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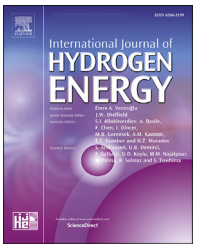

Enhanced hydrogen gas production from mixture of beer spent grains (BSG) and distiller's grains (DG) with glycerol by *Escherichia coli*

Satenik Mirzoyan [a,c], *Akerke Toleugazykyzy* [b,d], *Kairat Bekbayev* [b], *Armen Trchounian* [a,d], *Karen Trchounian* [a,c,d,*]

[a] *Scientific-Research Institute of Biology, Faculty of Biology, Yerevan State University, 0025, Yerevan, Armenia*

[b] *Department of Food Technology and Processing Products, Technical Faculty, S. Seifullin Kazakh Agro-Technical University, 62 Zhenis Avenue, Z11F9K5, Nur-Sultan, Kazakhstan*

[c] *Department of Biochemistry, Microbiology and Biotechnology, Faculty of Biology, Yerevan State University, 0025, Yerevan, Armenia*

[d] *Microbial Biotechnologies and Biofuel Innovation Center, Yerevan State University, 0025, Yerevan, Armenia*

HIGHLIGHTS

- Optimization of pretreatment and utilization parameters of the BSG and DG
- BSG and DG can be utilized sole or in mixture by *E. coli*
- BSG or DG in mixture with glycerol can enhance H_2 yield ~3 fold
- Application of multiple mutant enhances H_2 yield and prolong it till 120 h
- Hyd-3 and Hyd-4 are responsible for H_2 production during utilization of BSG and DG

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ABSTRACT

Escherichia coli perform mixed acid fermentation and produce hydrogen gas (H_2) as one of the fermentation end products. *E. coli* can ferment sugars like glucose, xylose and alcohols like glycerol. It has been shown that *E. coli* has the ability to utilize pretreated organic waste (BSG or DG) or mixtures of it with glycerol and H_2 can be produced. H_2 evolution was maximum when the concentration of BSG was 4% and DG - 10% yielding 1.4 mmol L^{-1} H_2. H_2 evolution was prolonged to ~24–120 h when mixtures of glycerol and DG or BSG wastes were applied. Moreover, in *hycE* (lacking large subunit of Hyd-3) or *hyfG* (lacking large subunit of Hyd-4) single mutants H_2 production was absent compared to wild type suggesting that Hyd-3 and Hyd-4 are responsible for H_2 generation. In addition, multiple mutant enhanced cumulative H_2 production ~3–4 fold. Taken together it can be proposed that BSG or DG wastes either together or in mixture with glycerol can be applied to obtain *E. coli* biomass and produce bio-H_2. The novel data can be used to further control effectively the application of organic waste resources as a feedstock for developing bio-H_2 production technology.

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

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Introduction

The stable growth of population in the world increases the demand on energy sources. It was shown that by 2050 the energy demand will be increased ~3 fold and the continuous usage of fossil fuels cannot cover all the requirements in energy [1,2]. As an additional energy source many countries are considering renewable and alternative energy sources. They can be produced with different methods and various substrates: e.g. from biomass as a feedstock which is known as first generation biofuels [3]. Nowadays, many industrial, agricultural and municipal solid wastes are generated and they can be a good feedstock for renewable energy production which is considered as a second-generation biofuel based on non-edible resources [4]. The use of this type of resources is promising for the implementation of bio-based circular economy strategies for waste management and further utilization. Among non-edible sources are wastes that contain lignocellulose residues, different industrial and agricultural production organic wastes and others [5,6].

During beer or ethanol production huge amount of organic waste is produced that is poured in the nature or in some cases used in agriculture after special treatment. It is reported that in 2016–2 billion hectoliters of beer is produced [7]. During production 85% of by-product is brewer's spent grain (BSG). It is estimated that ~39 million tons of BSG is produced annually [8]. Besides, beer, ethanol worldwide production is estimated ~100 billion liters [9]. Ethanol is produced via fermenting various feedstocks such as wheat, rice, corn, barley etc. During ethanol production, huge amounts of distiller's grains (DG) are generated [10]. Unlike BSG, DG contains high amount of proteins, water soluble vitamins and minerals and thus it is currently used as animal feed [11,12]. Previous studies revealed that sole BSG or DG can be pretreated and utilized by *E. coli* or in co-culture with *Rhodobacter sphaeroides* to produce bio-H_2 [13–15] but the mixtures of BSG and DG together or with glycerol and application of genetically engineered strains for enhanced yield of biomass and bioenergy has not been investigated yet.

Besides BSG and DG, glycerol as a waste is generated during biodiesel production. For production of 10 kg of biodiesel 1 kg of crude glycerol is generated [16]. In the last decade, biodiesel production grows exponentially and it is estimated that by 2020 world glycerol production will reach up to 2.6 billion kilograms [17,18]. From economic side it is very favorable, as crude glycerol prices (~8 cents kg^{-1}) has a tendency in decreasing [19]. Moreover, glycerol can be used as an effective feedstock or carbon source for production of various valuable chemicals and biofuels, such as organic acids, bio-H_2 etc. [20–24].

During fermentation of diverse carbon sources (glucose or glycerol) among end products hydrogen gas (H_2) is produced. H_2 is "eco-friendly" biofuel, as H_2 combustion does not generate any pollutants but water [25]. Moreover, the energy density for 1 g of H_2 is relatively high reaching ~140 kJ. It is 3–4 time higher than existing fossil fuels. Current trends of H_2 usage are demonstrated in governmental decisions for slowly changing towards H_2 economy [26,27]. H_2 is used mainly for fuel cells, portable electricity generators and automotive industry but the main challenge is to find cheap substrates or develop cheap technologies for H_2 generation [27].

H_2 can be produced by different methods or ways but biological one is the most promising [28]. Bio-hydrogen can be generated by applying microorganisms that are capable of encoding hydrogenase (Hyd) enzymes [29,30]. Besides microorganisms, it is possible to use either pure enzymes or make bio-augmentation but these technologies, as well as, whole cell ones are still to be developed [31]. Among well studied microorganisms is *Escherichia coli*. This bacterium has four [Ni–Fe] membrane bound reversible Hyd enzymes [29,32]. Hyd-3 and Hyd-4 are main H_2 producing Hyd enzymes during sugar (glucose) fermentation, whereas Hyd-1 and Hyd-2 can work in H_2 producing or uptake mode depending on external pH and fermentation substrate [33–35]. The differences in function of Hyd enzymes have been extensively studied by different groups: it has been shown that the differences might be related to distinguished values of proton-motive force generation (for reviews see Ref. [19,24]). Formate is the direct substrate for H_2 production during glucose fermentation and the redirection of formate metabolism pathways towards oxidation to H_2 will possibly increase the total volume of H_2 production [36,37].

Current problems and challenges for bio-H_2 production are: selection of appropriate fermentation substrate (various wastes or their mixtures), selection of bacteria that can effectively convert organic waste to bioenergy and last but not least possibility of enhancement and prolongation of H_2 production [38]. It has been shown that by genetic manipulations it was possible to significantly enhance H_2 production rate when sole glucose was applied [36]. In addition, during batch fermentation optimization of external parameters, as well as concentrations of single or mixtures of carbon sources, enhanced H_2 production and prolonged till ~240 h [21,39,40].

Present study describes the novel possibilities of using sole and mixture of beer and ethanol wastes and glycerol, optimized concentrations and pretreatment of waste, identification of responsible Hyd enzymes for H_2 production and enhancement of H_2 production via applying genetically engineered strains.

Materials and Methods

Bacterial strain cultivation and waste pretreatment procedures

The characteristics of *E. coli* BW25113 wild type and mutant strains with deletions of the genes coding Hyd-3 or Hyd-4 enzymes or multiple deletions are described in Table 1.

Bacteria from overnight cultures were inoculated into the pretreated beer spent grains (BSG) or distiller's grains (DG) waste medium. BSG was taken from Pavlodar beer factory (Pavlodar region, Kazakhstan) and DG waste was taken from Kokshetau alcohol producing factory (Akmolinsk region, Kazakhstan). The pH of the pretreated waste medium was adjusted to pH 7.5 with KOH. Acid hydrolysis with sulfuric acid was applied to pretreat the BSG or DG. Particularly, BSG and DG were treated by 1.5% sulfuric acid and autoclaved for 121 °C for 20 min, as done before [14]. The composition of BSG

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Table 1 – Characteristics of *E. coli* wild type and mutant strains used.

Strains	Genotype	References
BW25113	rrnB *ΔlacZ4787 HsdR514* Δ(araBAD)567 Δ(rhaBAD)568 rph-1 (old genotype: *lac* 1^q *rrnBT14* $\Delta lacZ_{WJ16}$ *hsdR514* $\Delta araBAD_{AH33}$ $\Delta rhaBAD_{LD78}$)	[35,41]
BW 25113 *ΔhycE*[a]	*ΔhycE* (defective Hyd-3)	[37,41]
BW 25113 *ΔhyfG*[a]	*ΔhyfG* (defective Hyd-4)	[35,41]
BW 25113 *ΔhyaB ΔhybC ΔhycA ΔfdoG ΔldhA ΔfrdC ΔaceE*[a]	BW 25113 *ΔhyaB, ΔhybC, ΔhycA, ΔfdoG, ΔfrdC, ΔldhA, and ΔaceE* (defective Hyd 1 and 2, defective FHL repressor, defective formate dehydrogenase-O, defective fumarate reductase membrane protein, defective D-lactate dehydrogenase, and defective pyruvate dehydrogenase)	[36]

[a] Resistant to kanamycin.

or DG was reported as before [8]. Sole BSG in final concentration of 4% or sole DG in 10% were applied. When mixture was applied–4% BSG and 10% DG in the final concentration were used. 10 g L^{-1} glycerol was added to the medium with BSG or DG when indicated [33]. Bacteria were grown in batch cultures in working volume of 500 mL sealed flasks with closed lids under fermentative conditions at 37 °C, as described earlier [35,37,42]. The medium or external pH was measured by a pH-meter (HI-3220, Hanna Instruments, Portugal) with a pH-electrode (HI–1110B, Hanna Instruments, Portugal) [37,42].

The bacterial biomass growth was studied with double beam UV-VIS spectrophotometer (Cary 60, Agilent Technologies, Germany) following the optical density (OD) readings of bacterial culture absorbance under the wavelength of 600 nm. The bacterial specific growth rate (μ) stated, as lg2/doubling time, was calculated where the logarithm of OD was grown linearly with time [43].

Agar, glycerol, peptone (Carl Roth GmbH, Sigma-Aldrich, Germany) and the other reagents of analytical grade were used.

Determination of hydrogen production and data processing

H_2 production determined by potentiometric method of applying redox (ORP) sensing system, using a pair titanium-silicate (Ti–Si) (EO-02, Gomel State Enterprise of Electrometric Equipment (GSEEE), Gomel, Belarus) and platinum (Pt) (EPB-1, GSEEE) ORP electrodes, as described previously [37,42,43,44]. The H_2 yield was calculated by the decrease of ORP to low negative values (≤–420 mV) in liquid and expressed in mmol H_2 per L of growth medium (mmol H_2 L^{-1}) [36,45,46]. Note, as a control experiment cells without any addition of carbon source were applied. In this case no H_2 generation has been detected. This is similar to Clark type electrode employed by Noguchi et al. [47] or H_2 electrode [48]. Cumulative H_2 yield was determined in 500 ml glass vessels under permanently stirring conditions; the gases (H_2 and CO_2) bubbled were treated by 1 M NaOH solution (to eliminate CO_2 from gas mixture); and further sole H_2 gas was collected and estimated by the water displacement volume and expressed in mL, as done before [14,33,40].

In addition, as a separate control experiment, H_2 gas during the growth of *E. coli* was visualized by the appearance of gas bubbles in the test tubes over the bacterial suspension using Durham tubes and verified by the chemical assay based on the bleaching of $KMnO_4$ solution in H_2SO_4, as described [35,37,42].

Each data point represented was averaged from independent triplicate cultures; the standard deviation, calculated as [35,45,46], was not more than 3% if they are not represented. The validity of differences between experimental and control data was evaluated by Student's criteria (p) [45,46]; $p < 0.01$ or less if this is not represented, otherwise $p > 0.5$ if the difference was not valid.

Results and discussion

H_2 production during utilization of beer spent grains (BSG) in E. coli wild type and Hyd-3 and Hyd-4 mutants

E. coli is able to anaerobically utilize different carbon sources (sugars, alcohols, organic acids etc.) and produce fermentation end products. One of them is H_2. It is well established that *E. coli* produces H_2 via multiple and reversible Hyd enzymes [29,35]. Current study describes the possibility of *E. coli* to produce H_2 via utilization of different wastes (BSG and DG) and their mixtures with or without glycerol and to identify which Hyd enzymes are responsible for H_2 production and the possibility to prolong and enhance H_2 generation.

First the BSG was treated by acid hydrolysis using sulfuric acid. Various concentrations (0.75–3%) of sulfuric acid were tested and the optimal one for further utilization of treated BSG and H_2 production was 1.5%. All experiments that were done with BSG have been treated with 1.5% sulfuric acid. After the treatment with sulfuric acid the pH was adjusted to pH 7.5 with KOH. Note, that when the pH was adjusted with K_2HPO_4 no H_2 production has been detected during *E. coli* growth (data not shown). It is important to mention that besides optimizing the treatment methodologies also concentrations of BSG were tested that could yield higher H_2 production. 4–30% of pretreated BSG was tested. In Figs. 1 and 2 data with 4% and 10% BSG only were shown, as higher concentrations repeated the data with 10%, demonstrating that no significant differences were found, and thus 4% of BSG was taken for further experiments as an optimal one. The optimal BSG concentration but not the sulfuric acid one used for pretreatment is in good conformity with previously obtained data [14].

It was shown that *E. coli* BW 25113 wild type strain grows very well on pretreated BSG with specific growth rate (μ) of

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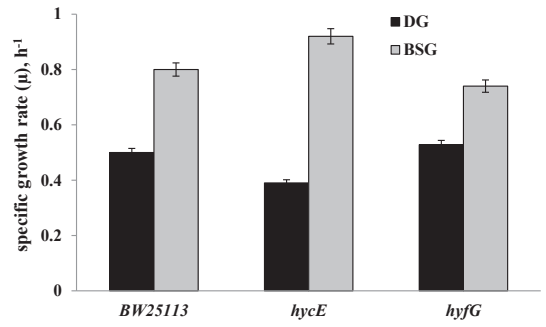

Fig. 1 – Specific growth rate (μ) of *E. coli* BW25113 wild type strain. Cells were grown in DG (10%) or BSG (4%) at 37 °C. For strains see Table 1, for others see Materials and Methods.

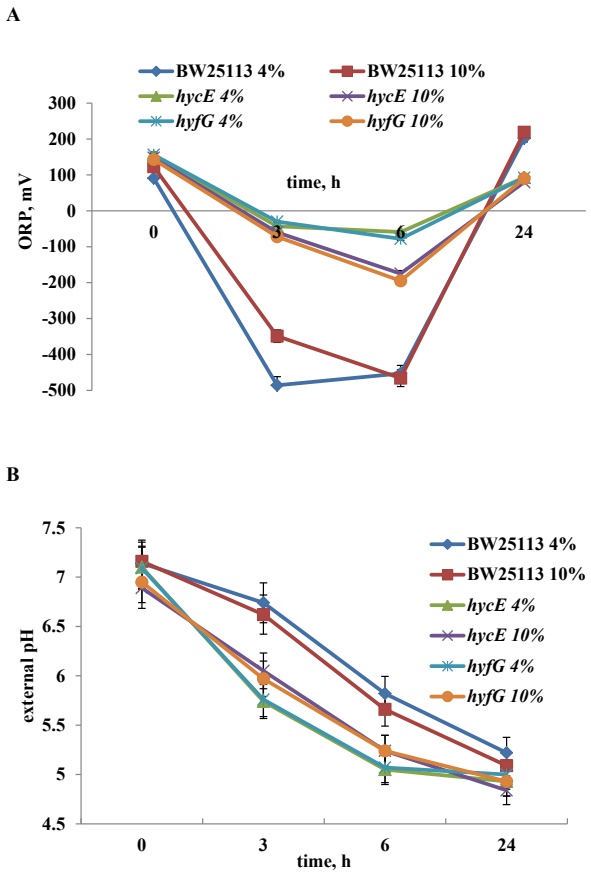

Fig. 2 – The ORP kinetics and H_2 production (A) and external pH (B) of *E. coli* wild type and mutants with defects in Hyd-3 and Hyd-4, grown in BSG medium with 4% and 10% at 37 °C at pH 7.0. ORP was measured by Pt redox electrode and expressed in mV (vs Ag/AgCl (saturated by KCl). For others see Materials and methods.

0.8 h^{-1} (Fig. 1). Earlier it has been detected that the same *E. coli* BW25113 μ was in the same range and can grow on BSG hydrolysate from Armenian beer factory [14]. Hyd-3 (*hycE*) and Hyd-4 (*hyfG*) single mutants (Table 1) had shown the same μ as in wild type. It can be suggested that in this conditions Hyd enzymes did not have significant impact on cell growth, as it was shown earlier on mixed carbon sources (glucose, glycerol and formate) fermentation where the cell growth was mostly dependent on Hyd-3 large subunit [39].

4% or 10% of BSG utilization in *E. coli* showed that H_2 production started from 3 h and lasted till 6 h of cell growth in batch cultures (Fig. 2A) with the yield of 1.4 mmol L^{-1}. Already on 24 h no H_2 production was detected which might be possible as the external or medium pH was decreased till pH 5.2 (Fig. 2B). It is important to mention that cell growth continued also after 24 h. Note, that at pH 5.2 or less H_2 generation in *E. coli* must be absent as the Hyd enzymes that are responsible for H_2 production are not active. It was experimentally determined that Hyd enzyme activity and function depends on external pH value [38,49,50] and thus it is suggested that when the pH of the external medium was adjusted, the H_2 production will be increased and could be prolonged. The differences between 4% or 10% BSG were absent and this suggests that by using less amount of BSG the same H_2 production rate can be achieved (Fig. 2) which is beneficial from economic point of view.

To identify which Hyd enzyme/s are responsible for H_2 production under the conditions tested Hyd-3 and Hyd-4 mutants were investigated as these two enzymes are main enzymes responsible for H_2 generation under different conditions during utilization of glucose [32,38]. The obtained data demonstrated that both enzymes are responsible for H_2 production (Fig. 2A). It is assumed that formate as an intermediate product is produced and further via these two enzymes is oxidized to H_2 and CO_2. For enhancement and prolongation of H_2 production it is important to regulate the formate metabolism and redirect it towards oxidation to H_2. The external pH of the medium in Hyd-3 and Hyd-4 mutants was lower by 0.2 units (Fig. 2B), compared to wild type, which might be due to produced weak acids such as formate that is not oxidized to H_2 and CO_2. During 24 h of growth cumulative H_2 production reached to 55 ml in wild type strain whereas in *hycE* or *hyfG* single mutants it was absent.

H_2 production during utilization of distiller grains (DG) in E. coli wild type and Hyd-3 and Hyd-4 mutants

Distiller grains (DG) are generated during alcohol production in huge amounts as BSG and it is important to try to apply it for production of high value products like biomass and bioenergy. For that purpose, DG was also pretreated in the same way as BSG and optimal concentration of sulfuric acid was again 1.5% but the DG concentration was optimal to be 10%.

When *E. coli* wild type strains were grown on DG μ was 0.5 h^{-1} (Fig. 1). This is lower by ~1.6 fold compared to the cells grown on BSG, and generally the obtained biomass on BSG was ~2–3 fold higher compared to DG. Interestingly, in *hycE* but not *hyfG* single mutant μ was 0.39 h^{-1} which is less by ~1.3 fold compared to wild type. Whereas when *hycE* was grown on BSG the μ was ~1.2 fold higher than in wild type (Fig. 1). The growth difference can be explained due to the composition of BSG and DG that affect Hyd enzymes and thus cell growth [8,11,12].

As was shown in Fig. 3, during growth of *E. coli* wild type cells on 4% DG, H_2 production was detected on 6 h but 10% or higher concentrations of DG had the same starting point of

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H_2 generation on 6 h and lasted till 24 h (Fig. 3A) and yielded 1.4 mmol L^{-1}. Hyd-3 and Hyd-4 mutants showed the same results as in the case of BSG. Both enzymes were responsible for H_2 generation under these conditions which suggest that besides Hyd-3 being major H_2 producing enzyme Hyd-4 also is active and contribute towards H_2 generation. Recently, it has been published that at pH 7.5 during 0.2% glucose fermentation Hyd-4 is not directly producing H_2 but contributing to it via supplying proton or electrons to Hyd-3 for H_2 generation [51]. It seems that either in BSG or DG the same principle is working for H_2 generation. When DG was applied cumulative H_2 production was less than BSG reaching up to 10 mL.

The external pH values also state that in Hyd-3 or Hyd-4 mutants it is less by 0.3 units when cells were grown on 4% DG and by 0.6 units when grown on 10% DG, compared to wild type. Again it is possible due to the weak acids produced such as formate and it is probable that general mechanism is the same for applying both wastes and producing H_2. As general mechanism exists for treating and further applying the BSG or DG wastes it could be good to apply the suggested technology for H_2 generation using mixtures of other industrial or agricultural wastes.

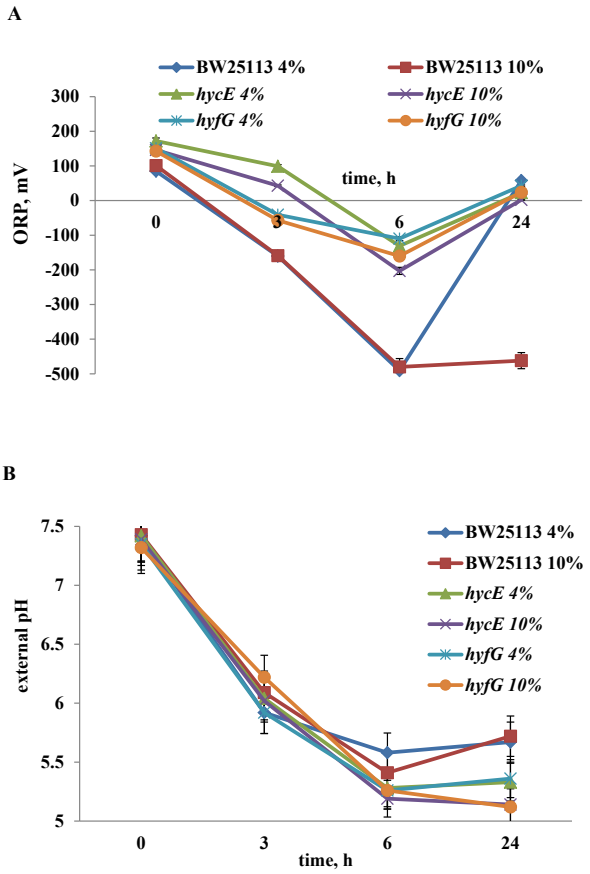

Fig. 3 – The ORP kinetics and H_2 production (A) and external pH (B) of *E. coli* wild type and mutants with defects in Hyd-3 and Hyd-4, grown in DG medium with 4% and 10% at 37 °C at pH 7.0. For others see Materials and methods and legends to Fig. 2.

Enhanced H_2 production during utilization of various mixtures of wastes in E. coli wild type and multiple mutant

To understand the possibility of H_2 production enhancement various mixtures of the wastes have been applied. Moreover, besides mixing the wastes multiple mutant was applied to see the possibility of optimization via genetic manipulations and mechanical mixing of wastes. It is important to mention that the multiple mutant was tested in sole glucose fermentative conditions [36] and this is the first time that it is applied in real waste to determine whether it can be used and enhanced H_2 production can be achieved. As in many productions different wastes are generated either they can be directly used in the same factories for bacterial biomass and bioenergy generation or transferred to the producing plants and as a feedstock mixed together [8]. For this DG and BSG wastes have been mixed and H_2 production was monitored in *E. coli* wild type strain.

Different concentrations of BSG and DG were mixed and increase of H_2 yield to 2.1 mmol L^{-1} (Fig. 5) in wild type cells was detected. Further the mixture of BSG or DG with glycerol was tested. In this case the multiple mutant and either BSG or BSG in mixture with glycerol has been applied (Fig. 4). It was shown that when multiple mutant was applied and BSG was utilized H_2 production was prolonged till 24 h compared to the wild type strain where it was produced till 6 h. Moreover, cumulative H_2 production was ~3.3 fold higher compared to

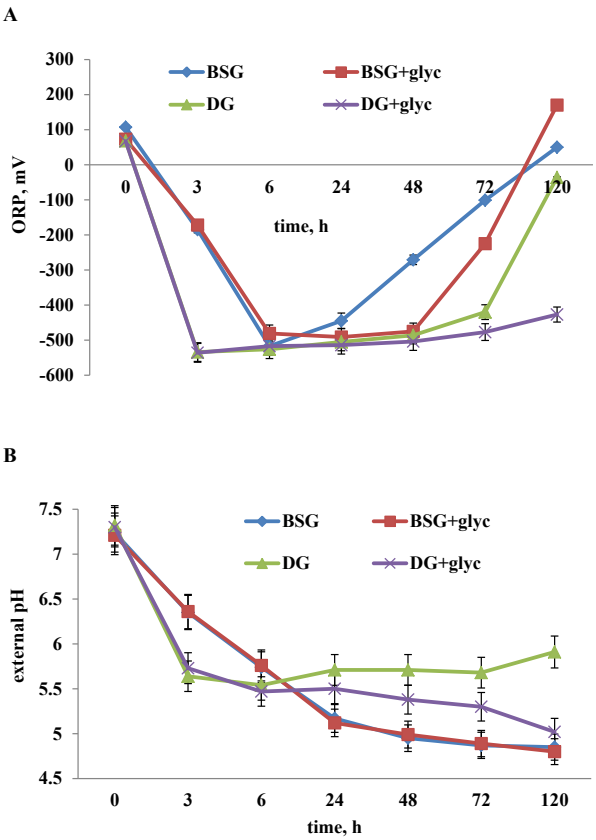

Fig. 4 – The ORP kinetics and H_2 production (A) and external pH (B) of *E. coli* multiple mutant (see Table 1) grown in BSG, DG medium or in mixture with glycerol. For others see Materials and methods.

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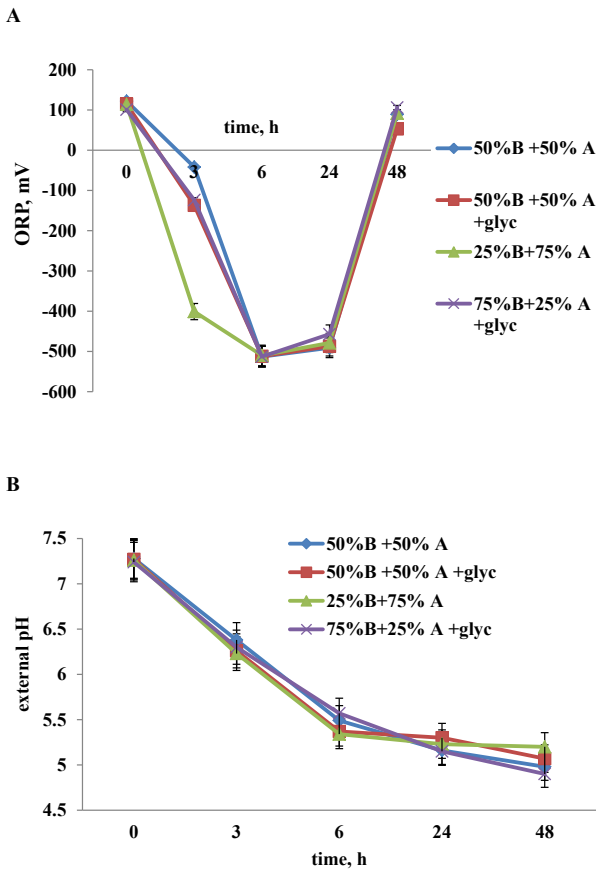

Fig. 5 – The ORP kinetics and H_2 production (A) and external pH (B) of *E. coli* wild type and mutants with defects in Hyd-3 and Hyd-4 grown in mix medium with BSG (4%) and DG (10%) at 37 °C at pH 7.5.50% B +50% A – is a mixture of 50% BSG and 50% DG; 50% B+50% A + glycerol (glyc) – is mixture of 50% BSG, 50% DG and 10 g L^{-1} glycerol; 25% B+75% A – is mixture of 25% BSG and 75% DG; 75% B+25% A + glyc – is mixture of 75% BSG, 25% DG and 10 g L^{-1} glycerol. For mixture variations stock solutions of 4% BSG and 10% DG were taken. For others see Materials and methods and legends to Fig. 2.

the wild type reaching up to 180 ml. Note, that external pH has the same values as in wild type.

To determine the effect of mixture of BSG and glycerol on H_2 production cells were grown on mixture of BSG and 10 g L^{-1} glycerol. The concentration of glycerol chosen has been shown to be optimal when applying only glycerol for H_2 production in *E. coli* wild type strains [33]. It was shown that in BSG + glycerol conditions in the multiple mutant H_2 production was prolonged and lasted till 48 h whereas cumulative H_2 production was less than in wild type. In wild type strain in BSG + glycerol conditions cumulative H_2 production reached 220 ml. The data might be explained with the differences of glucose and glycerol metabolism and further end products formation. The effect of different metabolites production and ratios might influence the yield and cumulative H_2 production which needs further separate study. As it was shown the multiple mutant strain is constructed for possible H_2 enhancement and redirection of formate towards H_2 generation when glucose is fermented [36]. The obtained data very well fits with the previously shown ones that during glycerol fermentative conditions Hyd-1 and Hyd-2 are the main enzymes responsible for H_2 generation and in multiple mutant Hyd-1 and Hyd-2 large subunits are absent [33].

When applying the multiple mutant with DG medium it was shown that H_2 production was prolonged till 72 h compared to the wild type where it was 24 h (Fig. 4A) and cumulative H_2 production was doubled reaching 20 ml. When DG or DG with glycerol was applied the H_2 production was prolonged till 72 or 120 h (Fig. 4A) respectively, compared to wild type strain lasting only 24 h. The external pH has shown significant difference in DG or DG with glycerol conditions compared to BSG or BSG with glycerol. The difference in multiple mutant between DG or DG with glycerol was 0.9 units (see Fig. 4B) which suggested that H_2 production in the mutant must be stopped. Moreover, cumulative H_2 production in wild type cells grown on DG with glycerol reached 80 ml and was 8 times higher compared to the cells grown only on DG. In contrast, when DG with glycerol mixture was applied H_2 production in the multiple mutant was the same as in DG conditions which is due to the lack of Hyd-1 and Hyd-2 as stated above [33,36]. Besides, the determined difference of the optimized conditions, multiple mutant application external pH changes, sole or mixtures of the organic substrates states that for each waste specific approach must be done: particularly when trying to mix glycerol or sugar containing wastes.

Conclusions and perspectives

Our results suggest that BSG and DG wastes or their mixtures with glycerol can be utilized by *E. coli* for production of bacterial biomass and bioenergy. The wastes need treatment prior to use by bacteria. Moreover, depending on the waste type H_2 production rate and time are different. The obtained data show the possibility of enhancement and prolongation of H_2 production by genetic manipulations and optimization of external parameters such as pretreatment, medium pH, mixtures of carbon sources and others. Particularly, the BSG or DG can be used for generation of biomass and further glycerol can be used in mixture with DG or BSG for production of H_2. The responsible Hyd enzymes (Hyd-3 and Hyd-4) for H_2 production have been determined and further application of multiple mutant enhanced and prolonged H_2 evolution ~3.3 fold when utilizing BSG. It is shown that BSG as feedstock is better than DG for biomass generation. When applying mixture of BSG or DG with glycerol, ~3.5 fold increase in cumulative H_2 production is determined. The data contribute to further developing and improving H_2 production technologies from organic wastes.

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