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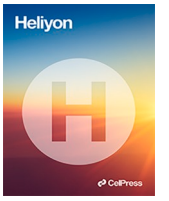

Research article

Production of biological hydrogen from Quinoa residue using dark fermentation and estimation of its microbial diversity

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ABSTRACT

Although they are one of the world's environmental problems, agricultural wastes or residues are carbohydrate-rich and low-cost, so they are used as raw materials for the manufacture of bio-hydrogen (bio-H_2). Among biological hydrogen manufacture methods, the dark fermentation method is suitable for processing waste or residues. In this regard, no study has been found in the literature on determining the potential of biological hydrogen manufacture from quinoa residue by the dark fermentation method. This work was carried out in a dark room at 36 ± 1 °C under different operating conditions in anaerobic batch bio-reactors fed with thermally pretreated anaerobic mixed bacteria + raw quinoa or quinoa extract liquid + nutrients. In the study, gas analyses were performed and biohydrogen production was detected in all the bio-reactors. Besides, taxonomic content analyses and organic acid analyses were executed. Maximum bio-H_2 production was found as follows: at pH 4.5, 14,543.10^{-4} mL in the bio-reactor fed with 1.00 g quinoa/L and 1880.10^{-4} mL in the bio-reactor fed with 0.50 g quinoa extract/L, and at pH 4.0, 61,537.10^{-4} mL in the bio-reactor fed with 1.00 g quinoa/L and 1511.10^{-4} mL in the bio-reactor fed with 0.75 g quinoa extract/L. In the bio-reactors fed with raw quinoa residue, *Clostridium butyricum* and *Hathewaya histolytica* were detected as the most dominant bacteria at pH 4.5 and 4.0, respectively, whereas in the bio-reactors fed with quinoa extract liquid, *Fonticella tunisiensis* were detected as the most dominant bacteria at both pH 4.5 and pH 4.0.

1. Introduction

The request for energy increases with the increment in world population. Developments in many sectors, such as industry, transportation, and agriculture, require energy. In the past years, fossil resources such as coal, natural gas, and oil were considered the only energy sources. The limitation of these resources in the last half century and their negative effects on the environment have necessitated the search for environmentally friendly and sustainable energy sources. Therefore, studies on renewable energy sources have become the focus of researchers. In this context, some of the renewable energy resources that are sustainable for the world's energy demand can be listed as biohydrogen, biomethane, bioethanol, biodiesel, and biochar. In the sustainable production of these resources, carbohydrate-rich waste or residues are especially used.

It has been reported that lignocellulosic biomass consisting of agricultural residues or wastes contains 70–80 % carbohydrates and that these carbohydrates may be utilized for biological hydrogen production when appropriately hydrolyzed [1]. Additionally, it has been reported that 10–20 % of human-induced greenhouse gas emissions occur in agricultural areas [2,3]. The increase in agricultural

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production resulting from the increasing world population and growing demand for agricultural products causes tons of waste or residue. Sustainable reduction of these environmentally harmful wastes or residues and their use for biological hydrogen production can both decrease greenhouse gas emissions by diminishing waste or residues and provide environmentally friendly biohydrogen production. Among the biohydrogen production methods, the dark fermentation method provides the advantage of continuously manufacturing hydrogen without requiring light, therefore it is considered the most promising method [4]. Therefore, various types of agricultural waste or residue can be considered as potential raw materials in biological hydrogen manufacture via dark fermentation.

Studies aiming at biological hydrogen production have generally applied certain pretreatment methods to organic waste or residual raw materials, singly or in combination. In addition, studies conducted in this context used both specific bacteria and mixed microorganisms. However, for reasons of cost and low accessibility, specific bacteria have been less preferred as inoculum. In studies aimed at hydrogen production, readily available mixed microorganisms have often been used after thermal pretreatment to inactivate methanogens. In one of the studies, a mixed culture from a bio-reactor that produced hydrogen by fermenting 1 mm wheat straw and waste sugar, pre-treated with H_2SO_4 at 120 °C for 90 min, was used [1]. In another work, raw wheat straw and wheat straw subjected to acid pre-treatment combined with cow manure compost pretreated in an infrared oven for 120 min were examined in terms of hydrogen production [5]. For this purpose, various raw materials and specific bacteria were used: hexose with *Clostridium* spp. [6], fruit waste with *Clostridium* strain BOH3 [7], sugarcane bagasse detoxified hydrolysate with *Enterobacter aerogenes* MTCC 2822 [8]. Lopez-Hidalgo et al. [9] examined the bio-H_2 manufacture potential of agro-industrial waste wheat straw hydrolyzate and whey mixture and anaerobic granular sludge under different operating conditions. In the study, tests using 0.11 L anaerobic bottles and 1 L and 4 L bio-reactors for biological hydrogen production gave results in the range of 4554.5-3685 mL H_2 L^{-1}. The study reported that the use of wheat straw hydrolyzate and whey both in combination and separately is possible for biohydrogen production. Li et al. [10] thermally pretreated the mixture of urban wastewater treatment plant sludge + pig manure + cow manure + camel manure at 100 °C for 10 min as inoculum and used the combination for biological hydrogen production after the culturing process. In the study where rice straw, corn straw, wheat straw, corn cob, and sorghum were used as raw materials, it was designated that the highest hydrogen manufacture (472.75 mL) was yielded by corn cob. In another work, Ren et al. [11] compared lipid and hydrogen manufacture from agricultural residues of corn stalks, rice straw, corn cobs, and wheat straw. In the work, it was designated that corn stalk residue was suitable for lipid manufacture and yielded maximum hydrogen production (762.3 mL L^{-1}). A study examining galactose for bio-H_2 production was conducted by Park et al. [12]. The work was carried out in batch mode at 150 rpm, 35 ± 0.1 °C for 21 h, and after this hour, the galactose medium containing nutrients was operated in the fermenter in continuous mode. In addition, in the bio-reactor operated at an initial pH value of 7.5, the pH value was above 5.5 ± 0.1 during the fermentation process. The study reported increased overall hydrogen production. Sydney et al. [13] determined the maximum biological hydrogen production as 1.59 ± 0.21 mol H_2 mol glucose^{-1} in a consortium half of which consisted of *Oxalobacteraceae* in a vinasse-based medium supplemented with sugar cane juice. In the work conducted by Oceguera-Contreras et al. [14], in which the inoculum was used without pretreatment, the hydrogen production potential from microorganisms obtained from the unpretreated *Eisenia foetida* earthworm and agro-industrial wastes was investigated in 120 mL volume bottles at 40 °C. In the study where butyric acid and acetic acid were the main metabolites, the best hydrogen manufacture was specified by molasses (1571.81 mL H_2 L^{-1}), bagasse (1246.36 mL H_2 L^{-1}), and vinasse (232.72 mL H_2 L^{-1}), respectively. In this present work, the potential for bio-H_2 production using quinoa residue was investigated. Quinoa has been reported to be little known even at the beginning of the 21st century. After this product was identified as the world's most nutritious grain, consumer interest in the product increased [15]. Quinoa has been reported to be adapted to growing at altitudes up to 4000 m above sea level. It can adapt to various environmental conditions and has significant production potential worldwide. FAO has selected quinoa as one of the products that can ensure food security in the next century [16]. This product is rich in minerals, vitamins, protein, essential fatty acids, carbohydrates, and dietary fibers, but does not contain gluten, which increases the demand for it. In addition, the quinoa plant, also known as the golden grain, is resistant to harsh climatic conditions, which has led to an increment in global request for quinoa production. Therefore, following the declaration of 2013 as the "International Year of Quinoa," quinoa production showed an increasing trend [17]. It has been reported that billions of tons of residues are obtained after growing and processing the products in the agricultural industry. It is also known that residues cause environmental problems such as pollution and, indirectly, economic problems. This situation requires developing strategies for using agricultural residues as high-value-added products [18]. Since it is predicted that the production of quinoa will become widespread worldwide and, accordingly, there will be an increase in quinoa residue, it was thought that it would be useful to examine the biohydrogen production potential of this residue in the context of research objectives. Considering the recommendation in the literature to use biomass with a dimension of less than 3 mm, quinoa residual biomass with a dimension of "∼1 mm" was used in this present study. In the litterateur on bio-H_2 production from agricultural residues, there are limited data comparing the use of raw biomass and liquid extracted from the raw residue, and there are limited implications in explaining the operating process output. Therefore, the effect of using raw biomass and extracted liquid on biohydrogen production needs to be elucidated. With this study, the relative significance of this situation is clarified. In literature, various studies focus on the manufacture of biohydrogen from various agricultural residues or waste biomass. However, no study has been encountered regarding the biological hydrogen production through dark fermentation using quinoa residue biomass with the utilization of anaerobic mixed bacteria.

This work aims to specify the impression of the use of raw quinoa residue and the liquid extracted from the quinoa residue after pretreatment in batch bio-reactors under different operating conditions on biohydrogen (bio-H_2) production by dark fermentation. Additionally, in the work, microbial community analysis of the pretreated mixed culture was performed and species-level rates were examined, depending on their biological hydrogen production potential in bio-reactors.

2. Materials and methods

2.1. Inoculum

For the purpose of biological hydrogen production, inoculum sludge was obtained from the anaerobic bio-reactor of a manufactory producing beet sugar. This mixed microorganism (sludge) was thermally pre-treated at 100 ± 1 °C for 60 min to hamper or absolutely extinguish the activity of methane-producing microorganisms. Thus, it was purposed to enhance the activity of hydrogen-manufacturing microorganisms.

2.2. Nutrient composition

Nutrient composition was endured on the work by Fang et al. [19]. Accordingly, nutrient compositions were $CuCl_2.H_2O$ 10 mg/L, $Na_2MoO_4.2H_2O$ 14.4 mg/L, $FeCl_3.6H_2O$ 20 mg/L, $CoCl_2.6H_2O$ 21 mg/L, $ZnCl_2$ 23 mg/L, $MnCl_2.4H_2O$ 30 mg/L, $NiSO_4$ 32 mg/L, KH_2PO_4 250 mg/L, $MgSO_4.7H_2O$ 320 mg/L, $CaCl_2.2H_2O$ 500 mg/L, and NH_4Cl 2500 mg/L.

2.3. Setup and operation of bio-reactors

The work was handled in laboratory-scale glass anaerobic batch reactors (ABRs) with a volume of 120 mL. The glass bio-reactors were wreathed with aluminum foil band to prevent the evolvement of phototrophic microorganisms. The content of the bio-reactors consisted of thermally pretreated anaerobic sludge, quinoa (raw waste or extracted liquid) biomass, and nutrients necessary for microbial growth. Bio-reactors were set up to contain 90 mL of material (quinoa or quinoa extract + nutrients + pretreated microorganism) and a gas collection zone of 30 mL. Biomass particle size has been notified to influence fermentation processes [20]. Moreover, biomass dimension has been reported to enable hydrolysis. For this reason, it has been recommended that biomass types with lignocellulosic structures should have a dimension of less than 3 mm [21]. In this context, the biological hydrogen production of two forms of "∼1 mm" quinoa waste was compared: (i) material without any pretreatment (raw waste) and (ii) liquid extracted after decoction pretreatment. Thus, the biological hydrogen production performance of batch bio-reactors fed with two dissimilar material forms and operated at dissimilar pH and organic loading rates (OLRs) was investigated. Anaerobic batch bio-reactors fed with raw quinoa waste included pretreated anaerobic mixed microorganisms, specified organic loading rates of waste, and nutrient composition added to tap water, whereas anaerobic batch bio-reactors fed with the extracted liquid after decoction of quinoa waste included pretreated anaerobic mixed microorganisms, the extracted liquid obtained and the nutritional composition added to this liquid. Decoction extraction involves adding cold water to small solids and subjecting them to heat treatment at 100 °C for 30 min. After this implementation, it is advised to cool the liquid-solid mixture at 40 °C and then filter it [22]. In this study, these operations were performed on quinoa biomass, and a small alteration was made in the chilling process. When the liquid-solid mixture reached room temperature, filtering with filter paper was performed. Following this process, the extracted liquid was used. Following the bio-reactor setup, pH was tuned with 1 M NaOH and 1 M HCl before closing the lids. After feeding the bio-reactors, their lids were closed, and the setup was completed. Nitrogen gas was introduced into the bio-reactors for 5 min, and it was aimed to remove oxygen from the bio-reactors through the injector tip. Thus, the bio-reactors were made ready for operation. Bio-reactors were operated in a temperature-controlled dark room at 36 ± 1 °C, in a 150 rpm shaking incubator. A shaking incubator was used to ensure a homogeneous mixing of mixed bacteria and rapid passage of gas into the bio-reactor headspace. Gas instancing was done with a glass injector with a gas-tight stopper. Gas measurements were made following sampling. Darkroom temperature (36 ± 1 °C), biological hydrogen production, and organic acids were monitored throughout the complete bio-reactor operations.

2.4. Analytical methods

To prepare the organic acid specimens for measuring, cellulose acetate syringe filters (0.45 μm) were used. Acetic acid, butyric acid, and propionic acid analyzes were actualized on high-press liquid chromatography with a Metacarb 87H (7.8 × 300 mm) column and a ProStar 330 PDA detector. Gas sampling was effected with a gas-tight glass injector with a stopper. Calibration was performed with high-purity CO_2, H_2, and CH_4 gases. In the study, He gas was used as the bearer gas. The column was operated at 35 °C, the injection at 200 °C, and the detector at 230 °C. Gas analyses were effected on a gas chromatograph (GC) with a thermal conductivity detector (TCD) and an RT-Msieve 5A (15 m, 0.53 mm ID, 50 μm DF) capillary column. COD measuring was carried out using the closed reflux method [23].

2.5. Microbial community analysis

For the production of bio-H_2 from quinoa residue (raw waste and extracted liquid) with anaerobic mixed microorganisms, ABRs were operated at different pH values and organic loading rates. Microbial communities were taken for analysis from the contents of bio-reactors with high biological hydrogen production. The samples were stored at −80 °C and then, after Next-Generation Sequencing, DNA insulation and quality check of the specimens were carried out to constitute a library. Subsequently, for this purpose, the V3–V4 region of the 16S rRNA gene was amplified with specific primers. Following this, purification was carried out. Illumina MiSeq Reagent Kit was employed. In the Index PCR phase, Illumina binary indexes and adapters were appended using the Nextera XT index kit. Subsequently, purification was effected. The concentration of libraries generated by real-time PCR was measured.

Additionally, they were diluted to 4 nM and normalized. Subsequently, the examples were unified using the pooling method. After library generation, each time a new deoxynucleoside triphosphate (dNTP) was added using the sequencing by the synthesis method, the fluorescence of the added base was optically monitored and registired. After the sequencing process, the manufactured data was converted to raw data (FASTA format) for analysis. 16S rRNA gene Illumina library reads were analyzed and processed using Quantitative Insights Into Microbial Ecology 2 (QIIME2) for prefiltering and preprocessing. Firstly, the read quality was controlled with FastQC, and barcodes were extracted in QIIME. Then, reads and metadata were transferred into QIIME2 [24–26]. Filtering of primers and barcodes of reads with a Phred score lesser than 20 and filtering of chimeric reads were done using DADA2. Taxonomic species were determined for each of the samples using the standard software package version QIIME2.

3. Results and discussion

3.1. Effect of using raw quinoa biomass on the production of biological hydrogen

Biohydrogen production potential of unpretreated quinoa residue biomass at initial pH of 4.5 and 4.0 and at organic loading rates (OLRs) of 0.25 (72.8 mg COD L^{-1} on average), 0.50 (81.3 mg COD L^{-1} on average), 0.75 (89.9 mg COD L^{-1} on average), and 1.00 (92.7 mg COD L^{-1} on average) g quinoa/L was investigated.

It was determined that no gas was produced in the first hours of operation in bio-reactors fed with unpretreated raw quinoa biomass and actuated at an initial pH of 4.5 (Fig. 1). As given in Fig. 1 A(1), the bio-reactor fed with 0.25 g quinoa/L gave biohydrogen manufacture of 114.10^{-4} mL at 12 h and 96.10^{-4} mL at 23 h. In this bio-reactor, maximum biohydrogen manufacture was determined as 634.10^{-4} mL at 36 h. In the bio-reactor operated fed with an OLR of 0.50 g quinoa/L and actuated at an initial pH of 4.5, biohydrogen production was specified as 92.10^{-4} mL at 13 h, 608.10^{-4} mL at 37 h, and maximum hydrogen manufacture was 623.10^{-4} mL at 44 h (Fig. 1 A(2)). After the maximum manufacture hour, hydrogen manufacture reduced to 409.10^{-4} mL, and at 93 h it increased back to 615.10^{-4} mL. Ninety-three hours later, hydrogen production dropped again. As presented in Fig. 1 A(3), in the bio-reactor actuated at the same initial pH and fed with an organic loading ratio of 0.75 g quinoa/L, 1014.10^{-4} mL of hydrogen was produced at 13 h and 1080.10^{-4} mL at 19 h. From this moment on, biohydrogen production of 1117.10^{-4} mL was achieved at 22 h, and after this hour the production evinced a decreasing trend, falling to 988.10^{-4} mL at 61 h. At 69 h, maximum biohydrogen production of

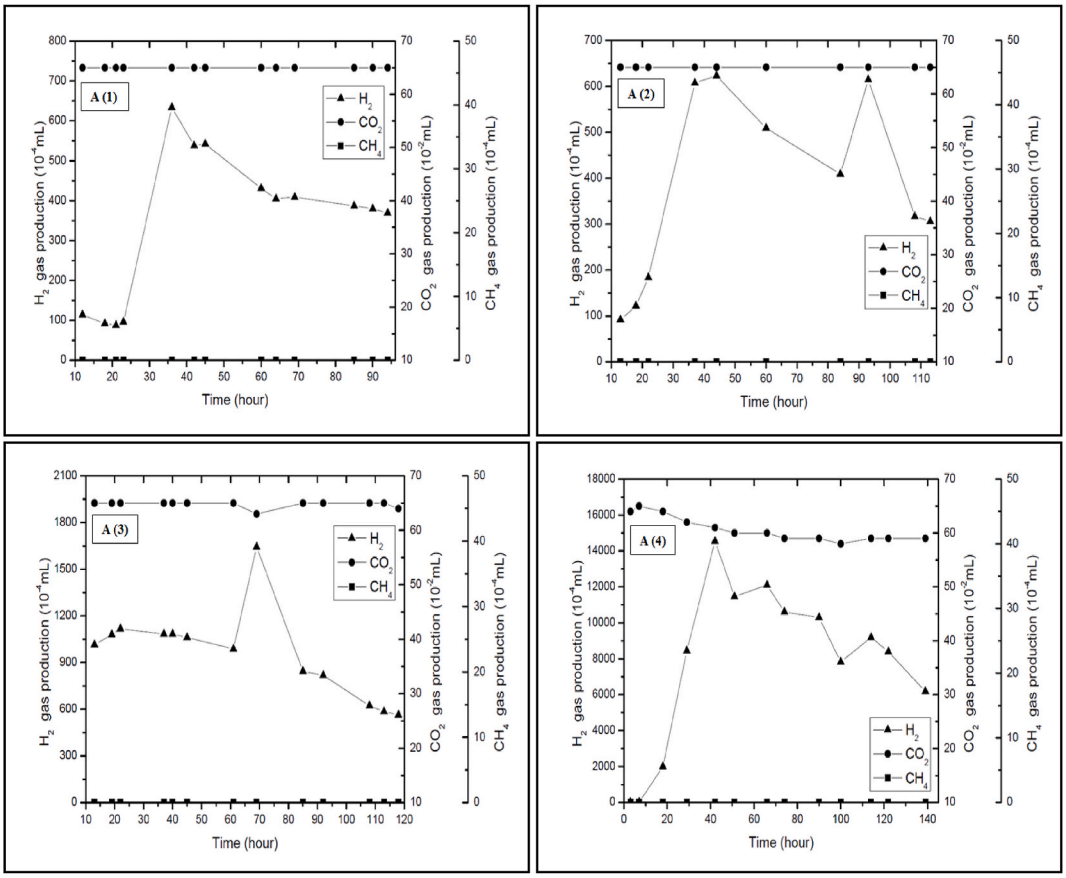

Fig. 1. Hydrogen gas production in bio-reactors fed with raw quinoa residual biomass and operated at pH 4.5.

1644.10^{-4} mL was achieved, and after this hour, hydrogen production began to decrease. As given in Fig. 1 A(4), the bio-reactor fed with an OLR of 1.00 g quinoa/L and actuated at an initial pH of 4.5 yielded bio-H_2 manufacture of 1994.10^{-4} mL at 18 h and 8447.10^{-4} mL at 29 h. Maximum bio-H_2 manufacture of 14,543.10^{-4} mL was reached at 42 h. After the hour of maximum biohydrogen manufacture, fluctuations were observed in hydrogen production, usually as a decrease.

The biohydrogen production potential of unpretreated raw quinoa residue was examined in the bio-reactors fed with OLRs of 0.25, 0.50, 0.75, and 1.00 g quinoa/L and operated at pH 4.0 (Fig. 2). In all the bio-reactors, no gas manufacture was detected in the first hours. Among the bio-reactors, the one fed with an OLR of 0.25 g quinoa/L yielded 92.10^{-4} mL of biohydrogen production at 12 h, 96.10^{-4} mL at 19 h, and 107.10^{-4} mL at 21 h. In this bio-reactor, maximum biohydrogen production was designated as 365.10^{-4} mL at 37 h. After the hour of maximum manufacture, biohydrogen tended to decrease over time (Fig. 2 B(1)). As presented in Fig. 2 B(2), in the bio-reactor actuated at pH 4.0 and fed with 0.50 g quinoa/L, hydrogen manufacture was 88.10^{-4} mL at 13 h, 122.10^{-4} mL at 19 h, and 1025.10^{-4} mL at 36 h, and maximum hydrogen manufacture was 1039.10^{-4} mL at 41 h. Biohydrogen production waned in the following hours. In the bio-reactor fed with 0.75 g quinoa/L (Fig. 2 B(3)), bio-H_2 production of 1043.10^{-4} mL at 13 h, 1846.10^{-4} mL at 22 h, and 2163.10^{-4} mL at 36 h was determined. In this bio-reactor, maximum hydrogen production (2241.10^{-4} mL) was reached at 42 h, and after this hour, hydrogen manufacture tended to decline until the eighty-fifth hour. In addition, hydrogen production increased to 1961.10^{-4} mL at the 92 h. After this time, biohydrogen tended to decrease again. As given in Fig. 2 B(4), in the bio-reactor fed with 1.00 g quinoa/L and operated at the same pH, bio-H_2 manufacture was 7.10^{-4} mL at 7 h, 21,044.10^{-4} mL at 19 h, and 31,301.10^{-4} mL at 29 h. In this bio-reactor, maximum biohydrogen production of 61,537.10^{-4} mL was determined at 43 h, after which time hydrogen production generally decreased. In the bio-reactors, COD values were found to be in the range of 92.7–72.8 mg/L at the beginning and 71.8–60.9 mg/L at the end of operation. Many studies have proven that anaerobic mixed microorganisms are one of the promising biological materials for biohydrogen manufacture by the dark fermentation method. In a work investigating the impact of pH value on biohydrogen manufacture [27], fermentative biohydrogen manufacture was examined in batch bio-reactors operated at 35 °C and an initial pH of 7.0 and fed with pretreated sludge and glucose. After hydrogen production, the pH value was reported to be in the range of 4.1 to 3.4. In this study, it was determined that the initial pH values of 4.5 and 4.0 were nearby to 3.2 at the end of the operation. Another work notified that initial pH values of 5.5–9.0 and temperature ranges of 30–40 °C were optimum conditions for mesophilic bacteria in fermentative hydrogen manufacture [28]. Liu et al. [29] enquired the influence of the initial pH value of alkaline-pretreated sludge on bio-H_2 manufacture. In the study, microorganisms were examined at initial pH values between 2.0 and

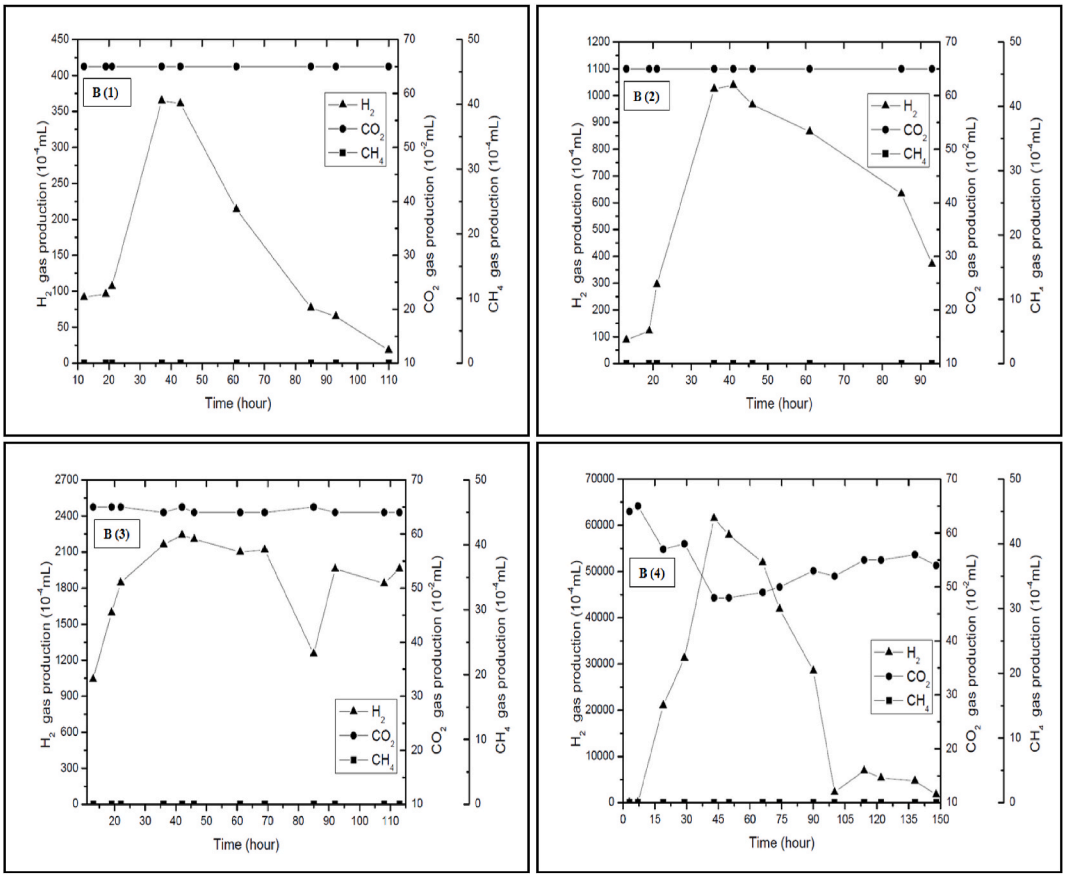

Fig. 2. Hydrogen gas production in the bio-reactors fed with raw quinoa residual biomass and operated at pH 4.0.

4.0, and it was reported that microorganisms were inhibited at these pH values, resulting in very limited hydrogen production. In addition, at an initial pH of 12.0, alkaline-pretreated sludge was notified to provide maximum hydrogen manufacture. The results in the current work displayed that biohydrogen production was not limited at the studied pH values. Differences with the results of studies in the literature are generally due to the raw material, the microbial community or specific bacteria used, and the pH value.

As displayed in Fig. 1 A(1) and Fig. 2 B(1), when pH 4.5 and 4.0 values were compared at an OLR of 0.25 g quinoa/L, it was designated that bio-H_2 production was higher at pH 4.5. Compared to other pH values, bio-reactors actuated at pH 4.0 and fed with an OLR of 0.50 gr kinoa/L as presented in Fig. 1 A(2) and Fig. 2 B(2), 0.75 g quinoa/L as presented in Fig. 1 A(3) and Fig. 2 B(3), and 1.00 g quinoa/L as presented in Fig. 1 A(4) and Fig. 2 B(4) produced more biological hydrogen. These results showed that pH was an important parameter depending on the OLR. Among the bio-reactors actuated at different OLRs but at the same pH values, maximum bio-H_2 manufacture was designated in the ones fed with 1.00 g quinoa/L (Fig. 1 A(4) and Fig. 2 B(4)). In all the bio-reactors actuated at an initial pH of 4.5 (Fig. 1) and pH 4.0 (as presented in Fig. 2 B(3) and Fig. 2 B(4)), hydrogen production increased over time, decreased after the increase, and then showed an increasing trend again. pH has been notified to influence hydrogen fermentation due to the event of the dominant species in the mixed culture [30]. This trend in the current study may be elucidated by the activity of the dominant species affecting pH. Additionally, it was designated in the study that biohydrogen production decreased over time. This may be a result of both the reduction in raw materials and the manufacture of volatile fatty acids (VFAs) during the hydrogen manufacture stage. In all of the bio-reactors, no methane production was detected as a result of gas measurements made during operation. On the other hand, it was observed that CO_2 manufacture was at similar levels in all the bio-reactors. In the bio-reactors fed with unpretreated quinoa biomass, biohydrogen production increased as the organic loading rate increased at both initial pH values.

Literature studies have generally reported that raw material type and pH value have a significant impact on bio-H_2 production. In this context, Sibiya et al. [31] noted that the activity of CH_4-producing bacteria was slow below pH 6.5, and Chen et al. [32] stated that hydrogen-producing microorganisms could be enriched by acid (pH 3.0) treatment and inhibit methanogens. Similarly, Ghimire et al. [33] notified that the activity of hydrogen-consuming bacteria decreased significantly below pH 6.0, depending on the organic loading rate. Biological hydrogen manufacture by dark fermentation is influenced by various factors, such as inoculum type, pre-treatment of the inoculum, raw material type, direct use of raw material or use of pretreated raw material, temperature, and pH. In another study, thermal pretreatment (at 100 °C for 15 min) was applied to anaerobic sludge from a natural river to explore the potential of hydrogen production from corn stalk waste in batch operation. The operation was performed at 36 °C, at an initial pH of 7.0, and eventuated in a maximum of 126.22 mL H_2/g biomass at a concentration of 20 g/L [34]. Sattar et al. [35] used rice straw, agricultural and industrial waste rice husk, rice bran, and sludge that had been thermally shocked under mesophilic and thermophilic temperature conditions and examined the biohydrogen manufacture potential in an anaerobic bio-reactor operated with no pH control and at an initial pH value of 7.5. The authors found that for all raw materials except rice waste, augmentation the temperature from mesophilic (37 °C) to thermophilic (55 °C) conditions boosted biohydrogen production. The study concluded that the optimum pH operating range of rice bran, rice husk, and rice straw for biological hydrogen production was between 7.0 and 6.0. Some studies have examined biological hydrogen production using specific bacteria rather than mixed microorganisms. Hu et al. [36] investigated the initial pH value and autoclaving effect in batch operations. The authors directly used raw food waste and examined its biological hydrogen production potential with some pure cultures (*Clostridium beijerinckii, Enterobacter aerogenes, Clostridium pasteurianum, Clostridium butyricum*). They found that pH value and autoclaving process had a positive effect on raw food waste. *Clostridium beijerinckii* and *Enterobacter aerogenes* bacteria produced hydrogen from unpretreated food waste. *Clostridium pasteurianum* and *Clostridium butyricum* cultures started to produce hydrogen after the optimum initial pH value was adjusted to 7.0. It was determined that the maximum biohydrogen manufacture was 38.9 mL H_2/g-VS_{added} by *Clostridium butyricum* bacteria at a pH value of 7.0 and after autoclaving of food waste. In another work, Qi et al. [37] examined peanut shells together with a new hydrogen producer, *Clostridium guangxiense* ZGM211^T, for fermentative hydrogen manufacture. In the work, the use of 10 mg/mL glucose with 40 mg/mL peanut shell powder gave maximum biohydrogen manufacture of 39.9 mL/g substrate under optimum fermentation conditions with a 4 % inoculation rate and initial pH value of 6.5. The results of these studies indicate that specific research needs to be expanded, as existing studies mainly carry out biological hydrogen production specific to temperature, pH value, and raw material type, depending on the use of mixed microorganisms or specific bacteria.

It has been enounced that the reduction in pH value may be occasioned by the accumulation of VFAs and carbon dioxide as a result of high organic loading [31]. Acetic acid was dominant in the entire fermentation, obtained in the hydrogen production phase carried

Table 1
Organic acid analysis in the bio-reactors fed with raw quinoa residual biomass.

Bioreactor-pH-Hour	Acetic acid (mg/mL)	Butyric acid (mg/mL)	Propionic acid (mg/mL)
A(4)-4.5-29	1.164 ± 0.021	0.292 ± 0.003	0.034 ± 0.004
A(4)-4.5-42	2.204 ± 0.000	1.135 ± 0.003	0.072 ± 0.001
A(4)-4.5-51	1.995 ± 0.011	1.080 ± 0.002	0.051 ± 0.000
A(4)-4.5-66	2.120 ± 0.002	1.128 ± 0.003	0.057 ± 0.001
A(4)-4.5-90	1.656 ± 0.004	0.738 ± 0.003	0.040 ± 0.001
B(4)-4.0-29	1.364 ± 0.021	0.352 ± 0.001	0.023 ± 0.002
B(4)-4.0-43	2.565 ± 0.004	1.117 ± 0.006	0.060 ± 0.001
B(4)-4.0-50	2.560 ± 0.002	1.130 ± 0.000	0.053 ± 0.005
B(4)-4.0-66	2.510 ± 0.000	1.145 ± 0.003	0.047 ± 0.001
B(4)-4.0-74	2.098 ± 0.010	0.720 ± 0.004	0.051 ± 0.003

out from raw quinoa biomass at an OLR of 1.00 g quinoa/L and initial pH values of 4.5 and 4.0, which can be seen in Table 1. This organic acid was followed by butyric acid. It has been reported that the biological hydrogen-producing metabolite is butyric acid and acetic acid, and the hydrogen-consuming metabolite is propionic acid [38]. Feng et al. [39] reported that acetic and butyric acid had a positive relation with biohydrogen yield and that the accumulation of propionic acid would consume the biological hydrogen produced. In the current study, it has been determined that within bio-reactors with different raw organic loading rates, maximum hydrogen production occurred in the bio-reactor actuated at pH 4.5 with 1.00 g quinoa/L, as shown in Fig. 1 A(4), and at pH 4.0 with 1.00 g quinoa/L, as depicted in Fig. 2 B(4). The results of the samples taken from these bio-reactors for organic acid analysis are presented in Table 1. Fig. 1 A(4) shows that maximum hydrogen production was obtained at 42 h at pH 4.5. As presented in Table 1, acetic acid was higher at 42 h than at other hours, which supports the literature. Fig. 2 B(4) demonstrates that maximum biohydrogen production was obtained at 43 h at pH 4.0. As presented in Table 1, acetic acid was higher at 43 h than at other hours, which supports the literature.

In biological hydrogen production studies, butyric, acetic, and propionic acids were generally examined among VFAs. Sillero et al. [40] examined the biological hydrogen and VFA production performance of wine vinasse, sewage sludge, and poultry manure co-fermentation. The authors reported that in addition to carbon dioxide and hydrogen, approximately 55 % acetic acid, 33 % butyric acid, and 10 % propionic acid were produced. They also reported that the presence and abundance of VFAs obtained varied depending on the operating parameters. Another study noted that acetic and butyric acid were produced during hydrogen production and reported that there was an increment in the manufacture of these acids in the first 24 h of the operations, after which time the manufacture of acetic acid increased in the medium containing bagasse acid rich in xylose, and that the manufacture of acids other than butyric acid remained almost constant in the medium containing standard xylose [41]. A work by Ai et al. [42] examined the anaerobic conversion of unpretreated and differently pretreated rice straw to butyric acid. The study found that the main products of mixed culture fermentation were acetic and butyric acid and that these two main products contributed 85 % to VFAs. Another study reported that methane-producing bacteria could directly convert acetic acid into CO_2 and CH_4, so the acetic acid concentration decreased to zero over time [43]. Similarly, the high acetic acid and butyric acid production and low propionic acid manufacture in the current study support the literature results.

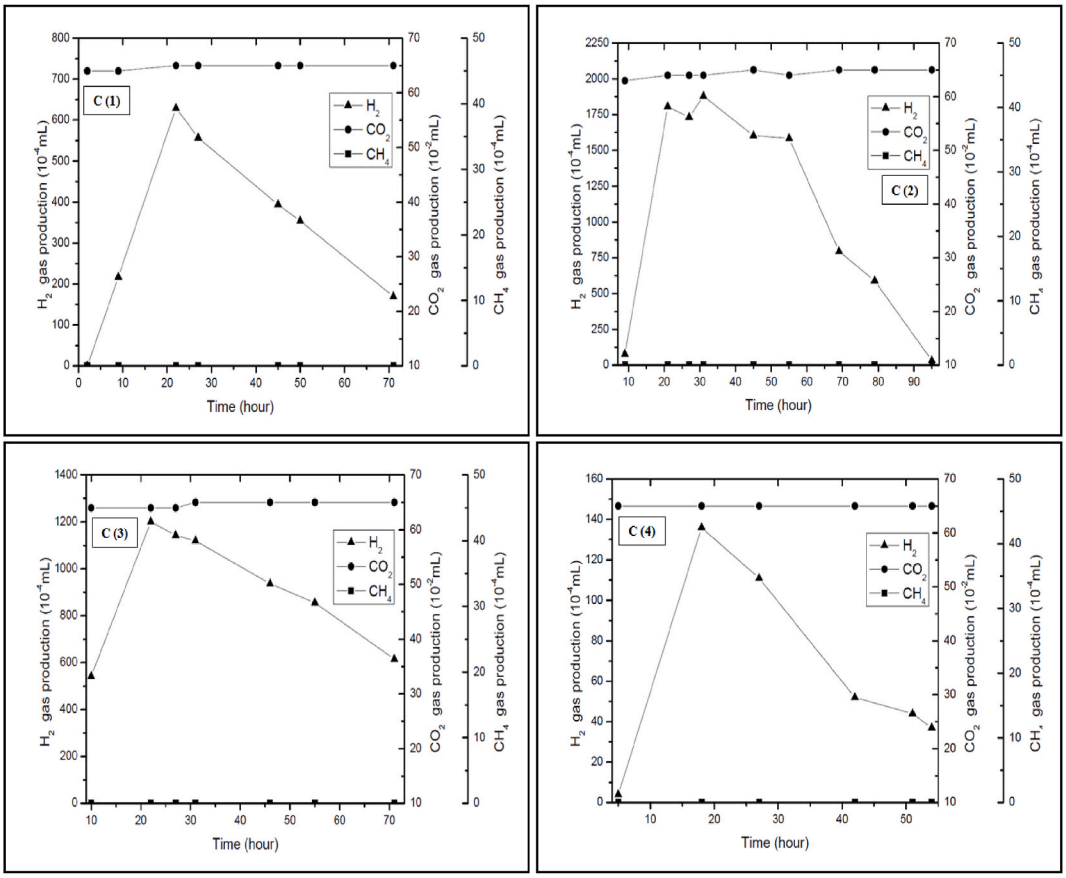

Fig. 3. Hydrogen gas production in the bio-reactors fed with quinoa residue extract liquid and operated at pH 4.5.

3.2. Effect of using quinoa biomass extract liquid on the production of biological hydrogen

Bio-reactors were operated in a dark room at 36 ± 1 °C and in a 150 rpm shaking incubator. The aim was to investigate the potential for biohydrogen production at OLRs of 0.25 (91.1 mg COD L^{-1} on average), 0.50 (102.3 mg COD L^{-1} on average), 0.75 (112.3 mg COD L^{-1} on average), and 1.00 (126.2 mg COD L^{-1} on average) g quinoa extract/L, and initial pH of 4.5 and 4.0.

It was designated that there was no gas manufacture during the first hours in the bio-reactors fed with liquid extracted from thermally pretreated quinoa biomass at different OLRs and actuated at an initial pH of 4.5 (Fig. 3). Maximum biological hydrogen manufacture was as follows: 630.10^{-4} mL at 22 h by 0.25 g quinoa extract/L (Fig. 3 C(1)), 1880.10^{-4} mL at 31 h by 0.50 g quinoa extract/L (Fig. 3 C(2)), 1201.10^{-4} mL at 22 h by 0.75 g quinoa extract/L (Fig. 3 C(3)), and 136.10^{-4} mL at 18 h by 1.00 g quinoa extract/L (Fig. 3 C(4)). After the hours when maximum biological hydrogen was produced, hydrogen production tended to decrease.

It was designated that no gas was produced in the first hours of operation in all the bio-reactors fed with OLRs of 0.25, 0.50, 0.75, and 1.00 g quinoa extract/L and actuated at an initial pH of 4.0 (Fig. 4). Maximum biological hydrogen manufacture was as follows: 557.10^{-4} mL at 8 h by 0.25 g quinoa extract/L (Fig. 4 D(1)), 170.10^{-4} mL at 22 h by 0.50 g quinoa extract/L (Fig. 4 D(2)), 1511.10^{-4} mL at 22 h by 0.75 g quinoa extract/L (Fig. 4 D(3)), and 192.10^{-4} mL at 18 h by 1.00 g quinoa extract/L (Fig. 4 D(4)). After these hours, bio-H_2 production generally tended to reduction.

It was designated that carbon dioxide production in the bio-reactors was at similar levels. No methane production was detected in all of the bio-reactors throughout the operation. Operating factors affect biological hydrogen production. In this context, temperature and raw material type and form affect hydrogen production. It has been reported that pH value significantly affects the activity of dominant species, especially in studies using mixed culture. Therefore, depending on the feeding solution and mixed bacteria, the pH value can vary within wide ranges [30,44]. In the study, bio-reactors fed with the same organic loading rates but actuated at initial pH of 4.5 and 4.0 were examined. Accordingly, the OLR of 0.25 g quinoa extract/L, as presented in Fig. 3 C(1) and Fig. 4 D(1), and the OLR of 0.50 g quinoa extract/L, as presented in Fig. 3 C(2) and Fig. 4 D(2), yielded higher biological hydrogen production in the bio-reactors actuated at pH 4.5, compared to pH 4.0. On the other hand, the OLR of 0.75 g quinoa extract/L, as presented in Fig. 3 C(3) and Fig. 4 D(3), and the OLR of 1.00 g quinoa extract/L, as presented in Fig. 3 C(4) and Fig. 4 D(4), yielded higher biological hydrogen production in the bio-reactors actuated at pH 4.0. Considering the bio-reactors actuated at the same pH values but fed with different organic loading rates, bio-reactors fed with 0.50 g quinoa extract/L (Fig. 3 C(2)) and 0.75 g quinoa extract/L (Fig. 4 D(3)) gave

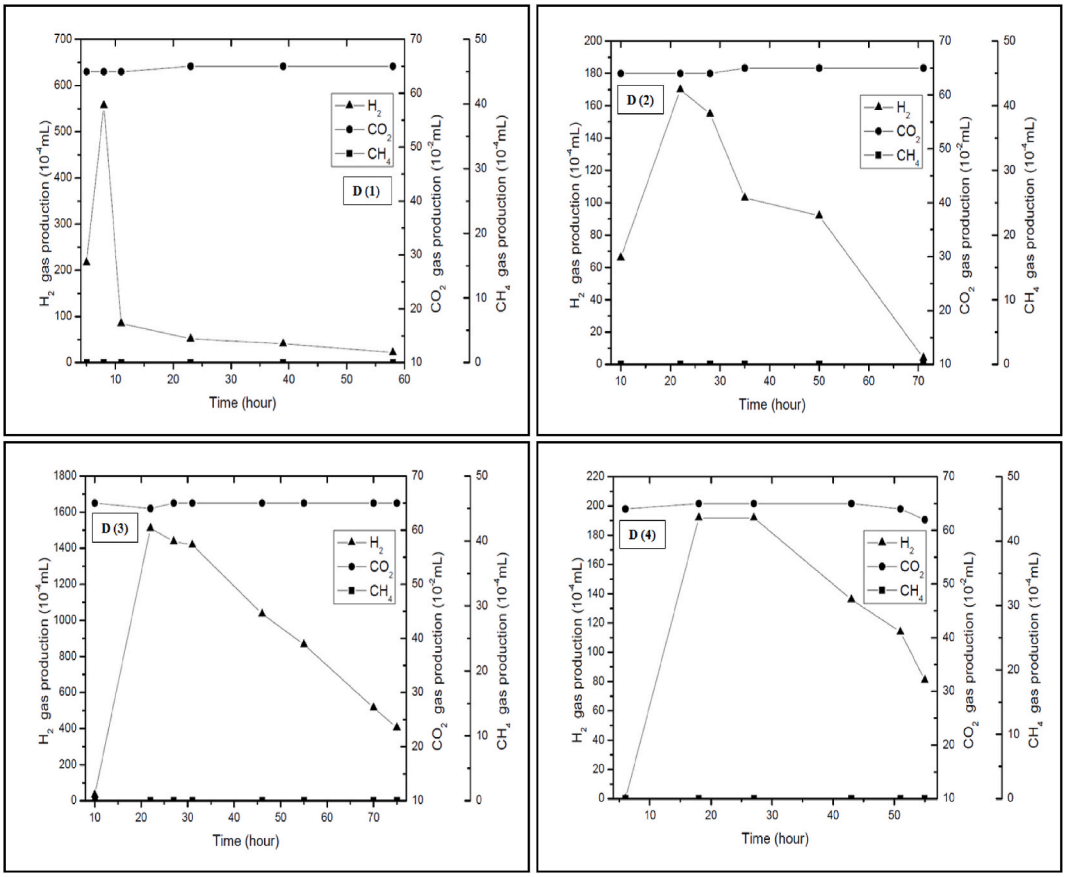

Fig. 4. Hydrogen gas production in the bio-reactors fed with quinoa residue extract liquid and operated at pH 4.0.

maximum bio-H_2 production. Therefore, in the study, pH, raw material type and form, and OLRs affected biohydrogen production. In the bio-reactors, COD values were determined to be in the range of 126.2–91.1 mg/L at the beginning and 99.7–72.3 mg/L at the end of the operation. Pretreatments in studies on pretreated raw materials and mixed microorganisms (sludge) are generally acid and alkaline processes, and the number of studies applying thermal pretreatment, which is used in this study, to raw materials and sludge is very limited. Sen et al. [45] pretreated bamboo park sludge (mixed culture) at 95–100 °C for 60 min. In addition, biological hydrogen production from pretreated (acid and autoclaving) rice straw was examined at an initial pH of 5.5, in batch serum vials, at 37 °C. The results of the study were reported to be in the range of 1.89–4.39 mL H_2/g biomass. Silva et al. [46] used *Clostridium roseum* bacteria and investigated biological hydrogen production from cashew apple bagasse raw material at 38 °C and 150 rpm. The raw material was subjected to grinding and chemical (alkali, acid) and enzymatic pretreatments. In the work, the results were found to be in the range of 0.08–1.89 mL H_2/g biomass. In another study, brewery spent grains were investigated for hydrogen production. To hydrolyze the lignocellulose structure of the raw material, the raw material was subjected to dilute acid and alkali pretreatments. The study reached the result of 100–175 mL H_2/g bacteria culture dry weight [47]. Pan et al. [48] subjected wheat bran to acid pre-treatment and examined the influence of initial pH on hydrogen manufacture under different operating conditions. In the study, the main VFAs were determined as butyric and acetic acid, and the dominant bacteria that produced maximum hydrogen at an initial pH of 5.0 were identified as *Clostridium* spp. In another work, Lee et al. [49] used sucrose in glass bottles and examined the impression of pH on hydrogen manufacture at 37 °C. The authors reported that no hydrogen was produced at pH values of 11.0 and 12.0, while hydrogen manufacture was maximum at an initial pH of 9.0. Similarly, in the current work, initial pH values of 4.5 and 4.0 decreased to approximately 3.2 pH at the end of the running. Based on the current study and previous studies in the literature, it has been determined that pH value and OLR significantly affect biological hydrogen production, depending on the inoculum and raw material used. Biohydrogen manufacture from sucrose by dark fermentation was carried out at 30 °C and without pH control in the study handled by Mota et al. [50]. The authors reported that biohydrogen production continued throughout the operation and the effluent pH value was 2.8 on average. The study result was found to be positive in terms of hydrogen production, contrary to litterateur data reporting inhibition below the 4.0 pH value in biological hydrogen production studies.

Table 2 presents the analysis results of organic acids in the bio-reactors fed with an OLR of 0.50 g quinoa extract/L and actuated at an initial pH of 4.5 and in the bio-reactors fed with an OLR of 0.75 g quinoa extract/L and actuated at an initial pH of 4.0. It was seen that acetic acid and butyric acid were dominant in the bio-reactors.

It has been reported that bio-H_2 production is accompanied by organic acids such as acetic acid and butyric acid [51] and that high acetic acid concentration can be associated with high biological hydrogen production [52]. On the other part, Alexandropoulou et al. [53] reported that the indicator of an efficient hydrogen production process is low propionic acid production. It has been notified that an increment in acetic acid concentration is associated with a diminishment in propionic acid concentration [54]. Additionally, it has been determined that carbohydrate-rich wastes generally turn into butyric acid and propionic acid, while protein-rich wastes support the manufacture of valeric acid and isovaleric acid [55]. In this study, among the bio-reactors fed with the extract liquid at different OLRs, maximum hydrogen production was designated in the bio-reactor fed with 0.50 g quinoa extract/L and operated at pH 4.5 Fig. 3C (2) and in the bio-reactor fed with 0.75 g quinoa extract/L and operated at pH 4.0 Fig. 4 D(3). The results of the samples taken from these bio-reactors for organic acid analysis are presented in Table 2. As presented in Fig. 3 C(2), maximum hydrogen production was obtained at 31 h at pH 4.5. As given in Table 2, acetic acid level is higher at 31 h than at other hours, which supports the literature. As presented in Fig. 4 D(3), maximum hydrogen production was obtained at 22 h at pH 4.0. As given in Table 2, acetic acid level is higher at 22 h than at other hours, which supports the literature.

3.3. Microbial community content

The role of the mixed microbial community on bio-H_2 manufacture has been surveyed in a limited number of studies. In this context, the species-level rates of mixed microorganisms that play an important role in biohydrogen production were examined using taxonomic content analysis. Since bio-H_2 manufacture is maximum in the bio-reactors fed with an OLR of 1.00 g raw quinoa/L and actuated at pH 4.5 and 4.0, the microbial community was taken from the contents of these bio-reactors. As shown in Fig. 5, in the bio-reactor actuated at an initial pH of 4.5 (Fig. 5 A(4)), *Clostridium butyricum* was the most dominant bacteria in hydrogen manufacture, with a rate of 10.63 %. It was followed by *Clostridium magnum* with 9.62 %, *Levilinea saccharolytica* with 8.76 %, and *Hathewaya histolytica* with 8.19 %. As shown in Fig. 5 B(4), in the bio-reactor operated at an initial pH value of 4.0, the dominant bacteria in hydrogen production were as follows: *Hathewaya histolytica* with 17.70 %, *Kosakonia cowanii* with 7.58 %, and *Levilinea saccharolytica* with 7.10 %. In the study, "others" was determined as 27.71 % (Fig. 5 A(4)) and 29.82 % (Fig. 5 B(4)), which represents the total of

Table 2
Organic acid analysis in the bio-reactors fed with quinoa residue extract liquid.

Bioreactor-pH-Hour	Acetic acid (mg/mL)	Butyric acid (mg/mL)	Propionic acid (mg/mL)
C(2)-4.5-21	0.518 ± 0.000	0.301 ± 0.001	0.068 ± 0.001
C(2)-4.5-31	0.565 ± 0.001	0.273 ± 0.000	0.055 ± 0.001
C(2)-4.5-45	0.394 ± 0.005	0.267 ± 0.005	0.034 ± 0.008
D(3)-4.0-22	0.394 ± 0.003	0.221 ± 0.002	0.009 ± 0.001
D(3)-4.0-27	0.361 ± 0.007	0.252 ± 0.005	0.034 ± 0.000
D(3)-4.0-31	0.311 ± 0.000	0.293 ± 0.001	0.047 ± 0.004

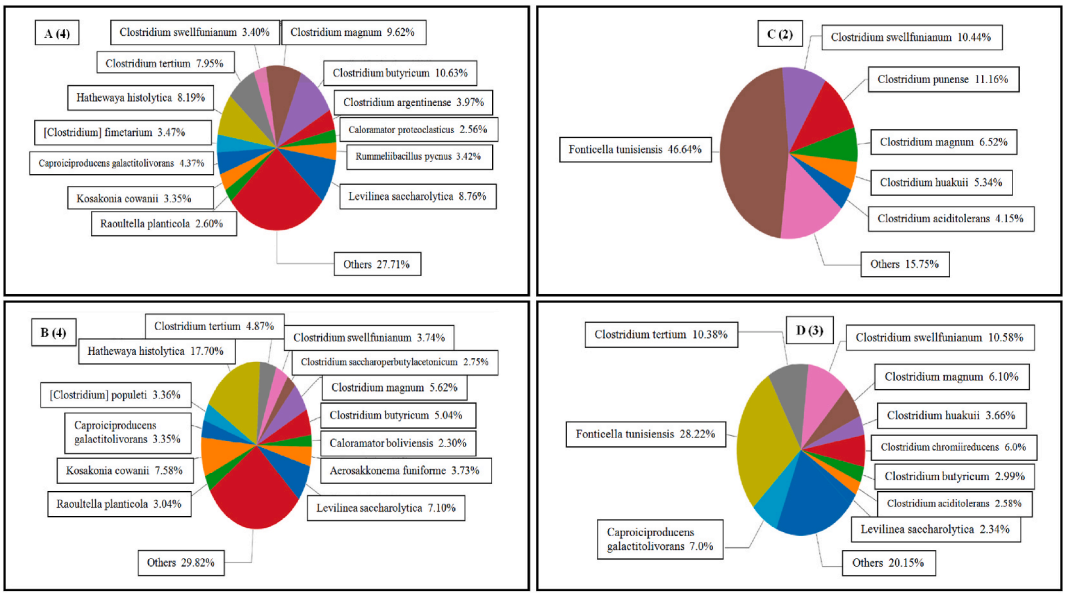

Fig. 5. Species-level rates in the bio-reactors.

microorganisms with a rate below 2 %. On the other hand, among the bio-reactors fed with liquid extracted from quinoa biomass, maximum biological hydrogen production was obtained from the bio-reactor (Fig. 5 C(2)) operated at an initial pH of 4.5 and fed with an organic loading rate of 0.50 g quinoa extract/L and from the bio-reactor actuated at an initial pH of 4.0 and fed with an OLR of 0.75 g quinoa extract/L (Fig. 5 D(3)). Therefore, the microbial community taken from these bio-reactors was used to determine species-level rates. In these bio-reactors, *Fonticella tunisiensis* was determined to be the most dominant species. It has been reported that the new genera with biohydrogen production potential are *Fonticella*, *Gracilibacteri*, and *Romboutsia* [56]. In this study, *Fonticella tunisiensis* was detected at a rate of 46.64 % in the bio-reactor actuated at an initial pH of 4.5 and a rate of 28.22 % in the bio-reactor actuated at an initial pH of 4.0. In the study, "others" was determined as 15.75 % (Fig. 5 C(2)) and 20.15 % (Fig. 5 D(3)), which represents the total of microorganisms with a rate below 2 %.

In studies where specific bacteria were preferred for hydrogen production, *Clostridium* species were generally used. The raw materials and bacteria used included glucose monohydrate and *Clostridium butyricum* CWBI1009 [57], food waste and *Clostridium butyricum* [58], non-sterile food waste and *Clostridium butyricum* TISTR 1032 [59], vine shoots and *Clostridium butyricum* [60], glucose and molasses and *Clostridium butyricum* TM-9A [61], and tequila vinasses and *Ethanoligenens harbinense* and *Clostridium tyrobutyricum* [62]. The majority of hydrogen-producing bacteria can grow in wide pH ranges. Additionally, hydrogen-producing bacteria have been reported to grow much faster than methane-producing bacteria [63]. Tang et al. [64] analyzed the microbial community used for biohydrogen production and examined the influence of the initial pH value. The authors reported that a significant alteration in initial pH value did not cause a alteration in the dominant population at the bio-H_2 manufacture stage. Similarly, the results of the present work displayed that despite the change in pH value, there was no important alteration in the proportions of the dominant population at the biohydrogen production stage. In addition, in the current work, it was designated that the use of raw residue and extracted liquid at the initial pH value of 4.5 and 4.0 at mesophilic temperature was suitable for the activity of hydrogen-manufacturing bacteria.

4. Conclusions

In this study, raw quinoa residue and extracted liquid were investigated in batch bio-reactors operated under different operating conditions. Biohydrogen production was detected in all the bio-reactors. In the bio-reactors fed with unpretreated quinoa biomass, biohydrogen production increased as the organic loading rate increased at both initial pH values. On the other hand, in the bio-reactors fed with the extract liquid obtained after thermal pre-treatment of quinoa biomass, maximum bio-H_2 production was obtained from the bio-reactor actuated at an initial pH value of 4.5 and fed with 0.50 g quinoa extract/L and from the bio-reactor actuated at an initial pH value of 4.0 and fed with 0.75 g quinoa extract/L. Among the bio-reactors operated at pH 4.5, maximum bio-H_2 production was $14{,}543.10^{-4}$ mL in the bio-reactor fed with 1.00 g quinoa/L and 1880.10^{-4} mL in the bio-reactor fed with 0.50 g quinoa extract/L, whereas among the bio-reactors operated at pH 4.0, maximum biological hydrogen production was $61{,}537.10^{-4}$ mL in the bio-reactor fed with 1.00 g quinoa/L and 1511.10^{-4} mL in the bio-reactor fed with 0.75 g quinoa extract/L. It was determined that species-level rates did not change significantly in the bio-reactors actuated at different pH values. In the bio-reactors fed with raw quinoa residue, *Clostridium butyricum* with a ratio of 10.63 %, and *Hathewaya histolytica* with a ratio of 17.70 % were designated as the most dominant bacteria at pH 4.5 and 4.0, respectively. On the other hand, in the bio-reactors fed with extracted liquid, *Fonticella tunisiensis* was appointed to be the most dominant bacteria with a ratio of 46.64 % and 28.22 % at pH 4.5 and 4.0, respectively. These bacteria can be

used as specific bacteria for bio-H2 manufacture. This work demonstrated that quinoa residue could be utilized for biohydrogen production.

5. Limitations and future research scope

There is a need for further research examining the effects of factors such as temperature, using specific bacteria or mixed bacteria, raw material type and form, and organic loading rates on bio-H_2 production from waste or residues. In the study, a problem with any of these key factors can limit biohydrogen production. Researchers have become more interested in biohydrogen production using residues or waste in recent years. In this context, the fact that the studies are new and limited in number requires comprehensive optimization studies to be carried out in the future for the wastes whose biohydrogen production potential has been identified. Additionally, due to the high diversity of residues from agricultural activities, it is recommended to investigate residues with unknown biohydrogen production potential.

Data availability

The author confirms that the data supporting the findings of this study are available within the article.

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CRediT authorship contribution statement

Nesrin Dursun: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The author declares no conflicts of interest.

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