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Research Letter

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Roasted coffee wastes as a substrate for *Escherichia coli* to grow and produce hydrogen

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One sentence summary: Roasted coffee wastes can be applied for biomass and biohydrogen production by *Escherichia coli*. Responsible hydrogenase enzymes and enhanced hydrogen production have been detected.

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ABSTRACT

After brewing roasted coffee, spent coffee grounds (SCGs) are generated being one of the daily wastes emerging in dominant countries with high rate and big quantity. *Escherichia coli* BW25113 wild-type strain, mutants with defects in hydrogen (H_2)-producing/oxidizing four hydrogenases (Hyd) (Δ*hyaB* Δ*hybC*, Δ*hycE*, Δ*hyfG*) and septuple mutant (Δ*hyaB* Δ*hybC* Δ*hycA* Δ*fdoG* Δ*ldhA* Δ*frdC* Δ*aceE*) were investigated by measuring change of external pH, bacterial growth and H_2 production during the utilization of SCG hydrolysate. In wild type, H_2 was produced with rate of 1.28 mL H_2 (g sugar)$^{-1}$ h^{-1} yielding 30.7 mL H_2 (g sugar)$^{-1}$ or 2.75 L (kg SCG)$^{-1}$ during 24 h. In septuple mutant, H_2 production yield was 72 mL H_2 (g sugar)$^{-1}$ with rate of 3 mL H_2 (g sugar)$^{-1}$ h^{-1}. H_2 generation was absent in *hycE* single mutant showing the main role of Hyd-3 in H_2 production. During utilization of SCG wild type, specific growth rate was 0.72 ± 0.01 h^{-1} with biomass yield of 0.3 g L^{-1}. Genetic modifications and control of external parameters during growth could lead to prolonged and enhanced microbiological H_2 production by organic wastes, which will aid more efficiently global sustainable energy needs resulting in diversification of mobile and fixed energy sources.

Keywords: roasted coffee wastes; waste pretreatment; hydrogenases; biomass and bio-H_2 production

INTRODUCTION

Coffee is the most preferred drink worldwide and by its consumption rebates only petroleum (Murthy and Naidu 2012). It is grown in more than 70 countries and produces to over 16 billion pounds of coffee beans every year (Blinova *et al.* 2017), which is consumed as a soluble or instant coffee production, accompanied with by-products generation. While using them once and throwing, a huge amount of waste is generated and the presence of organic material is pollutant leftovers and furnishes a part of toxic nature (Campos-Vega *et al.* 2015).

Spent coffee grounds (SCGs) are the main coffee industry residues, with coffee silverskin (CS), obtained from different stages of coffee bean treatment (Mussatto *et al.* 2011a). SCG residue is generated from both soluble and instant coffee making, and waste quantity reaches over 6 million tons per year (Kovalcik, Obrucaa and Marova 2018). It is worth to note that ∼650 kg of SCGs are generated from 1 ton green coffee, and ∼2 kg

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from 1 kg of soluble coffee is produced (Murthy and Naidu 2012). After brewing, roasted coffee wastes (the solid residue emerged during the treatment of coffee with hot water or steam) can be considered as SCG (Getachew and Chun 2017). Its hydrolysate contains fermentable sugars, such as glucose, galactose, mannose, arabinose and cellobiose, which are polymerized into lignocellulose structures (cellulose 5–12 wt%, hemicellulose 40 wt%). It is richer than CS-containing material. Also proteins and fat constitute a significant fraction of SCGs (Scully, Jaiswal and Abu-Ghannam 2016) and have great applications in different scope of life starting from cosmetics to wastewater treatment possibilities, or as nutritional supplement in animal feed (Kovalcik, Obrucaa and Marova 2018), but still making some value from SCGs is needed, so studies are ongoing (Mussatto *et al.* 2011a). Besides the organic compounds, a variety of mineral elements are present in SCGs and CS ashes (Mussatto *et al.* 2011b).

It has been shown that raw materials or wastes can be applied to generate biogas, bioethanol, biomethane or other valuable chemicals (Batista *et al.* 2016). Besides methane, bio-H_2 can also be produced from various industrial or agricultural organic wastes (Poladyan *et al.* 2018; Prabakar *et al.* 2018). Biological H_2 production is found to be more environmentally friendly, renewable and less energy consuming compared to water electrolysis, natural gas reforming and coal gasification (Lee, Vermaas and Rittmann 2010; Trchounian, Sawers and Trchounian 2017). H_2 is used widely in fuel cells to generate electricity (Rahman *et al.* 2016), which results in decreasing greenhouse gases, and by increasing the use of raw material (organic waste) the positive impact for environment will be multiplied. H_2 can be produced by well-studied *Escherichia coli* via utilization of pretreated brewery spent grains (BSG) or distiller's grains (DG) (Sargsyan *et al.* 2016; Poladyan *et al.* 2018). In addition, glycerol is generated as a waste product during biodiesel production, which made it much more inexpensive in recent years (Clomburg and Gonzalez 2013; Trchounian and Trchounian 2015) and could be used solely or in mixture with various wastes as a feedstock for biomass and bioenergy or other products with added values (Trchounian, Sawers and Trchounian 2017). Interestingly, *E. coli* can utilize mixture of sugars and glycerol simultaneously (Wang *et al.* 2019). All this is important for applying in bio-based circular economy to control the application and usage of various organic wastes (D'Amato *et al.* 2017).

Escherichia coli performs mixed-acid fermentation and can convert sugars (glucose, arabinose, galactose, xylose etc.) or alcohols (glycerol) into different organic acids (e.g. lactic, succinic, acetic, formic acids), ethanol, H_2 and CO_2 (Trchounian, Sawers and Trchounian 2017; Zhu, San and Bennett 2020). In lower stages of glycolysis, H_2 is generated from formate via reversible membrane-bound hydrogenases (Hyd), which have been extensively studied (Hollinshead *et al.* 2016; Sargent 2016; Benoit *et al.* 2020). *Escherichia coli* has four hydrogenase (Hyd) enzymes (Hyd-1 to 4), where Hyd-1 (*hya*) induced in the presence of formate in stationary growth phase is responsible for pH switch from alkaline to acidic values (Trchounian *et al.* 2011, 2012), and Hyd-2 is active in exponential growth phase in anaerobic conditions and also responsible for H_2 production during glycerol fermentation at slightly alkaline pH (Trchounian and Trchounian 2009, 2015; Trchounian, Sawers and Trchounian 2017). Hyd-3 (*hyc*) is mainly responsible for H_2 production during sugar utilization (Maeda, Sanchez-Torres and Wood 2007a; Redwood *et al.* 2008; Petrosyan *et al.* 2020). H_2 is generated via Hyd-3 and Hyd-4 (*hyf*) forming formate hydrogen lyase (FHL) complexes with formate dehydrogenase H (FDH-H), but it depends on external pH and redox potential values (Mnatsakanyan, Bagramyan and Trchounian 2004; Trchounian 2015; Trchounian, Sawers and Trchounian 2017). By manipulating *E. coli* genes encoding different components of FHL complex, it was possible to enhance H_2 production yield from formate by ~2.8-fold with high *E. coli* cell density of 93 g L^{-1} with productivity of 300 L H_2 h^{-1} at 37°C in continuous reactor (Yoshida *et al.* 2005). Moreover, it has been shown that by introducing multiple mutations, it was possible to create septuple mutant (BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*fdoG* Δ*ldhA* Δ*frdC* Δ*aceE*; Table 1) for enhanced H_2 production from glucose. The strategy that has been applied was to redirect glucose mainly to formate generation that can be further oxidized by FHL complex to H_2 and CO_2 (Maeda, Sanchez-Torres and Wood 2007b).

Current paper describes the possibility of using SCG hydrolyaste solely or in the mixture with glycerol as a feedstock for bio-H_2 production by *E. coli* and generation of bacterial biomass. Moreover, Hyd enzymes responsible for H_2 evolution have been identified, and enhanced H_2 production has been obtained by applying appropriate genetically engineered mutant. This is significant for developing bio-based technologies for application of available various organic wastes and simultaneous generation of two different beneficial products—biomass and by-products such as H_2 (Ghimire *et al.* 2015; Maurya, Singla and Negi 2015).

MATERIALS AND METHODS

Bacterial strains, cell specific growth rate and yield, H_2 production determination

Escherichia coli wild type (wt) BW25113, Hyd mutants with defects in different Hyds and septuple mutant were used (see Table 1).

Bacteria were cultivated in anaerobic conditions in 500-mL glass bottles with pretreated SCGs growth medium. 3% (v/v) of night culture was added to same concentrations of growth medium. Bacterial optical density at 600-nm wavelength (OD_{600}) was measured by spectrophotometer (UV–VIS spectrophotometer, Cary 60, Agilent Technologies, USA) and biomass yield was estimated by determining the dry weight of culture and expressed in g L^{-1} (Poladyan *et al.* 2018). Bacterial specific growth rate (μ) was calculated as $\ln(OD_{600})$ h^{-1} taking the doubling values of exponential phase. Redox potential (ORP in mV) was determined by ORP electrode Pt BNC (HI3131, HANNA Instruments, Portugal), and pH by selective pH meter (HI1131, HANNA Instruments, Romania), as before (Trchounian *et al.* 2011). ORP electrode was calibrated by 16.13 g L^{-1} $K_3[Fe(CN)_6]$ and 21.12 g L^{-1} $K_4[Fe(CN)_6]$ (pH 6,86) reference solution, where the ORP was 254 ± 10 mV at 25°C (Trchounian and Trchounian 2009). Pt electrode is sensitive to H_2, and drop of ORP was detected during the utilization of substrate, and mineral composition does not affect ORP change and addition of H_2 does not change the external pH value (Piskarev *et al.* 2010). When ORP drops to strictly negative values (≥−400 mV), H_2 production is determined. This method is similar to Clark-type electrode, which was shown by Noguchi *et al.* (2010).

Cumulative H_2 production 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 (Poladyan *et al.* 2018). H_2 production rate was expressed as mL H_2 per g sugar utilized per h [mL H_2 (g sugar)$^{-1}$ h^{-1}] and H_2 yield—in mL per g

Table 1. Characteristics of bacterial strains used.

Strains	Genotype	Subunits lacking	Reference
BW25113	*laclq rrnBT*$_{14}$ Δ*lacZW116 hsdR514* Δ*araBAD*$_{AH33}$ Δ*rha BAD*$_{LD78}$	WT parental strain	Maeda, Sanchez-Torres and Wood 2007b
[a]MW1000	BW25113 Δ*hyaB* Δ*hybC*	Large subunits of Hyd-1, Hyd-2	Maeda, Sanchez-Torres and Wood 2007b
[a]JW2917	BW25113 Δ*hycE*	Large subunit of Hyd-3	Baba *et al.* 2006
[a]JW2472	BW25113 Δ*hyfG*	Large subunit of Hyd-4	Trchounian *et al.* 2011
[a]BW25113 *hyaB hybC hycA fdoG ldhA frdC aceE*	BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*fdoG* Δ*ldhA* Δ*frdC* Δ*aceE*	Large subunit of Hyd-1 and 2, repressor of FHL, α-subunit of formate dehydrogenase-N, lactate dehydrogenase, fumarate reductase, pyruvate dehydrogenase	Maeda, Sanchez-Torres and Wood 2007b

[a]Resistant to kanamycin.

sugar utilized [mL H_2 (g sugar)$^{-1}$]. H_2 yield was also calculated as L H_2 per kg SCG used and expressed as L H_2 (kg SCG)$^{-1}$.

SCG treatment and determination of total carbohydrates

SCGs generated from coffee preparation containing mainly Robusta with a little amount of Arabica have been obtained from Zanussi Necta Zenith (Italy) coffee making machines in Yerevan, Armenia. Afterward, SCGs (with slightly acidity and humidity of 70%) have been dried at 100°C till constant mass observed and suspended in distilled water. Mixture was exposed to pretreatment with acidic hydrolysis in an autoclave (WiseClave WACS-1100, Daihan Scientific, South Korea) at 121°C for 45 min (Fig. 1) Various concentrations of SCG (40–100 g L^{-1}) were tested, and optimal was detected to be 40 g L^{-1}. For pretreatment, 180 mg H_2SO_4 per g SCGs was applied (Kovalcik, Obrucaa and Marova 2018; Poladyan *et al.* 2018; Cesaro *et al.* 2020). The carbohydrate (sugars) content was determined with the method according to Dubois *et al.* (1956).

The pH of the SCG hydrolysate was adjusted to pH 7.0 by addition of 24 g L^{-1} K_2HPO_4. 13.6 g L^{-1} glycerol was added as a substrate in the SCG medium when indicated.

Reagents and data processing

Glycerol, K_2HPO_4 and H_2SO_4 (98%) and other reagents with analytical grade were used. The average data was figured by at least three experiments performed, the standard errors were considered, and Student criteria (P) were calculated to confirm the difference in average data between experiments performed; the difference was considered valid when $P < 0.05$ (Sargsyan *et al.* 2016; Poladyan *et al.* 2018).

RESULTS AND DISCUSSION

Escherichia coli growth during utilization of SCG

It is well known that *E. coli* can grow and perform mixed-acid fermentation on various organic carbon sources that depend on diverse environmental conditions such as pH, concentration of carbon source, ORP etc. *Escherichia coli* wt and all mutants tested were able to utilize SCGs and generate biomass. The growth and H_2 production by *E. coli* in SCG medium were studied till 96 h in batch cultures.

During assays, higher H_2SO_4 concentrations were found to inhibit both bacterial growth (Fig. 2) and H_2 production. Moreover, when SCG medium pH was adjusted by KOH, bacteria did not grow >6 h because of low pH (pH 4.9) generated (data not shown), and thus for further experiments K_2HPO_4 was used. Also 1, 2 and 5 times SCG hydrolysate dilutions were investigated, and 2 times dilution was revealed to be optimal (data not shown). The optimal conditions for both bacterial growth and H_2 production were found to be 40 g L^{-1} SCGs treated with 180 mg H_2SO_4 per g SCGs hydrolyzed in 121°C for 45 min, pH adjusted by 24 g L^{-1} K_2HPO_4 and final medium diluted twice (Fig. 1). Note that many pretreatment methods and technologies of organic wastes exist, and depending on the method chosen the yield of end product will be different (Kumari and Singh 2018).

Wt specific growth rate (μ) during SCG and glycerol co-fermentation was 0.68 ± 0.02 h^{-1} compared to that without glycerol where μ was 0.72 ± 0.01 h^{-1} (Fig. 3). Indeed, bacteria distinguish between the substrates to utilize at first due to activation of appropriate genes and metabolic networks (Wang *et al.* 2019). *hyaB hybC* double mutant demonstrated similar μ as in wt, suggesting that Hyd-1 and Hyd-2 role for bacterial growth was absent in the conditions used. This was in good conformity with previously obtained data from different groups for glucose fermentative conditions (King and Przybyla 1999; Pinske *et al.* 2012). However, *hyfG* and septuple mutants showed significantly lower μ ~2.8 and 2-fold decrease, respectively, in both conditions with and without glycerol, compared to wt (see Fig. 3). In *hycE*, single mutant μ was 0.42 ± 0.02 h^{-1}, which was ~1.7-fold lower compared to wt. Such a role of Hyd-3 and Hyd-4 enzymes might be interpreted if Hyd-3 and Hyd-4 are contributing toward H_2 cycling via Hyd enzymes and proton transport through proton ATPase (Trchounian and Sawers 2014) and their absence disturbs the H_2 cycling and proton transport and thus reduces μ. Note that similar reduction of μ in *hycE* mutant during fermentation of glucose, glycerol and formate was experimentally shown at pH 5.5 (Mirzoyan *et al.* 2018). Further study is required to elucidate the exact role of Hyd enzymes depending on the presence of carbon sources mixtures or organic wastes and change of external parameters.

H_2 production during utilization of SCG by *E. coli*

Escherichia coli wt produced 30.7 mL H_2 (g sugar)$^{-1}$ ($P < 0.03$) starting at 24 h pending not longer than 48 h with the rate of ~ 1.3 mL H_2 (g sugar)$^{-1}$ h^{-1}. To reveal how much H_2 was produced per kg

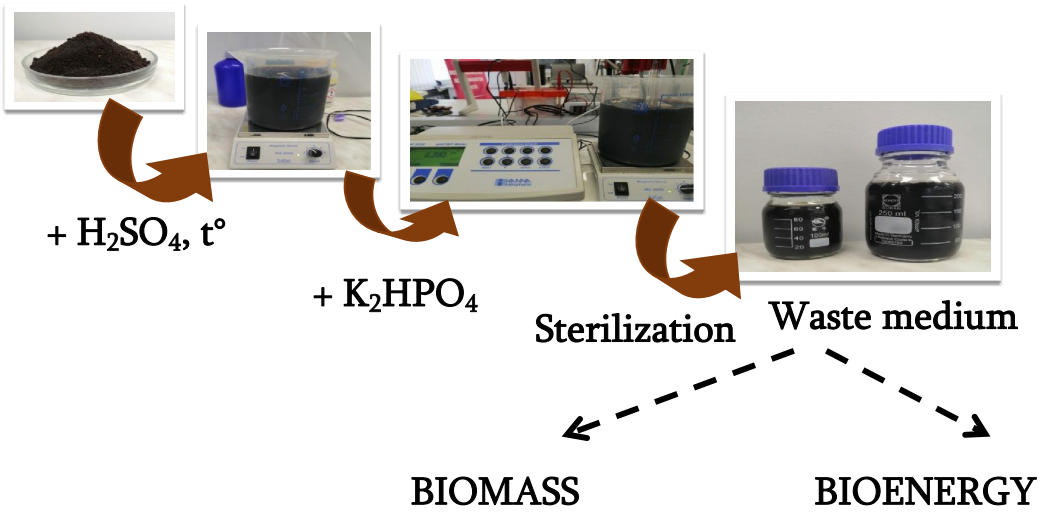

Figure 1. Pretreatment steps of SCG for biomass and bioenergy production. For details, see the Materials and methods section.

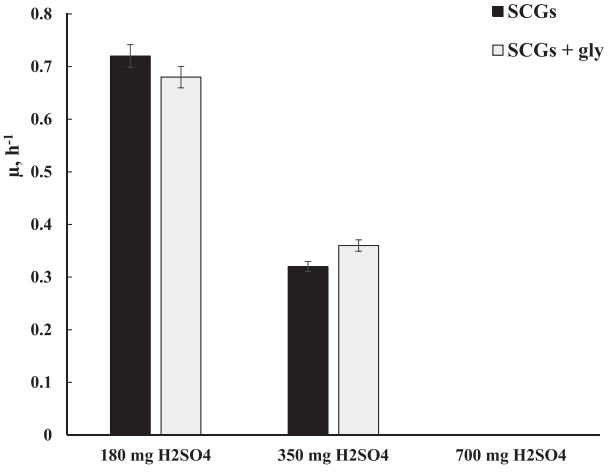

Figure 2. Specific growth rate of *E. coli* BW25113 wild type during utilization of 40 g L^{-1} of SCGs treated with 180 mg H_2SO_4 for 45 min, 350 mg H_2SO_4 for 20 min and 700 mg H_2SO_4 for 20 min, where H_2SO_4 concentrations were calculated for 1 g SCGs; gly is glycerol.

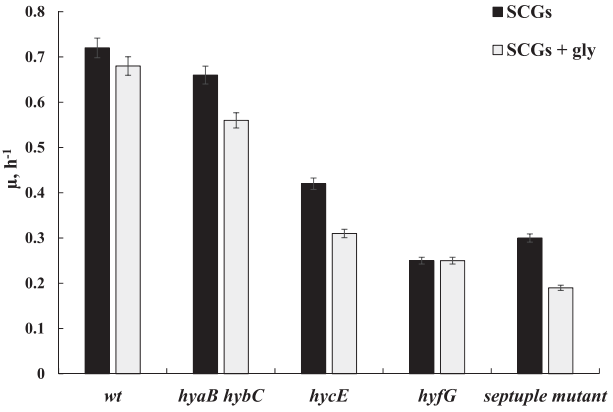

Figure 3. Specific growth rate of *E. coli* BW 25113 wild type (wt) and mutants during utilization of SCG. Septuple mutant: *hyaB hybC hycA fdoG ldhA frdC aceE* (see Table 1); for other details, see the Materials and methods section and legends to Fig. 2.

SCG used, it was calculated that *E. coli* wt cells yielded 2.75 L H_2 (kg SCG)$^{-1}$.

To understand which Hyd enzyme is responsible for H_2 production during utilization of SCG, different Hyd mutants (see Table 1) have been applied. This was important since Hyd enzymes are reversible and can work in different directions under various conditions including substrates (Mnatsakanyan, Bagramyan and Trchounian 2004; Trchounian 2015; Trchounian, Sawers and Trchounian 2017). Actually, similar maximum yields were observed in mutants used, besides *hycE* where H_2 production was absent. These data clearly showed that during anaerobic utilization of SCG Hyd-3 is the main Hyd enzyme responsible for H_2 production, which was demonstrated for sole glucose fermentation (Maeda *et al.* 2018; Petrosyan *et al.* 2020). In *hyfG* mutant, H_2 production was determined till 48 h, as in wt (Fig. 4). Addition of glycerol to SCG did not enhance H_2 production that has similar yields and rates as in SCG utilization only. It should be noted that various organic wastes and different bacteria have been applied to produce H_2 when different yields and rates have been obtained (Kapdan and Kargi 2006; Kumari and Singh 2018). Particularly, mixture of inoculum from anaerobic sludge that was applied has been reported to yield 56.47 mL H_2 (g VS)$^{-1}$ when using mixture of corn stalk, bean husk and municipal solid waste (Sekoai and Kana 2013). Recently, it was also shown that from food waste by anaerobic mixed cultures it was possible to enhance the H_2 production yield till 71.34 mL (g VS)$^{-1}$ (Rafieenia, Pivato and Lavagnolo 2019).

Furthermore, in septuple mutant, during the utilization of SCGs hydrolysate H_2 production yield was enhanced ~2-fold and reached 72 mL H_2 (g sugar)$^{-1}$ or 5.5 L H_2 (kg SCG)$^{-1}$ ($P < 0.03$) with H_2 production rate of ~ 3 mL H_2 (g sugar)$^{-1}$ h^{-1} at 48 h of growth. The H_2 yields observed in septuple mutant are higher compared to different bacteria and conditions, where mixed cultures were used. These data seem to be very promising as it will be possible to further increase the H_2 yield by optimizing the pretreatment methods and external parameters. Indeed, almost similar maximum H_2 yields were observed during SCGs and glycerol co-fermentation. The use of mixture with glycerol did not enhance H_2 generation in septuple mutant, as Hyd-1 and Hyd-2 were responsible for H_2 generation during glycerol fermentation (Trchounian and Trchounian 2009). It can be suggested that the

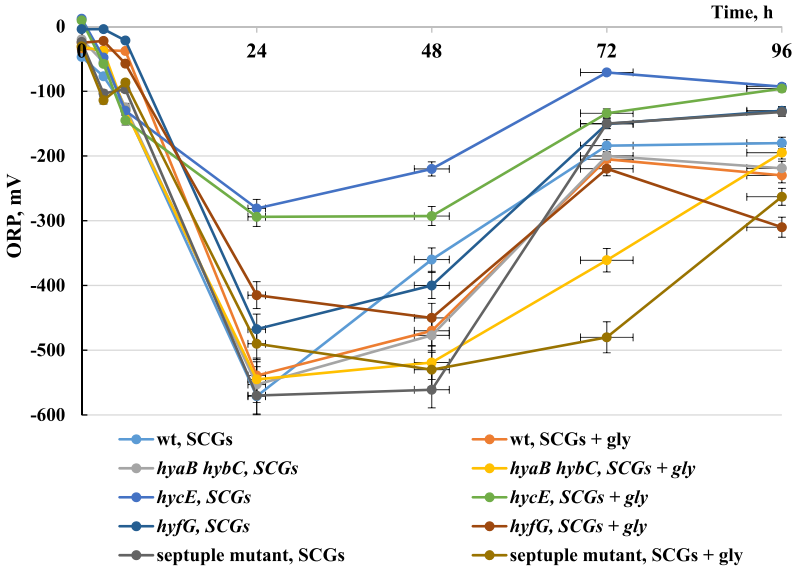

Figure 4. ORP and H_2 production during growth of *E. coli*. Bacteria were grown upon utilization of 40 g L^{-1} SCGs or SCGs + glycerol (SCGs + glyc) where indicated for 96 h at 37°C. For more details, see the Materials and methods section.

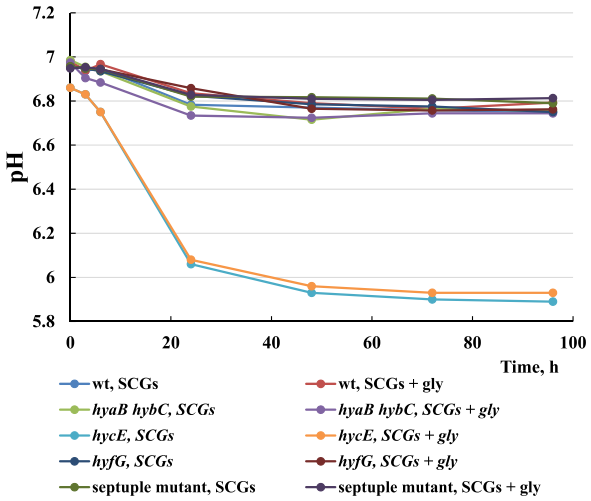

Figure 5. External pH during growth of *E. coli*. For more details, see the legends to Fig. 4.

engineered septuple mutant can be applied when sugars are utilized. The results are in good conformity with the initial strategy to construct the septuple mutant for enhanced H_2 production from sugars (Maeda, Sanchez-Torres and Wood 2007b). Further study is required to optimize the pretreatment methods and test them during scale-up.

The external pH in *hycE* mutant was dropped significantly by 0.9 units till pH of 5.9 compared to wt, and in *hyaB hybC* double and *hyfG* single mutants where the medium was acidified by 0.2–0.3 units till pH of 6.7 (Fig. 5). As it was shown, Hyd-3 was responsible for H_2 generation during fermentation and mainly formate, as one of the end products is formed and secreted contributing to the acidification of the external pH, whereas in *hycE* mutant the formate cannot be oxidized to H_2 and CO_2 or alternatively it can affect the secretion of formate. In addition, Hyd enzymes contribute to proton motive force generation during fermentation (Trchounian, Blbulyan and Trchounian 2013), and secretion of organic acids can generate pH gradient and/or membrane potential (Michels *et al.* 1979). It is suggested that one of the mechanisms of intra- and extracellular formate concentration regulation is related with maintenance of proton motive force. Such idea about the role of Hyd enzymes in addition to the existing one for organic acids had been reviewed recently (Trchounian and Trchounian 2019). The role of different Hyd enzymes on the generation of formate gradient and other organic acids during fermentation needs separate and more comprehensive study.

It is important to mention that the main goal of the research was focused on evaluating the possibility of utilization of SCG for biomass and biohydrogen production by *E. coli* wild type and further application of the engineered strain/s for enhancement of H_2 generation in the waste used. In addition, the obtained results were promising; thus, the next step will be the determination of fermentation end products that will add new valuable information for regulating cell growth and biohydrogen production.

CONCLUSIONS

Taken together, the results obtained have shown that SCGs hydrolyzed with H_2SO_4 for 45 min and with 2 times dilution were optimal conditions for *E. coli* to grow and produce H_2 with yield of ~31 mL H_2 (g sugar)$^{-1}$ or 2.75 L (kg SCG)$^{-1}$. Hyd-3 and Hyd-4 had influenced specific growth rate during utilization of SCG, which might be due to change of bioenergetic properties. *Escherichia coli* Hyd-3 was responsible for H_2 production, where deletion of Hyd-1 and Hyd-2 had no significant difference. H_2 production yield and rate were enhanced by ~2-fold in septuple mutant reaching ~72 mL H_2 (g sugar)$^{-1}$ or 5.5 L H_2 (kg SCG)$^{-1}$.

Bioconversion of organic wastes like SCGs that are generated in huge amounts, and the utilization of these sources for production of added-value products such as bio-H_2, is of great interest. The data can be used for further technological

optimization of coffee wastes as a source for not only biomethane and bioethanol but also biohydrogen production.

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REFERENCES

Baba T, Ara T, Hasegawa M *et al*. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* 2006;**2**:2006.0008.

Battista F, Fino D, Mancini G. Optimization of biogas production from coffee production waste. *Bioresour Technol* 2016;**200**: 884–90.

Benoit SL, Maier RJ, Sawers RG *et al*. Molecular hydrogen metabolism: a widespread trait of pathogenic bacteria and protists. *Microbiol Mol Biol Rev* 2020;**84**:e00092–19.

Blinová L, Sirotiak M, Bartošová A *et al*. Review: utilization of waste from coffee production. *Res Pap* 2017;**25**:91–101.

Campos-Vega R, Loarca-Pina G, Vergara-Castaneda H *et al*. Spent coffee grounds: a review on current research and future prospects. *Trends Food Sci Tech* 2015;**45**:24–36.

Cesaro A, Conte A, Carrère H *et al*. Formic acid pretreatment for enhanced production of bioenergy and biochemicals from organic solid waste. *Biomass Bioenerg* 2020;**133**:105455.

Clomburg J, Gonzalez R. Anaerobic fermentation of glycerol: a platform for renewable fuels and chemicals. *Trends Biotechnol* 2013;**31**:20–8.

D'Amato D, Droste N, Allen B *et al*. Green, circular, bio economy: a comparative analysis of sustainability avenues. *J Clean Prod* 2017;**168**:716–34.

Dubois M, Gilles KA, Hamilton JK *et al*. Colorimetric method for determination of sugars and related substances. *Anal Chem* 1956;**28**:350–6.

Getachew AT, Chun BS. Influence of pretreatment and modifiers on subcritical water liquefaction of spent coffee grounds: a green waste valorization approach. *J Clean Prod* 2017;**142**:3719–27.

Ghimire A, Frunzo L, Pirozzi F *et al*. A review on dark fermentative biohydrogen production from organic biomass: process parameters and use of by-products. *Appl Energy* 2015;**144**: 73–95.

Hollinshead WD, Rodriguez S, Martin HG *et al*. Examining *Escherichia coli* glycolytic pathways, catabolite repression, and metabolite channeling using Δ*pfk* mutants. *Biotechnol Biofuel* 2016;**9**:212.

Kapdan IK, Kargi F. Bio-hydrogen production from waste materials. *Enz Microb Technol* 2006;**38**:569–82.

King PW, Przybyla AE. Response of *hya* expression to external pH in *Escherichia coli*. *J Bacteriol* 1999;**181**:5250–6.

Kovalcik A, Obrucaa S, Marova I. Valorization of spent coffee grounds: a review. *Food Bioprod Process* 2018;**110**:104–19.

Kumari D, Singh R. Pretreatment of lignocellulosic wastes for biofuel production: a critical review. *Renew Sust Energy Rev* 2018;**90**:877–91.

Lee HS, Vermaas WF, Rittmann BE. Biological hydrogen production: prospects and challenges. *Trends Biotechnol* 2010;**28**: 262–71.

Maeda T, Sanchez-Torres V, Wood T. Enhanced hydrogen production from glucose by metabolically engineered *Escherichia coli*. *Appl Microbiol Biotechnol* 2007b;**77**:879–90.

Maeda T, Sanchez-Torres V, Wood T. *Escherichia coli* hydrogenase 3 is a reversible enzyme possessing hydrogen uptake and synthesis activities. *Appl Microbiol Biotechnol* 2007a;**76**: 1035–42.

Maeda T, Tran KT, Yamasaki R *et al*. Current state and perspectives in hydrogen production by *Escherichia coli*: roles of hydrogenases in glucose or glycerol metabolism. *Appl Microbiol Biotechnol* 2018;**102**:2041–50.

Maurya D, Singla A, Negi S. An overview of key pretreatment processes for biological conversion of lignocellulosic biomass to bioethanol. *Biotechnol* 2015;**5**:597–609.

Michels PA, Michels JP, Boonstra J *et al*. Generation of an electrochemical proton gradient in bacteria by the excretion of metabolic end products. *FEMS Microbiol Lett* 1979;**5**: 357–64.

Mirzoyan S, Vassilian A, Trchounian A *et al*. Prolongation of H_2 production during mixed carbon sources fermentation in *E. coli* batch cultures: new findings and role of different hydrogenases. *Int J Hydrogen Energy* 2018;**43**:8739–46.

Mnatsakanyan N, Bagramyan K, Trchounian A. Hydrogenase 3 but not hydrogenase 4 is major in hydrogen gas production by *Escherichia coli* formate hydrogenlyase at acidic pH and in the presence of external formate. *Cell Biochem Biophys* 2004;**41**:357–66.

Murthy P, Naidu M. Sustainable management of coffee industry by-products and value addition: a review. *Resour Conserv Recycl* 2012;**66**:45–58.

Mussatto S, Carneiro L, Silva J *et al*. A study on chemical constituents and sugars extraction from spent coffee grounds. *Carbohydr Polym* 2011b;**83**:368–74.

Mussatto S, Machado E, Martins S *et al*. Production, composition and application of coffee and its industrial residues. *Food Bioprocess Tech* 2011a;**4**:661–72.

Noguchi K, Riggins D, Eldahan K *et al*. Hydrogenase-3 contributes to anaerobic acid resistance of *Escherichia coli*. *PLoS One* 2010;**5**:e10132.

Petrosyan H, Vanyan L, Trchounian A *et al*. Defining the roles of the hydrogenase 3 and 4 subunits in hydrogen production during glucose fermentation: a new model of a H_2-producing hydrogenase complex. *Int J Hydrogen Energy* 2020;**45**: 5192–201.

Pinske C, McDowall JS, Sargent F *et al*. Analysis of hydrogenase 1 levels reveals an intimate link between carbon and hydrogen metabolism in *Escherichia coli* K-12. *Microbiology* 2012;**158**: 856–68.

Piskarev I, Ushkanov V, Aristova N *et al*. Establishment of the redox potential of water saturated with hydrogen. *Biophysics* 2010;**55**:13–7.

Poladyan A, Trchounian K, Vassilian A *et al*. Hydrogen production by *Escherichia coli* using brewery waste: optimal pretreatment of waste and role of different hydrogenases. *Renew Energy* 2018;**115**:931–6.

Prabakar D, Manimudi VT, Sampath S *et al*. Advanced biohydrogen production using pretreated industrial waste: outlook and prospects. *Renew Sust Energy Rev* 2018;**96**:306–24.

Rafieenia R, Pivato A, Lavagnolo MC. Optimization of hydrogen production from food waste using anaerobic mixed cultures pretreated with waste frying oil. *Renew Energy* 2019;**139**:1077–85.

Rahman S, Masdar M, Rosli M *et al*. Overview biohydrogen technologies and application in fuel cell technology. *Renew Sust Energ Rev* 2016;**66**:137–62.

Redwood MD, Mikheenko IP, Sargent F *et al*. Dissecting the roles of *Escherichia coli* hydrogenases in biohydrogen production. *FEMS Microbiol Lett* 2008;**278**:48–55.

Sargent F. The model [NiFe]-hydrogenases of *Escherichia coli*. *Adv Microb Physiol* 2016;**68**:433–507.

Sargsyan H, Trchounian K, Gabrielyan L *et al*. Novel approach of ethanol waste utilization: biohydrogen production by mixed cultures of dark- and photo-fermentative bacteria using distillers grains. *Int J Hydrogen Energy* 2016;**41**:2377–82.

Scully D, Jaiswal A, Abu-Ghannam N. An investigation into spent coffee waste as a renewable source of bioactive compounds and industrially important sugars. *Bioengineer* 2016;**3**:33.

Sekoai PT, Kana EG. A two-stage modelling and optimization of biohydrogen production from a mixture of agro-municipal waste. *Int J Hydrogen Energy* 2013;**38**:8657–63.

Trchounian A, Sawers GR. Novel insights into the bioenergetics of mixed-acid fermentation: can hydrogen and proton cycles combine to help maintain a proton motive force? *IUBMB Life* 2014;**66**:1–7.

Trchounian A, Trchounian K. Fermentation revisited: how do microorganisms survive under energy-limited conditions? *Trends Biochem Sci* 2019;**44**:391–400.

Trchounian A. Mechanisms for hydrogen production by different bacteria during mixed-acid and photo-fermentation and perspectives of hydrogen production biotechnology. *Crit Rev Biotechnol* 2015;**35**:103–13.

Trchounian K, Blbulyan S, Trchounian A. Hydrogenase activity and proton-motive force generation by *Escherichia coli* during glycerol fermentation. *J Bioenerget Biomembr* 2013;**45**:253–60.

Trchounian K, Poladyan A, Vassilian A *et al*. Multiple and reversible hydrogenases for hydrogen production by *Escherichia coli*: dependence on fermentation substrate, pH and the F_OF_1-ATPase. *Crit Rev Biochem Mol Biol* 2012;**47**:236–49.

Trchounian K, Sanchez-Torres V, Wood T *et al*. *Escherichia coli* hydrogenase activity and H_2 production under glycerol fermentation at a low pH. *Int J Hydrogen Energy* 2011;**36**:4323–31.

Trchounian K, Sawers GR, Trchounian A. Improving biohydrogen productivity by microbial dark-and photo-fermentations: novel data and future approaches. *Renew Sust Energy Rev* 2017;**80**:1201–16.

Trchounian K, Trchounian A. Hydrogenase 2 is most and hydrogenase 1 is less responsible for H_2 production by *Escherichia coli* under glycerol fermentation at neutral and slightly alkaline pH. *Int J Hydrogen Energy* 2009;**34**:8839–45.

Trchounian K, Trchounian A. Hydrogen production from glycerol by *Escherichia coli* and other bacteria: an overview and perspectives. *Appl Energy* 2015;**156**,174–84.

Wang X, Xia K, Yang X *et al*. Growth strategy of microbes on mixed carbon sources. *Nat Commun* 2019;**10**:1–7.

Yoshida A, Nishimura T, Kawaguchi H *et al*. Enhanced hydrogen production from formic acid by formate hydrogen lyase-overexpressing *Escherichia coli* strains. *Appl Environ Microbiol* 2005;**71**:6762–8.

Zhu F, San K, Bennett G. Improved succinate production from galactose-rich feedstocks by engineered *Escherichia coli* under anaerobic conditions. *Biotechnol Bioengineer* 2020;**117**:1082–91.