 oxidation properties, improved levels of H_2 accumulation can be achieved. Significant amounts of BSG are annually produced from breweries [16]. Thus, providing wet BSG with the cheapest (low-value) price to local consumers, like biogas stations or farmers (for use as cattle feed, etc.) will be a good solution for the breweries to eliminate BSG, since this is a cheap "fuel" alternative that avoids the energy spent needed for drying of BSG. However, the costs of transporting BSG are still significant and should be considered [36]. Usually, on average 16 USD per ton of wet BSG is the transport cost over a distance of 5 miles (~8 km) [16,36]. So, comparing the prices for glucose [37] and BSG [16,36] and yields of H_2 produced by the WT strain and the Δ*hyaB* Δ*hybC* double mutant under different conditions suggests the possibility of BSG application for low-priced H_2 generation (see Table 2).

4. Conclusions and significance

Taken together, our results demonstrated that use of BSG consisting of cellulose, hemicellulose and lignin needs a treatment process to release the substrates to achieve bacterial growth and sufficient H_2 production by *E. coli*. Thus, the optimal treatment of BSG has been developed and *E. coli* growth and H_2 production have been shown using BSGH as substrate. Moreover, the responsible Hyd enzymes for H_2 production – Hyd-3 and Hyd-4 were determined for bacterial growth on BSGH. The mutations in *E. coli* genes encoding Hyd-1 and Hyd-2 resulted in enhanced H_2 production.

These findings on bio-hydrogen production by bioconversion of organic waste are novel and of great interest for renewable energy supply. They should lead to development of a strategy for sustainable and renewable energy production from available and cheap wastes like BSG. In addition, they have ecological and economic advantages. Therefore, the results might lead to evaluation of new approaches to produce H_2 simultaneously with waste treatment and offer the opportunity to effectively obtain H_2 by bacteria.

Conflict of interest

The authors have no conflict of interests.

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