Bio-hydrogen production by co-digestion of domestic wastewater and biodiesel industry effluent

Jyotsana Prakash¹,²,³, Rakesh Sharma², Sanjay K. S. Patel¹, In-Won Kim¹*, Vipin Chandra Kalia¹,²,³*

¹ Department of Chemical Engineering, Konkuk University, Seoul, Republic of Korea, ² CSIR–Institute of Genomics and Integrative Biology (IGIB), Delhi University Campus, Delhi, India, ³ Academy of Scientific & Innovative Research (AcSIR), Anusandhan Bhawan, New Delhi, India

* vckaliaku@gmail.com (VCK); inwon@konkuk.ac.kr (IWK)

Abstract

The increasing water crisis makes fresh water a valuable resource, which must be used wisely. However, with growing population and inefficient waste treatment systems, the amount of wastewater dispelled in rivers is increasing abominably. Utilizing this freely available waste-water along with biodiesel industry waste- crude glycerol for bio-hydrogen production is being reported here. The bacterial cultures of Bacillus thuringiensis strain EGU45 and Bacillus amyloliquefaciens strain CD16 produced 2.4–3.0 L H₂/day/L feed during a 60 days continuous culture system at hydraulic retention time of 2 days. An average H₂ yield of 100–120 L/L CG was reported by the two strains. Recycling of the effluent by up to 25% resulted in up to 94% H₂ production compared to control.

Introduction

Availability of clean water is a worldwide crisis. Despite our earth surface being covered with 70% of water, only 2% is a freshwater, of which 3/4th is frozen and unavailable for human consumption [1, 2]. Thus, billions of people live with severe water scarcity and poor sanitation. The small amount of available fresh water faces an allocation and competition in agricultural, industrial and municipal sectors. As a result, allocating this sparsely available fresh water to bioenergy production is a very costly affair [3]. During bioenergy production, the substrate occupies 10% of the medium while the rest is water. This water used in most of the studies is distilled and the medium is sterilized [4–6]. Since most of the population struggles for fresh water for their daily basic needs, it would be unethical to divert it towards increasing energy demands. The possible solution would be to use wastewater that is generated from domestic and industrial sources. As per the 2016 report published by International Institute of Health and Hygiene, in metro cities like New Delhi (India) about 6.1×10⁴ million liters (ML) of wastewater is generated every day. The treatment capacity is around 50% only (http://www.sulabhenvis.nic.in/Database/STST_wastewater_2090.aspx). The rest of the wastewater is drained into the rivers or can meet a less dreadful fate if utilized e.g., for bioenergy production.
The surplus availability of wastewater makes it a timeless resource for the researchers struggling with cheap and steady bioenergy generation [7].

Bioenergy being a sustainable alternative to fossil fuels has attracted a huge worldwide support. Hydrogen (H₂), Methane (CH₄), ethanol, bio-diesel are amongst the most widely studied bio-fuels. H₂ however has gained immense favors owing to its high calorific value and cleaner combustion [8, 9]. Most extensively studied technique for biological H₂ production is dark fermentation and is most likely to be commercialized in near future [10]. A variety of organic wastes have been used successfully as substrate for H₂ production. This substrate is mostly present along with some minerals in distilled water [11]. The challenge is thus to replace the valuable clean water with readily available domestic wastewater for H₂ production. H₂ production from various industrial wastewaters such as cassava starch processing wastewater, brown sugar wastewater, paperboard mill wastewater, ethanol wastewater, etc. have been reported [12–18]. Sugar rich wastewaters such as molasses wastewater, sugarbeet wastewater and sugarcane vinasse have the ability to produce high H₂ yields of around 3.2 mol/mol substrate. Starchy wastewaters generally result in relatively lower H₂ yields of around 1.9 mol/mol substrate [17, 19, 20]. Wastewaters from biodiesel industry, which are rich in glycerol also have a potential to produce bioenergy [21, 22]. Use of crude glycerol (CG) as feed prepared in distilled water resulted in 165 L H₂/L CG by an immobilized biofilm forming bacteria B. amyloliquefaciens [23]. The use of different industrial wastewaters as medium may not be available throughout the year and may thus hinder the continuity of bio-H₂ production. In contrast, domestic wastewater which is generated everyday throughout the globe may be a better option to counter this problem. Therefore, in the present study we have used freely available domestic wastewater as the medium and biodiesel industry waste- CG as the substrate for an economic bioenergy generation. To further improve the production efficiency, recycling of the effluent is also reported.

Material and methods
Organisms and growth conditions

Hydrogen producers. Bacillus amyloliquefaciens strain CD16 (KX348272) and B. thurin-giensis strain EGU45 (DQ508971) were isolated in our laboratory [23]. These were grown on Himedia nutrient broth (NB) at 37˚C with stirring at 200 rpm for 16 h. The media also contained CG (2%, v v⁻¹) and the cultures were thus adapted to the substrate for 5 cycles. The cultures were then used as inoculum at the rate of 10 μg cellular protein mL⁻¹ [6].

Hydrogen production

Immobilization of cells on lignocellulosic support material. Coconut coir (CC) was dried and packed in PVC tubes to prepare cartridges (3 x 2 cm) containing 3 g coir each, as reported earlier [6]. These cartridges were used as support material for bacterial immobilization. Aspirator bottles (1.2 L) with working volume of 1.0 L were used to perform the experiments. In order to allow the growth of biofilm on cartridges, casein enzyme hydrolyzate (CEH) was used as a biofilm forming media [21]. In bottles containing different amounts of cartridges (5–15%, vv⁻¹), 100 mL of CEH was added. Each cartridge occupied ~10 mL of the 1.0 L working volume used. Thus, 5, 10 and 15 cartridges were used for 5%, 10% and 15% CC reactors. Free floating (FF) cultures were used as controls. Inoculation with CG acclimatized cultures was done at the rate of 10 μg cell protein mL⁻¹. Anaerobic conditions were maintained by flushing the reactors with argon gas. The bottles were incubated at 37˚C for 24 h without shaking, to allow biofilm formation. Domestic waste water diluted with tap water in the ratio of 3:1 was used as H₂ producing media (DWW). Minimal salts (KH₂PO₄- 1.5 g/L, NaCl- 0.25 g/L, NH₄Cl- 0.5 g/L, MgSO₄(1.0 M)- 0.5 mL/L, CaCl₂(1.0 M)- 0.5 mL/L) were added to the
DWW media. After 24 h biofilm growth on cartridges in aspirator bottles, DWW containing CG (2%, v v⁻¹) was used to complete the remaining working volume.

**Batch culture.** The aspirator bottles containing hydrogen producers immobilized on CC (5–15%, v v⁻¹) in CG supplemented DWW media were made air tight using glass stoppers after adjusting the pH to 7.0. The pH was adjusted using NaOH (2.0 N) or HCl (2.0 N) after which argon flushing was given to maintain anaerobic environment inside the aspirator bottles. These were incubated at 37˚C. A provision for gas outlet and liquid sampling was provided in the bottles. On a daily basis, the gas evolved was collected and analyzed. Adjustment of pH and argon flushing was also done on a daily basis until the gas production ceased. After this the fermentation was switched to continuous mode.

**Continuous culture.** For the continuous culture digestion, a hydraulic retention time (HRT) of 2 days was used. On a daily basis, 500 mL of the effluent was removed from each reactor and was replenished with fresh DWWTW media containing CG (2%, v v⁻¹). Adjustment of pH and argon flushing was done daily, and the evolved gas was analyzed. Incubation was done at 37˚C and the process was continued for 60 days to obtain steady gas production. The experiments were performed in triplicates.

**Recycling of effluent.** During the continuous H₂ production effluent was generated daily. This effluent was further used for H₂ production by mixing it with fresh DWW medium indifferent ratios: (i) 1:3, (ii) 1:1, and (iii) 3:1. The gas production was compared with the controls and the process was continued for an additional 60 days. The support material used in all these reactors contained 15%(v v⁻¹) CC.

**Analytical methods**

**Gas analysis.** Water displacement method was used to determine the volume of biogas produced. The composition of gas was analyzed using gas chromatography (Nucon GC5765, India) equipped with molecular sieve and Porapak-Q columns (1.8 m long and 2 mm inner diameter) and a thermal conductivity detector, as reported earlier [6, 24]. For the daily fed culture experiments, H₂ yields were calculated on the glycerol fed basis.

**Glycerol estimation.** The amount of residual glycerol in the fermented medium was estimated by taking 1 mL of the sample. It was centrifuged at 10,000 g for 5 min. Supernatant (1μL) was injected into Gas chromatograph (Nucon GC5765, India) and analyzed under standard conditions as described earlier [6].

**Results and discussion**

The effectiveness of biofilm forming *B. amyloliquefaciens* strain CD16 for a high and steady continuous H₂ production has been reported [23]. However, the medium used in these studies is sterile distilled water. This increases the production cost for bio-energy generation. The medium thus used in present study is unsterile domestic waste diluted with tap water.

**Cell immobilization**

Several support materials have been widely used for bacterial immobilization. These may include activated carbon, alginate gel, polyester fiber, porous glass beads, egg shells and lignocellulosic wastes such as banana leaves, groundnut shells, coconut coir, bamboo stem, etc.[25–28]. Apart from these, biofilms as natural cell entrapment strategy has also gained importance. Biofilms although have been extensively utilized for bioremediation is also gaining interest with bioenergy production [29, 30]. With the availability of medium that can screen biofilm formers, biofilm forming H₂ producers have been isolated [21, 23]. One of these biofilm formers has been utilized for H₂ production using wastewater in the present study. After a 24 h
incubation, in the reactors inoculated with *B. amyloliquefaciens* strain CD16, biofilm formation was observed on CC cartridges. While in case of *B. thuringiensis* strain EGU45 no biofilm was formed. The cells immobilized in biofilm are resistant to environmental stresses and thus, may provide a better robust environment for gas production.

**Batch culture H₂ production**

The total biogas produced during 5 days batch fermentation by *B. thuringiensis* strain EGU45 ranged from 2.0 L to 3.1 L. The biogas constituted a mixture of H₂ and CO₂. The H₂ in the produced gas constituted 56.2–70.2%. With *B. amyloliquefaciens* strain CD16, 2.4 L - 3.3 L biogas was produced which consisted of 58.3–60.0% H₂ (Table 1). When comparing the H₂ yield, 55 L H₂/L CG to 110 L H₂/L CG was produced by *B. thuringiensis* strain EGU45 while with *B. amyloliquefaciens* strain CD16, 70 L H₂/L CG—100 L H₂/L CG was obtained. The results obtained with DWWTW were strikingly similar to that obtained with sterile M-9 medium [23]. This shows that there are no deteriorating effects of using unsterile waste water as medium for biogas production under batch conditions.

**Continuous culture H₂ production**

For an economical large-scale bioenergy production, continuous culture fermentation is required. However, despite being an ideal system for higher product yields, cell washout is a major concern of this mode of fermentation. To deal with the problem, a number of bacterial support materials have been utilized. Recently, 1.18-fold increase in H₂ production by using biofilm immobilized on lignocellulosic wastes has been reported [23].

Similar strategy when applied to prevent cell washout from waste water medium in the current work resulted in encouragingly higher H₂ yields during 60 days continuous fermentation. Without any cell immobilization, i.e. FF conditions, the biogas production showed a significant decline with both the strains (Figs 1 and 2, and S1 and S2 Tables). In case of *B. thuringiensis* strain EGU45, from an average of 0.6 L H₂/0.5 L feed/day during initial 10 days of

| Support material (%) | Biogas (mL) | Hydrogen Volume (mL) | Yield |
|----------------------|-------------|----------------------|-------|
|                      |             |                      | %     |
| *Bacillus thuringiensis* EGU45 |
| 0                    | 2000        | 1125                 | 56.2  | 0.22 |
| 5                    | 2850        | 1755                 | 61.5  | 0.35 |
| 10                   | 2945        | 1775                 | 60.2  | 0.35 |
| 15                   | 3140        | 2205                 | 70.2  | 0.44 |
| *Bacillus amyloliquefaciens* CD16 |
| 0                    | 2400        | 1400                 | 58.3  | 0.28 |
| 5                    | 3180        | 1930                 | 60.0  | 0.38 |
| 10                   | 3370        | 2015                 | 59.7  | 0.40 |
| 15                   | 3000        | 1925                 | 64.1  | 0.38 |

*a*coconut coir.

*b*mixture of H₂ + CO₂.

*c*mol mol⁻¹ crude glycerol utilized.

Feed: Sewage water medium diluted with Tap water in 3:1 ratio (M-9 salts: 0.5 X) with crude glycerol (2%, v/v).

Inoculum: 10 µg cell protein mL⁻¹ feed. Values represent 5 days of batch fermentation. All experiments were performed in triplicate. The standard deviation was less than 10%

https://doi.org/10.1371/journal.pone.0199059.t001
fermentation, the production declined to 0.06 L H\(_2\) / 0.5 L feed/day at the end of 30 days of fermentation. The gas production thereafter became so low that the reactors had to be terminated (Table 2). Similar was the case observed with biofilm forming \(B. amyloliquefaciens\) strain CD16, where the production declined from 0.8 L H\(_2\) / 0.5 L feed/day to 0.07 L H\(_2\) / 0.5 L feed/day during 30 days of fermentation and ceased thereafter (Table 2).

Effect of support material on biogas production could be clearly seen with its increasing quantity from 5–15% CC (v/v\(^{-1}\)). At 5% CC, non-biofilm former \(B. thuringiensis\) strain EGU45 produced 0.7 L H\(_2\) / 0.5 L feed/day for 30 days after which it reduced to 0.4 L H\(_2\) / 0.5 L feed/day and continued till 60 days maintaining a stable yield of 0.16–0.18 mol H\(_2\) / mol CG. On increasing the support material to 10% CC, 0.8 L H\(_2\) / 0.5 L feed/day was observed during initial 10 days of continuous culture. It increased thereafter and maintained a stable value of around 0.95–1.2 L H\(_2\) / 0.5 L feed/day during 60 days fermentation. The average H\(_2\) production with 10% CC was 2.28-fold higher than with 5% CC (Table 3). On increasing the support material to 15% CC, a higher and stable H\(_2\) of 1.2–1.3 L / 0.5 L feed/day was produced during 60 days continuous fermentation. This corresponds to 0.48–0.53 mol H\(_2\) / mol CG which was 1.23-fold higher than with 10%CC. Similar effect of increasing gas production with support material was also seen with biofilm former \(B. amyloliquefaciens\) strain CD16. At 5% CC, 1.0–1.2 L H\(_2\) / 0.5 L feed/day was produced during 0–30 days of fermentation which achieved a steady 0.7–0.8 L H\(_2\) / 0.5 L feed/day during 31–60 days of fermentation. At 10% CC, the gas production maintained a steady biogas production constituting 61.1–62.9% H\(_2\) throughout 60 days of fermentation. The amount of H\(_2\) varied from 1.1–1.3 L /0.5 L feed/day. This was 1.55-fold higher
Table 2. Hydrogen production from sewage water and crude glycerol by different *Bacillus* species immobilized on lignocellulosic waste and the effect recycling of the effluent: Continuous culture.

| Support material (%) | 0     | 5     | 10    | 15    |
|----------------------|-------|-------|-------|-------|
|                      | DAI   | Vol[^a] | %     | Yield[^b] | Vol[^a] | %     | Yield[^b] | Vol[^a] | %     | Yield[^b] |
| **Bacillus thuringiensis EGU45** |       |        |       |         |       |        |         |       |        |         |
| 0–10                 | 595   | 41.0   | 0.23  | 755     | 58.7   | 0.30   | 865     | 62.4   | 0.34   | 1200    | 65.2   | 0.48   |
| 11–20                | 215   | 37.0   | 0.08  | 755     | 58.3   | 0.30   | 1165    | 64.0   | 0.46   | 1330    | 64.4   | 0.53   |
| 21–30                | 60    | 57.1   | 0.02  | 780     | 56.1   | 0.31   | 1245    | 65.6   | 0.50   | 1225    | 59.4   | 0.49   |
| 31–40                | -     | -      | -     | 545     | 54.5   | 0.21   | 1115    | 65.0   | 0.44   | 1305    | 66.5   | 0.52   |
| 41–50                | -     | -      | -     | 415     | 44.6   | 0.16   | 1085    | 64.3   | 0.43   | 1265    | 59.1   | 0.50   |
| 51–60                | -     | -      | -     | 465     | 56.3   | 0.18   | 955     | 64.0   | 0.38   | 1265    | 63.5   | 0.50   |
| **Bacillus amyloliquefaciens CD16** |       |        |       |         |       |        |         |       |        |         |
| 0–10                 | 860   | 60.7   | 0.34  | 1015    | 59.7   | 0.40   | 1130    | 62.9   | 0.45   | 1255    | 62.5   | 0.50   |
| 11–20                | 550   | 67.4   | 0.22  | 1260    | 63.3   | 0.50   | 1230    | 62.5   | 0.49   | 1395    | 64.2   | 0.56   |
| 21–30                | 75    | 62.5   | 0.03  | 1065    | 59.4   | 0.42   | 1300    | 61.1   | 0.52   | 1580    | 65.8   | 0.63   |
| 31–40                | -     | -      | -     | 810     | 57.8   | 0.32   | 1330    | 62.4   | 0.53   | 1475    | 63.8   | 0.59   |
| 41–50                | -     | -      | -     | 790     | 64.2   | 0.31   | 1280    | 62.7   | 0.51   | 1510    | 63.7   | 0.60   |
| 51–60                | -     | -      | -     | 760     | 72.0   | 0.30   | 1120    | 61.8   | 0.45   | 1450    | 66.2   | 0.58   |

**Effluent recycling (%)**

| DAI | 0     | 25    | 50    | 75    |
|-----|-------|-------|-------|-------|
| **Bacillus thuringiensis EGU45** |       |       |       |       |
| 0–10| 1280  | 61.7  | 0.50  | 1345  | 65.7  | 0.54  | 1040  | 71.2  | 0.41  | 620   | 74.2  | 0.24  |
| 11–20| 1310  | 62.5  | 0.52  | 1170  | 58.7  | 0.47  | 680   | 57.6  | 0.27  | 265   | 60.2  | 0.10  |
| 21–30| 1280  | 61.6  | 0.51  | 1135  | 58.2  | 0.45  | 345   | 57.5  | 0.13  | 100   | 55.5  | 0.04  |
| 31–40| 1180  | 61.6  | 0.47  | 955   | 48.9  | 0.38  | 300   | 58.0  | 0.12  | 90    | 45.0  | 0.03  |
| 41–50| 995   | 60.3  | 0.40  | 910   | 58.5  | 0.36  | -     | -     | -     | -     | -     | -     |
| 51–60| 985   | 63.3  | 0.39  | 840   | 60.0  | 0.33  | -     | -     | -     | -     | -     | -     |
| **Bacillus amyloliquefaciens CD16** |       |       |       |       |
| 0–10| 1445  | 60.4  | 0.58  | 1460  | 57.4  | 0.58  | 1185  | 54.6  | 0.47  | 840   | 54.3  | 0.33  |
| 11–20| 1480  | 62.1  | 0.59  | 1265  | 57.2  | 0.50  | 940   | 56.4  | 0.37  | 370   | 52.4  | 0.14  |
| 21–30| 1285  | 61.0  | 0.51  | 1110  | 58.2  | 0.44  | 390   | 53.4  | 0.15  | 130   | 52.0  | 0.05  |
| 31–40| 1395  | 61.0  | 0.56  | 1030  | 59.7  | 0.41  | 55    | 55.0  | 0.02  | 200   | 45.9  | 0.08  |
| 41–50| 1230  | 61.8  | 0.49  | 980   | 59.0  | 0.39  | -     | -     | -     | -     | -     | -     |
| 51–60| 1200  | 64.0  | 0.48  | 1015  | 63.0  | 0.40  | -     | -     | -     | -     | -     | -     |

[^a]: coconut coir.
[^b]: mixture of H\_2 + CO\_2 in mL.
[^c]: mol mol\(^{-1}\) crude glycerol utilized.

DAI: Days after incubation. Feed: Sewage water medium diluted with Tap water in 3:1 ratio (M-9 salts: 0.5 X) with crude glycerol (2%, v/v). Inoculum: 10 \(\mu\)g cell protein \(mL\(^{-1}\) feed. Values represent 5 days of batch fermentation. All experiments were performed in triplicate. The standard deviation was less than 10%.

https://doi.org/10.1371/journal.pone.0199059.t002

than with 5% CC. On increasing the support material to 15% CC, 1.2–1.5 L \(H_2\)/0.5 L feed/day was produced during 60 days of fermentation, which was 1.23-fold higher than with 10% CC. On comparing the \(H_2\) producing abilities of two strains, without any support material both the strains produced an abysmal low \(H_2\) (Figs 1 and 2). Immobilizing *B. thuringiensis* strain EGU45 on CC increased the \(H_2\) production by 4- to 10- fold. With biofilm forming *B. amyloliquefaciens* strain CD16, the increase in \(H_2\) production with CC was 3- to 5- fold. Biofilm forming strain at 15% CC resulted in 1.17 times more \(H_2\) as compared to non-biofilm forming strain (Table 3).
Effect of effluent recycling

To increase the overall process efficiency and economy, effluent generated from the H₂ production stage was recycled. With, *B. thuringiensis* strain EGU45, at 75% and 50% effluent recycling a very sharp decline in gas production was recorded (Figs 3 and 4 and S3 and S4 Tables). After an initial production of 0.6–1.0 L H₂/0.5 L feed/day in these cases, the H₂ production declined to 0.09–0.3 L H₂/0.5 L feed/day and ceased thereafter. However, at 25% effluent recycling a significant difference was not observed with respect to control during 60 days of fermentation. During initial 20 days of recycling, 1.2–1.4 L H₂/0.5 L feed/day was observed. After this a small decline in gas production was observed which maintained an average of 0.9 L H₂/0.5 L feed/day till 60 days of recycling (Table 2). Considering the average gas produced, a 6% drop in H₂ was observed with 25% effluent recycling as compared to controls This corresponded to 0.36 mol H₂/mol CG as compared to controls which produced 0.41 mol H₂/mol CG.

![Fig 3. Effect of recycling of effluent on hydrogen production from sewage water and crude glycerol by Bacillus thuringiensis immobilized on coconut coir (CC): Recycling of effluent —25% (●, red filled square), 50% (▲, green filled triangle), 75% (♦, blue filled diamond) and control (●, violet filled circle). Feed: 500 mL of Sewage water + Tap water in 3:1 ratio (0.05X M-9 salts) supplemented with crude glycerol (2%, v/v) in case of control and 125–375 mL Sewage water + Tap water in 3:1 ratio (0.05X M-9 salts) made up to 500 mL with effluent in other cases.](https://doi.org/10.1371/journal.pone.0199059.g003)
CG (Table 3). With *B. amyloliquefaciens* strain CD16, a similar trend of sharp decline in gas production was observed at 75% and 50% recycling. The gas production declined to 0.2 L H₂/0.5 L feed/day and 0.05 L H₂/0.5 L feed/day at 75% and 50% recycling respectively within 40 days of recycling and ceased thereafter. However, at 25% recycling the gas production did not show any significant reduction. An average of 1.0 L H₂/0.5 L feed/day was produced with 25% recycling which was a 10% decline as compared to controls (Table 3). The reactors with 15% CC support material were run for 120 days (an additional 60 days during recycling of effluent) for the entire duration of which an average H₂ yield of 100–120 L/L CG was maintained by both the strains (Tables 2 and 3).

**Conclusion**

Waste generation is an integral part of our routine activities. Under natural environmental conditions, microbes metabolize the organic matter content and release gases into the atmosphere. This contributes significantly to environmental pollution. Another contributor to environmental pollution is the burning of fossil fuels. Efforts to treat biowastes through microbial activity have revealed that this concept can be exploited to produce energy rich gases (H₂ and methane, CH₄) through fermentation. A wide range of biowastes have the potential to produce H₂ and CH₄. A major limitation in the use of H₂ producers is the risk of contamination which emanates from bacteria present in the unsterile biowastes. So to avoid contamination, sterilization become imperative, this obviously results in lowering economic efficiency. Secondly, in most biological processes, the substrate concentrations vary from very low of 0.1% to a maximum 10%. It implies that 90 to 99.9% is water or medium. In this study, we have circumvented almost all the issues related to biological H₂ production: (i) use of unsterile conditions, (ii) use of sewage water as medium, (iii) use crude glycerol, which otherwise cause heavy pollution, (iii) use of a single bacterium with abilities to form biofilm and produce H₂, (iv) recycling of the effluent for further enhancing the process efficiency, (v) continuous culture conditions enable easy operation (vi) no stirring required, (vii) independent of light, and (viii) used organism i.e. *Bacillus*, which has been categorized as GRAS (Generally Regarded As Safe) organism [31]. Biofilm forming bacteria *B. amyloliquefaciens* CD16 immobilized on CC utilized CG in domestic wastewater medium to produce 120 L H₂/L CG during 120 days continuous fermentation. Under similar conditions, the non-biofilm forming *B. thuringiensis* strain EGU45 produced around 100 L H₂/L CG fed (This study). In contrast, there are only a limited number of studies where wastewater has been co-digested with CG (1% w/v) by *Klebsiella* sp. It generated around 9.8 L H₂/g substrate consumed, with H₂ constituting only 44% of the total
Using activated sludge from biodiesel industry effluent, 75L H₂/L glycerol consumed was reported in anaerobic sequencing batch reactors, with H₂ constituting only 33.4% of the total biogas produced [22]. Further, we have observed that on recycling the effluent up to 25%, no drastic changes in gas production were observed. In comparison to the use of sterile distilled water as medium, the use of wastewater did not result in any adverse effect on H₂ production. A similar response on recycling of effluent from H₂ production stage was shown in our previous study, where sterile distilled water was used for preparing the slurry [23]. Another interesting aspect of this study is the possibility of further utilization of effluent of H₂ production stage for producing value added products such as polyhydroxyalkanoates and CH₄ [14, 18].

Supporting information

S1 Table. Effect of support material on continuous culture hydrogen production by Bacillus thuringiensis EGU45. (DOCX)

S2 Table. Effect of support material on continuous culture hydrogen production by Bacillus amyloliquefaciens CD16. (DOCX)

S3 Table. Effect of Effluent recycling on continuous culture hydrogen production by Bacillus thuringiensis EGU45. (DOCX)

S4 Table. Effect of Effluent recycling on continuous culture hydrogen production by Bacillus amyloliquefaciens CD16. (DOCX)

Acknowledgments

The authors are thankful to the anonymous reviewers and the academic editor. JP is thankful to University Grants Commission and Jung-Kul Lee.

Author Contributions

Conceptualization: In-Won Kim, Vipin Chandra Kalia.
Formal analysis: Jyotsana Prakash, In-Won Kim, Vipin Chandra Kalia.
Funding acquisition: In-Won Kim, Vipin Chandra Kalia.
Investigation: Jyotsana Prakash, Sanjay K. S. Patel, In-Won Kim.
Project administration: In-Won Kim.
Resources: In-Won Kim.
Supervision: In-Won Kim.
Validation: In-Won Kim.
Writing – original draft: Jyotsana Prakash, Rakesh Sharma, Sanjay K. S. Patel, In-Won Kim, Vipin Chandra Kalia.
Writing – review & editing: Jyotsana Prakash, Rakesh Sharma, Sanjay K. S. Patel, In-Won Kim, Vipin Chandra Kalia.
References

1. Sonune A, Ghate R. Developments in wastewater treatment methods. Desalinat. 2004; 167:55–63. https://doi.org/10.1016/j.desal.2004.06.113

2. Kiran B, Pathak K, Kumar R, Deshmukh D. Phycoremediation: An Eco-friendly Approach to Solve Water Pollution Problems. In: Kalia VC, Kumar P, editors. Microbial Applications. Springer; 2017, Volume 1, pp. 3–58. https://doi.org/10.1007/978-3-319-52666-9_1

3. Sato T, Qadir M, Yamamoto S, Endo T, Zahoor A. Global, regional, and country level need for data on wastewater generation, treatment, and use. Agric. Water Manag. 2013; 130:1–13. https://doi.org/10.1016/j.agwat.2013.08.007

4. Patel SKS, Kumar P, Mehariya S, Purohit HJ, Lee JK, Kalia VC. Enhancement in hydrogen production by co-cultures of Bacillus and Enterobacter. Int. J. Hydrogen Energy. 2014; 39:14663–8. https://doi.org/10.1016/j.ijhydene.2014.07.084

5. Kumar P, Ray S, Patel SKS, Lee JK, Kalia VC. Bioconversion of crude glycerol to polyhydroxyalkanoate by Bacillus thuringiensis under non-limiting nitrogen conditions. Int. J. Biol. Macromol. 2015; 121:1–9. https://doi.org/10.1016/j.ijbiomac.2015.05.046 PMID: 26057930

6. Kumar P, Sharma R, Ray S, Mehariya S, Patel SKS, Lee JK et al. Dark fermentative bioconversion of glycerol to hydrogen by Bacillus thuringiensis. Bioprocess. Technol. 2015; 172:283–8. https://doi.org/10.1016/j.biortech.2015.01.138 PMID: 26057930

7. Kalia VC. Microbial treatment of domestic and industrial wastes for bioenergy production. Appl. Microbiol. 2007(e-Book). National Science Digital Library NISCAIR, New Delhi, India. http://nsdl.niscair.res.in/bitstream/123456789/650/1/DomesticWaste.pdf

8. Mohan SV, Sarkar O. Waste to biohydrogen: addressing sustainability with biorefinery. In: Raghavan K, Ghosh P, editors. Energy Engineering, Springer; 2017, pp. 29–37. https://doi.org/10.1007/978-981-10-3102-1_4

9. Patel SKS, Lee JK, Kalia VC. Dark-fermentative biological hydrogen production from mixed biowastes using defined mixed cultures. Indian J. Microbiol. 2017; 57:171–6. https://doi.org/10.1007/s12088-017-0643-7 PMID: 28611494

10. Hallenbeck PC, Abo-Hashesh M, Ghosh D. Strategies for improving biological hydrogen production. Bioprocess. Technol. 2012; 110:1–9. https://doi.org/10.1016/j.biortech.2012.01.103 PMID: 22342581

11. Silva S, Rodrigues AC, Ferraz A, Alonso J. An integrated approach for efficient energy recovery production from livestock and agro-industrial wastes. In: Singh L, Kalia VC, editors. Waste biomass management–A holistic approach. Springer; 2017, pp. 339–66. https://doi.org/10.1007/978-3-319-49595-8_15

12. Li N, Zhao J, Liu R-N, Li Y-F, Ren N-Q. Biological fermentative methane production from brown sugar wastewater in a two-phase anaerobic system. J. Fundam. Renewable Energy Appl. 2015; 5:181. https://doi.org/10.4172/2090-4541.1000181

13. Farghaly A, Tawfik A, Danial A. Inoculation of papermill mill sludge versus mixed culture bacteria for hydrogen production from papermill mill wastewater. Environ. Sci. Pollut. Res. 2016; 23:3834–46. https://doi.org/10.1007/s11356-015-5652-7 PMID: 26498965

14. Intanoo P, Chaimongkol P, Chavadej S. Hydrogen and methane production from cassava wastewater using two-stage upflow anaerobic sludge blanket reactors (UASB) with an emphasis on maximum hydrogen production. Int. J. Hydrogen Energy. 2016; 41:6107–14. https://doi.org/10.1016/j.ijhydene.2015.10.125

15. Hemalatha M, Sravan JS, Yeruva DK, Mohan SV. Integrated ecotechnology approach towards treatment of complex wastewater with simultaneous bioenergy production. Bioprocess. Technol. 2017; 242:60–7. https://doi.org/10.1016/j.biortech.2017.03.018 PMID: 28434787

16. Jaikeaw S, Chavadej S. Separate production of hydrogen and methane from ethanol wastewater using two-stage UASB: micronutrient transportation. Int. J. Chem. Mol. Eng. 2017; 11:1. https://doi.org/10.1999/1307-6892/66190

17. Khongkliang P, Kongjan P, Chavadej S. Hydrogen and methane production from cassava starch processing wastewater by two-stage thermophilic dark fermentation and microbial electrolysis. Int. J. Hydrogen Energy. 2017; 42:27584–92. https://doi.org/10.1016/j.ijhydene.2017.06.145

18. Prakash J, Sharma R, Ray S, Koul S. Wastewater: A potential bioenergy resource. Indian J. Microbiol. 2018; 58:127–37. https://doi.org/10.1007/s12088-017-0703-z PMID: 29651171

19. Júnior ADNF, Etchebehere C, Zaiat M. Mesophilic hydrogen production in acidogenic packed-bed reactors (APBR) using raw sugarcane vinasse as substrate: Influence of support materials. Anaerobe. 2015; 34:94–105. https://doi.org/10.1016/j.anaerobe.2015.04.008 PMID: 25891935
20. Wang S, Zhang T, Su H. Enhanced hydrogen production from corn starch wastewater as nitrogen source by mixed cultures. Renew. Energy. 2016; 96:1135–41. https://doi.org/10.1016/j.renene.2015.11.072

21. Kalia VC, Prakash J, Koul S. Biorefinery for glycerol rich biodiesel industry waste. Indian J. Microbiol. 2016; 56:113–25. https://doi.org/10.1007/s12088-016-0583-7 PMID: 27570302

22. Tangkathitipong P, Intanoo P, Butpan J, Chavadej S. Separate production of hydrogen and methane from biodiesel wastewater with added glycerin by two-stage anaerobic sequencing batch reactors (ASBR). Renew. Energy. 2017; 113:1077–85. https://doi.org/10.1016/j.renene.2017.06.056

23. Prakash J, Gupta RK, Priyanka XX, Kalia VC. Bioprocessing of biodiesel industry effluent by immobilized bacteria to produce value-added products. Appl. Biochem. Biotechnol. 2017; 1–12. https://doi.org/10.1007/s12100-017-2637-7 PMID: 29101733

24. Kalia VC, Joshi AP. Conversion of waste biomass (pea-shells) into hydrogen and methane through anaerobic digestion. Bioreourc. Technol. 1995; 53:165–8. https://doi.org/10.1016/0960-8524(95)00077-R

25. Akinbomi J, Wikandari R, Taherzadeh MJ. Enhanced fermentative hydrogen and methane production from an inhibitory fruit-flavored medium with membrane-encapsulated cells. Membranes. 2015; 5:616–31. https://doi.org/10.3390/membranes5040616 PMID: 26501329

26. Andreani CL, Torres DG, Schultz L, de Carvalho KQ, Gomes SD. Hydrogen production from cassava processing wastewater in an anaerobic fixed bed reactor with bamboo as a support material. Eng. Agric. 2015; 35:578–87. https://doi.org/10.1590/1809-4430-Eng.Agric.v35n3p578-587/2015

27. Kirli B, Kapdan IK. Selection of microorganism immobilization particle for dark fermentative biohydrogen production by repeated batch operation. Renew. Energy. 2016; 87,697–702. https://doi.org/10.1016/j.renene.2015.11.003

28. Gokfiliz P, Karapinar I. The effect of support particle type on thermophilic hydrogen production by immobilized batch dark fermentation. Int. J. Hydrogen Energy. 2016; 42:2553–61. https://doi.org/10.1016/j.ijhydene.2016.03.041

29. Al-Mailem DM, Kansour MK, Radwan SS. Bioremediation of hydrocarbons contaminating sewage effluent using man-made biofilms: effects of some variables. Appl. Biochem. Biotechnol. 2014; 174:1736–51. https://doi.org/10.1007/s12010-014-1067-z PMID: 25146193

30. Ercan D, Demirci A. Current and future trends for biofilm reactors for fermentation processes. Crit. Rev. Biotechnol.2015; 35:1–14. https://doi.org/10.3109/07388551.2013.793170 PMID: 23919241

31. Porwal S, Lal S, Cheema S, Kalia VC. Phylogeny in aid of the present and novel microbial lineages: Diversity in Bacillus. PLoS ONE. 2009; 4:e4438. https://doi.org/10.1371/journal.pone.0004438 PMID: 19212464

32. Chookaew T, Sompong O, Prasertpsan P. Biohydrogen production from crude glycerol by immobilized Klebsiella sp. TR17 in a UASB reactor and bacterial quantification under nonsterile conditions. Int J Hydrog Energy. 2014; 39:9580–7. https://doi.org/10.1016/j.ijhydene.2014.04.083