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# Evaluation of industrial waste black cumin (*Nigella Sativa*) for biohydrogen production without pretreatment

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**Abstract**
The depletion of fossil fuels and the fact that fossil fuels pollute the environment accelerated the trend toward environmentally friendly energy alternatives such as biohydrogen. As one of the hydrogen production methods, biological hydrogen production processes are clean, sustainable, cost-effective and operation-easy processes that eliminate a series of disadvantages in the other hydrogen production processes. This study examined usability of black cumin obtained as waste from vegetable oil industry in the production of biohydrogen with dark fermentation without any pretreatment under different operating conditions (pH: 4.0, 5.0, 6.0 and hydraulic retention time (HRT): 36, 24 h) in a completely stirred-tank reactor and fluidized bed reactor. The best hydrogen production was established at pH 5.0 during the operating period. In completely stirred-tank reactor and fluidized bed reactor, the maximum hydrogen production measured at pH 5.0 was 25.3 and 11.1 mL/day, respectively. In the study that used mixed culture, the predominant microbial population was found to be *Enterobacteria*, *Salmonella bongori*, *CFB group bacteria*, *Brenneria goodwinii*, *Erwinia amylovora*, *Thiofractor thiocaminus*, *Sulfurospirillum sp.* and *Hydrogenimonas thermophila* at pH 5.0. Within the scope of the study, it was concluded that waste black cumin, which has not been tried before in any biohydrogen research, can be used successfully for biohydrogen production.

**Keywords** Black cumin · Mixed culture · Biological hydrogen production · Dark fermentation · Bioreactor

## 1 Introduction

Fossil fuels, which are still being used throughout the world but about to be depleted, can be listed as coal, wood, petroleum and natural gas. Most of the energy need is provided by fossil fuels. Toxic gases such as SOx, NOx and COx, which are generated by combustion after combining, by the aforementioned fossil fuels oxygen gas, cause air pollution. These toxic gases that are emitted into the atmosphere cause adverse effects such as melting of

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glaciers, floods and climate changes due to the greenhouse effect as well as warming by the world. The depletion of fossil fuels and the fact that fossil fuels pollute the environment have become one of the major problems in the world. The rapid depletion of fossil energy resources has accelerated the process for developed countries to turn toward agriculture-based alternative energy resources such as bioethanol, biodiesel and biohydrogen. Hydrogen energy, which is among alternative energy sources, can be easily adapted to existing energy infrastructure changes, as it has similar characteristics with fossil fuels in the field of transportation, storage and consumption. The fact that the combustion product is toxic gases in fossil fuels and water in hydrogen gas makes it more attractive to prefer hydrogen gas as fuel (Dursun & Gülşen, 2019). It is possible to use biogas (hydrogen and methane) energy in all areas with energy consumption (houses, factories, automobiles, aircraft, rockets, etc.) (Dursun & Gülşen, 2019; Abdelsalam et al., 2020). Hydrogen gas can be produced with various production technologies. Biological hydrogen production processes have several advantages in terms of being clean, sustainable, low cost, easy to operate, using organic waste and eliminating the risk of bacterial contamination. Dark fermentation, which is the most advantageous one of these processes, is the most effective process among biohydrogen production methods since it does not require sunlight or artificial light as an energy source (Dursun & Gülşen, 2019). Despite these advantages, there are few studies on biohydrogen production by dark fermentation. Most of the studies have examined biohydrogen production by pretreatment of wastes. The fact that there are limited studies in the literature on the use of waste directly in biohydrogen production without any pretreatment indicates the research gap on this subject.

One of these limited studies was conducted by Sivagurunathan et al. (2018). In the study, hydrogen produced by co-digestion of macroalgal and microalgal biomass was examined using bottles with a working volume of 65 mL and a total volume of 150 mL. The top spaces of the bottles were filled with high pure $N_2$ gas to provide an anaerobic environment. The red macroalgal biomass and green mixed microalgal biomass were mixed in a ratio of 8:2 using an initial substrate concentration of 10 g/L, and various additions of inoculum ranging between 3 and 15%. The results of the research revealed that co-digestion with 6% inoculum addition to the 45 mL/g dry biomass with a high hydrogen content of 24% provided the maximum hydrogen yield. On the other hand, Oceguera-Contreras et al. (2019) studied hydrogen production from the substrates of agricultural industrial wastes, such as molasses, sugarcane bagasse and vinasse, using dark fermentation activated sludge consortia and vermihumus-associated microorganisms inoculum. The study was carried out using bottles of 120 mL under anaerobic conditions at 40 °C. Hydrogen production from molasses, sugarcane bagasse and vinasse was found to be 1571.81, 1246.36 and 232.72 $mlH_2L^{-1}$, respectively. The results revealed that vermihumus-associated microorganisms produced hydrogen using untreated sugarcane bagasse. Another study using organic waste directly without pretreatment was conducted by Kim et al. (2012). This study investigated biological hydrogen production using the potential substrate of carbon-rich rice straw milled up to 2 mm and sewage sludge with high nitrogen content in batch reactors at 55 °C. Both untreated sludge and heat-treated sludge (15 min at 100 °C) were used. Hydrogen production at the C/N ratio of 25 in untreated sludge was found to be 33% higher compared to heat-treated sludge. Moreover, high and stable hydrogen content was found to be 58% under the same conditions. The researchers concluded that the mixture of untreated sludge and rice straw could be used as an inoculum for efficient hydrogen production. On the other hand, Cheng and Liu (2011) examined biohydrogen production using specific bacteria utilizing organic matter directly without any pretreatment. In this study, the hydrogen yield of untreated cornstalk powder was investigated using *Clostridium*

*thermocellum 7072* in a 100-L continuous stirred-tank reactor (CSTR) under thermophilic conditions (55 °C). The hydrogen yield was found to be 61.4 ml $H_2$/g of the cornstalk. This research indicated that direct microbial conversion of lignocellulosic waste via *C. thermocellum* was a promising method for biohydrogen production. In another study, Talluri et al. (2013) investigated the potential of hydrogen fermentation by dark fermentation using the extremely thermophilic bacterium *Caldicellulosiruptor saccharolyticus DSM 8903* and untreated switchgrass. The research was conducted using 100 ml batch serum bottles containing 25 ml culture medium and 3% switchgrass at 65 °C. The hydrogen yield after fermentation was found to be 11.2 mmol $H_2$/g biomass.

Black cumin seed oil was tested in certain foods such as Domiati and Kermanian cheese for its inhibitory effect against some pathogenic bacteria (Ramadan Hassanien et al., 2014; Taherkhani et al., 2015). Another study reported that black cumin oil was used in natural therapies and mainly to prevent spoilage in foods. In this study, five different black cumin oils were examined in terms of preventing spoilage in food products, and it was concluded that black cumin oil can be used as antimicrobial agents in food products. (Arici et al., 2005). The black cumin oil was also reported to be used in the field of health. In this context, the black cumin oil was examined in clinical studies for the treatment of moderate chronic generalized periodontitis. The study revealed that clinical improvements were observed in 2–3 days after the beginning of the treatment in patients who used black cumin oil and that there were improvements in 8–10 days after the beginning of the treatment in patients who did not use black cumin oil (those having traditional treatment) (Gaybullaev, 2020).

Studies that investigated biological hydrogen production using biomass with a high amount of carbohydrates became successful. Black cumin waste is obtained as a residue of the black cumin oil industry. Considering the use of black cumin oil in natural therapies, preventing food spoilage and clinical studies as reported by the studies on black cumin oil, it is anticipated that there will be an increase in demand for this oil in the future. Due to the anticipation of an increase in black cumin oil demand in the future, it is expected that there will be an indirect increase in black cumin waste. In this regard, black cumin waste was obtained from a company (in Sanliurfa, Turkey) engaged in the production of black cumin oil, and its biological hydrogen generation potential was investigated. Many biomass were tested on biohydrogen production by dark fermentation using mixed bacterial culture, and in this context, no study was found on black cumin biomass in the literature. Black cumin, which has high carbohydrate content, is promising in biological hydrogen production. Some studies reported that 100 g of black cumin contained 30.5 ~ 39.04 g of carbohydrate, 21.67 ~ 37.33 g of oil, 20.02 ~ 24.05 g of protein, as well as moisture, ash and fiber (Ali et al., 2012; Javed et al., 2012; Khoddami et al., 2011). The composition (%) of de-oiled nigella sativa waste obtained as waste from black cumin oil production industry waste used in this study was found to be 43.5% carbohydrate, 15.5% fat, 35% protein and 6% ash.

This study is important in terms of both using the wastes generated by the industries producing black cumin oil, which was reported to be used in various fields, and researching renewable energy potentials for sustainability. In this study, black cumin waste was used for biohydrogen production for the first time. Moreover, the waste used in this study was directly used without any pretreatment.

The purpose of this study was to determine the usability of industrial black cumin waste in biohydrogen production through dark fermentation under different operating conditions without any pretreatment. The study also aimed to establish the appropriate process design basics for the wastes obtained in the black cumin industries to be utilizable for biohydrogen production. Microbial community structures were also investigated according to different

pH operating conditions in the completely stirred-tank reactor and fluidized bed reactor, depending on the use of mixed inoculation.

## 2 Materials and methods

In this section, the steps of the heat treatment application to anaerobic sludge, trace elements used, installation of bioreactors, operating conditions of bioreactors and measurement method of the gas obtained are described in detail. The framework of this research is presented in Fig. 1.

### 2.1 Bioreactors and operating conditions

For the inoculation process, sludge was taken from the upflow anaerobic sludge bed reactor of the biological wastewater treatment plant of Malatya Sugar Factory. Before the sludge was added to the reactor, it was subjected to heat pretreatment (45 min at $93 \pm 2$ °C) to increase the activity of hydrogen-producing microorganisms in the mixed microorganism content and inhibit or completely destroy the activity of methanogenic microorganisms. Reactors were operated according to the amounts of trace elements studied by Fang et al. (2006). The trace elements used included $NH_4Cl$, $KH_2PO_4$, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, $NiCl_2.6H_2O$, $Na_2MoO_4.2H_2O$, $ZnCl_2$, $CoCl_2.6H_2O$, $CuCl_2.H_2O$, $MnCl_2.4H_2O$, $FeCl_3.6H_2O$ and $H_3BO_3$. In order to prevent the growth of phototrophic microorganisms, the reactors were covered with aluminum foil and operated in a temperature-controlled dark room

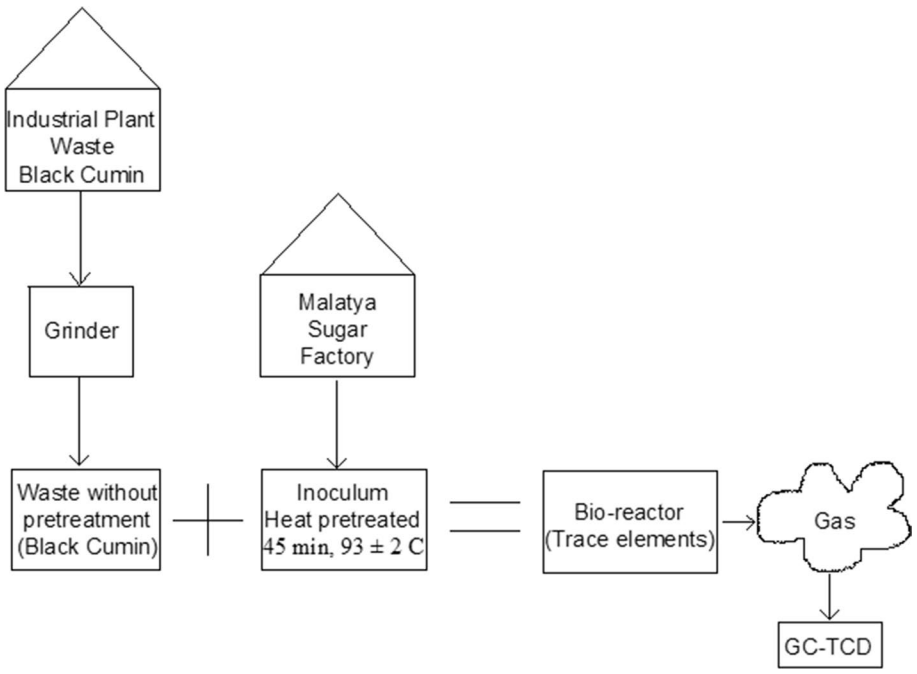


**Fig. 1** Application framework of the study

where the temperature was kept constant at 35 ± 2 °C. To prevent biological activity, nutrient tanks were kept in a refrigerator operating at + 4 °C. Freshly prepared nutrient solution was deoxygenated by passing through $N_2$ gas for 25 min and then fed into reactors.

Detailed information is given below about anaerobic bioreactors and operating conditions.

### 2.1.1 Continuous stirred-tank reactor

A continuous stirred-tank reactor made of glass material with a total volume of 5000 mL and an empty bed volume of 2250 mL was used (Fig. 2). Continuous stirring was achieved by adding magnetic fish into the reactor and dropping the reactor on the magnetic stirrer. An additional 5000-mL reactor was added to this reactor to provide gravity flow. With this added reactor, it was aimed to prevent the filling material, bacteria and waste black cumin in the reactor from leaving the system and to collect the effluent. Activated carbon (1.5–2.0 mm) and non-pretreated waste black cumin (0.5–1.0 mm) were added to the continuous stirred-tank reactor. The total volume of material added to the reactor was 1500 mL. Half of this volume was filled with granular activated carbon, one third with sludge and one sixth with waste black cumin. Activated carbon granules were preferred to increase and accelerate biofilm formation. In the reactor, waste black cumin (before being fed into the bioreactor system) was used by passing through the grinder system in order to ensure homogeneous organic matter balance. Thus, the fragmentation of black cumin particles is dependent on the surface area, and the process efficiency can be significantly

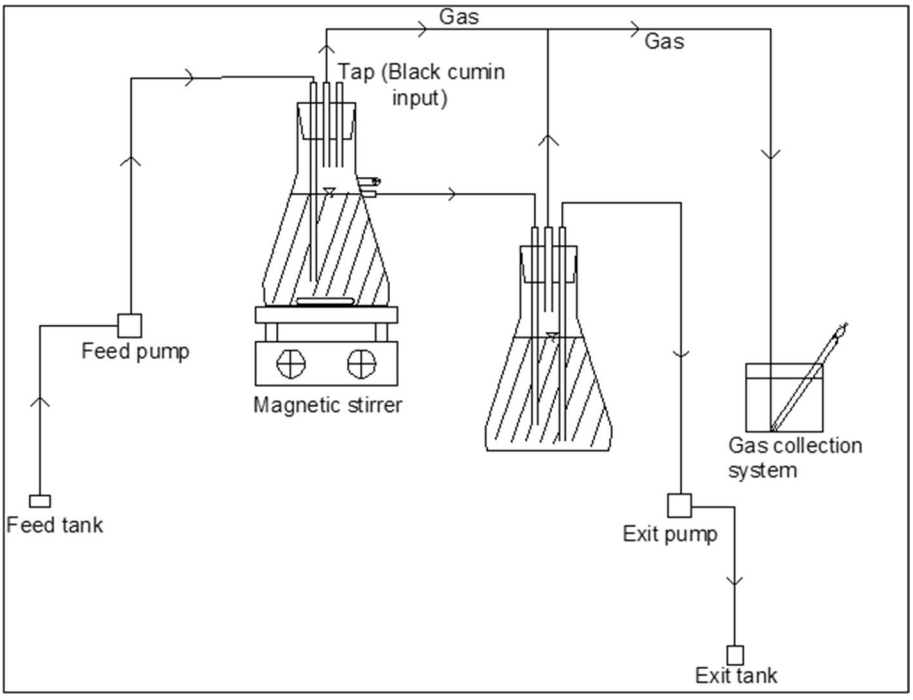


**Fig. 2** Pilot-scale continuous stirred-tank reactor design

increased due to the easy fragmentation of small particles. The heat-pretreated inoculum was added to the reactor and mixed intermittently for 5 days. Then, it was operated with continuous batch feed as completely stirred at 160 rpm stirring rate at 35 ± 2 °C (in a dark room where the temperature was kept constant). In order to prevent the waste black cumin, bacteria and active carbon in the reactor from leaving the reactor with the effect of stirring, the stirring was stopped 60 min before the feeding time, so that the reactor content could subside. Besides, the bioreactor was stirred again at 160 rpm after 5 min following the end of this period, with no stirring during the feeding period. Reactor feeding was performed keeping the nutrient tank in a refrigerator operated at + 4 $^{0}$C to prevent biological activity. Waste black cumin was added to the reactor from the one-way cock located at the top of the reactor, according to the determined organic loading amount. The reactor was fed with the feeding solution prepared by adding trace elements studied by Fang et al. (2006) to the raw tap water. Before the nutrient solution prepared by adding trace elements to the raw tap water was fed into the reactor, the pH was adjusted with 1 M HCl and 1 M NaOH every day before feeding. Operating conditions of the continuous stirred-tank reactor are shown in Table 1.

### 2.1.2 Fluidized bed reactor

A fluidized bed column reactor made of glass material with a total volume of 1000 mL and an empty bed volume of 400 mL, filled with activated carbon (1.5–2.0 mm) and non-pretreated waste black cumin (0.5–1.0 mm), was used (Fig. 3). The total volume of material added to the reactor was 600 mL. One-fourth of this volume was filled with granular activated carbon, one-fourth with sludge, and two-fourth with waste black cumin. Activated carbon granules were used to increase/accelerate biofilm formation. The reactor was operated by returning. In the reactor, waste black cumin (before being fed into the bioreactor system) was used by passing through the grinder system in order to ensure homogeneous organic matter balance. Similar to the completely stirred-tank reactor, the reactor was covered with aluminum foil and operated and inoculated in a dark room. The reactor was returned intermittently for 5 days and operated with continuous batch feeding at 35 ± 2 °C. No return was made during the feeding process in order to prevent the passage of the reactor contents to the return pump or reactor exit tank due to return. After the feeding was completed, return was resumed in the reactor. The reactor return is in the range of 50–100 times the flow rate of the nutrient solution. Reactor feeding was performed keeping the nutrient tank in a refrigerator operated at + 4 °C to

**Table 1** Operating conditions of continuous stirred-tank reactor filled with non-pretreated waste black cumin and activated carbon

| Periods | Acclimation phase | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| Days | 0–35 | 36–47 | 48–58 | 59–79 | 80–97 |
| pH | 5.0 | 4.0 | 4.0 | 5.0 | 6.0 |
| HRT (h) | 24.0 | 36.1 | 24.1 | 24.0 | 24.0 |
| Loading* (g.black cumin/L) | 2.22–4.44 (1–10) | 6.66 (36) | 6.66 (48) | 6.66 (59) | 6.66 (80) |
| Maximum hydrogen* (mL/day) | – | 1.2 (36) | 8.0 (52) | 25.3 (78) | 32.1 (90) |

*Values in parentheses indicate the days when the organic loading rate was changed and the days when the hydrogen concentration was maximum

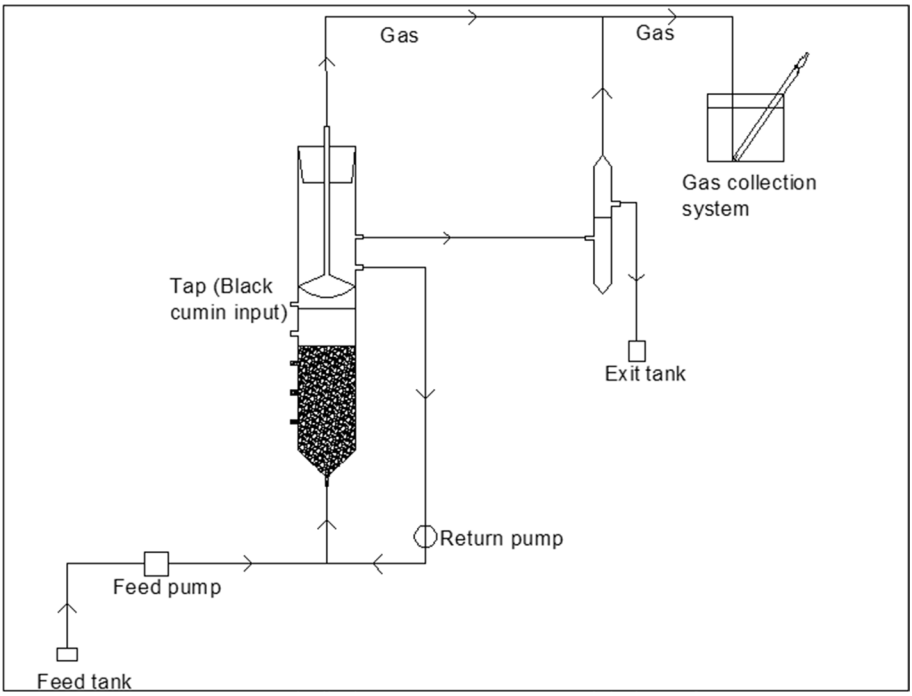


**Fig. 3** Pilot-scale fluidized bed reactor design

prevent biological activity. Non-pretreated waste black cumin was added to the reactor from the cock under the gas collection funnel in the reactor according to the determined organic loading amount. Feed solution prepared by adding trace elements and pre-feed pH adjustment are the same as in the continuous stirred-tank reactor. Operating conditions of the fluidized bed reactor are shown in Table 2.

**Table 2** Operating conditions of fluidized bed reactor filled with non-pretreated waste black cumin and activated carbon

| Periods | Acclimation phase | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| Days | 0–35 | 36–48 | 49–58 | 59–83 | 84–97 |
| pH | 5.0 | 4.0 | 4.0 | 5.0 | 6.0 |
| HRT (h) | 23.8 | 36.6 | 24.3 | 24.0 | 23.9 |
| Loading* (gr.black cumin/L) | 2.22–4.44 (1–10) | 6.66 (36) | 6.66 (49) | 6.66 (59) | 6.66 (84) |
| Maximum hydrogen* (mL/day) | – | 0.2 (48) | 6.4 (52) | 11.1 (68–78) | 7.8 (97) |

*Values in parentheses indicate the days when the organic loading rate was changed and the days when the hydrogen concentration was maximum

### 2.1.3 Batch reactors

In order to determine the biohydrogen production activity that changed depending on the pH in continuous batch fed reactors, sludge samples taken from these reactors on the 26th day of the acclimation phase were added to 120-mL serum bottles (reactors). In the study, non-pretreated waste black cumin was tested for different organic loading rates and pH values. The feed solution prepared by adding it to the raw tap water and the pH adjustment of this solution are the same as in the completely stirred-tank reactor. The installation was made by filling three-fourth of the reactor volume. The reactors were covered with aluminum foil and operated in a dark room. Sludge samples taken from continuous batch fed reactors were added to batch reactors, then, their lids were closed, and then, the reactors were passed through $N_2$ gas for 5 min to deoxidize the reactor environment. Finally, the installed reactors were placed in the shaker incubator running at 170 rpm and operated at $35 \pm 2$ °C.

## 2.2 Analytical methods

Gas analyses were conducted in a gas chromatography (Shimadzu-brand Nexis GC-2030) of HUBTAM in Harran University (Turkey) by taking samples using glass syringes with gas-tight cock. Thermal conductivity detector (TCD) and capillary column RT®-Msieve 5A (0.53 mmID, 50 µm df) were used in the GC. Calibration was carried out with high-purity methane, carbon dioxide and hydrogen gases. He was preferred as the carrier gas. Column, detector and injection temperature values were 35 °C, 230 °C and 200 °C, respectively. Liquid samples were prepared for the tests using cellulose acetate syringe (0.45 µm pore size) filters prior to measurement. The reactor effluent sample was taken for measurement of organic acid at certain time intervals using the HPLC device in the Chromatography and Fermentation Laboratory of the Middle East Technical University (Turkey). Chemical oxygen demand (COD) was measured using the closed reflux method according to Standard Methods (APHA, 2005).

In the COD test, firstly, potassium dichromate ($K_2Cr_2O_7$) was dried in an oven set at 150 $^0$C for two hours to prepare a standard $K_2Cr_2O_7$ solution. Then, the sulfuric acid solution was prepared by adding silver sulfate. The solution was kept for 1–2 days to allow the silver sulfate to completely dissolve in the acid. These two solutions were added to the samples, then the samples were boiled at 148 °C for 2 h in the COD set. At the end of this process, the samples were taken from the COD set, and the test was finished by titration after cooling. The suspended solid test was performed according to the Standard Methods. In order to determine the dominant species in each different pH operation in CSTR and FBR, PCR-DGGE analysis was performed in the Innovative Technologies Application and Research Center of Suleyman Demirel University (Turkey). DNA insulation of the samples was performed using Qiagen QIAamp DNA Stool Mini Kit (cat. no. 51504) according to the kit protocol. After the obtained DNAs were evaluated in terms of concentration A260/230 and A260/280 rates in a Microvolume Spectrophotometers, mySPEC (VWR) device, the suitability of all samples for later analyses was determined. DNAs were amplified in PCR using primers capable of amplifying the bacterial V3 gene region. Bio-Rad T100 PCR system was used for amplification. The PCR products obtained with primers in amplification were run in a 2% agarose gel electrophoresis system to ensure that the samples were amplified and that the appropriate amount of amplification was achieved for

DGGE. Then, the samples were loaded into DGGE Gel, and the gel image was analyzed. After the PCR products were sequenced in a sequence device, the species of the samples (microorganisms) were determined using NCBI Blast program.

## 3 Results and discussion

In this section, the effect of pH and hydraulic retention time on gas production is examined and discussed using continuous stirred-tank reactors (CSTR), fluidized bed reactors (FBR) and batch reactors.

### 3.1 The effect of pH and hydraulic retention time on gas production in completely stirred-tank reactor (CSTR)

The reactor was operated for approximately 100 days with the acclimation phase and different operating (1–4 period) conditions. There are very limited number of studies that are partially similar to the completely stirred-tank reactor in terms of waste organic matter use and reactor operation. In their study, Koutroulı et al. (2006) investigated hydrogen production from olive pomace by means of culture that produced hydrogen in mesophilic conditions at 35 °C in the completely stirred-tank reactor. The reactor was periodically stirred for 15 min, twice per hour, and the effect of periodic stirring on the system performance was tested in batch tests. The reactor was operated anaerobically for an average of 30, 14.5 and 7.5 h of hydraulic retention time, and it was fed periodically with olive pomace for 1 min every 6 h, 2 min every 6 h and 2 min every 3 h, respectively. Continuous feeding was not possible because the high solid content of the raw olive pomace diluted with water (1:4) prevented the efficient operation of peristaltic pumps (low feeding flows blocking the pump hose). Hydrogen production efficiency was found to be 4.5 and 2.8 mmol $H_2$ at 30- and 7.5-h HRT, respectively. With the decrease of HRT, hydrogen production decreased. Thus, the effect of continuous stirring in batch reactors on hydrogen production was compared with the effect of periodic stirring in continuous stirred-tank reactors, and it was reported by Koutroulı et al. (2006) that continuity in stirring had no impact on the hydrogen production.

In completely stirred-tank reactor (CSTR), the acclimation phase of the bacteria lasted 35 days. Gas production was not observed in the first 18 days, and un-balanced gas production was observed after then. In this acclimation phase, the reactor was operated with an organic loading ratio of 4.44 g.black cumin/L (186 mg COD/L), hydraulic retention time of 24 h and at pH 5.0. On day 35, it was determined that there was continuous hydrogen fermentation in gas production under steady state conditions. Furthermore, batch reactors were installed in order to predict possible effects before changing the operating conditions in the completely stirred-tank reactor. In batch reactors, non-pretreated waste was tested at different pH values (4.0, 5.0 and 6.0) and organic loading rates (2.22, 4.44 and 6.66 g.black cumin/L). The study found that the optimum hydrogen production in all reactors operated at different pH values was in reactors with an organic loading rate of 6.66 g.black cumin/L. Accordingly, it was decided to operate the completely stirred-tank reactor at an organic loading rate of 6.66 g.black cumin/L in all periods. Operating conditions in the first period were changed to organic loading ratio of 6.66 g.black cumin/L (average 197 mg COD/L), hydraulic retention time of 36 h and pH 4.0. With this change, it was aimed to accelerate the gas production in the process. However, it was determined that this change stopped gas

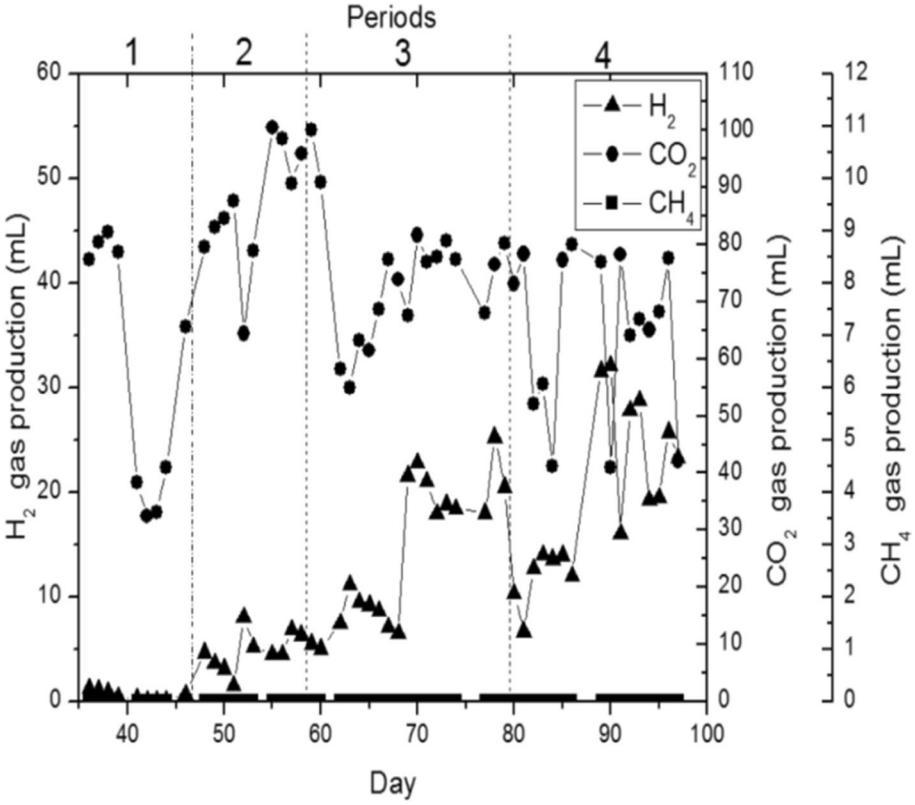


**Fig. 4** Hydrogen gas production in the completely stirred-tank reactor in different operating periods

production (Fig. 4). The study by Kumar et al. (2016) reported that propionic acid production and hydraulic retention time (HRT) limited the hydrogen production. The study investigated hydrogen production in the hydraulic retention time range of 48–8 h using jatropha waste pretreated with acid as the substrate. The study was initiated with a hydraulic retention time of 48 h, then it was gradually reduced to 12 h of HRT, and accordingly, a gradual increase was observed in hydrogen production performance. However, when the operation was resumed with 8 h of HRT after 12 h of HRT, hydrogen production performance was greatly reduced and therefore, the reactor operation was returned to 12 h of HRT so that the reactor could reach a stable performance. Optimum hydrogen production performance was found to be 3.65 L $H_2$/L.per day under the operating conditions of 24.6 g/L nutrient concentration and 12-h HRT. In the present study, it was evaluated that the unstable hydrogen production in the completely stirred-tank reactor (CSTR) in the first period was associated with 36 h of hydraulic retention time and it should have been operated at 24-h HRT again (Fig. 4). In hydrogen production, organic acids (acetic acid, butyric acid) are observed as the main fraction of total soluble metabolic products. Biohydrogen studies suggest that propionic acid decreases hydrogen production, while acetic acid and butyric acid thermodynamically support and increase hydrogen production. Organic acid analyses performed at certain time intervals in the completely stirred-tank reactor are shown in Table 3. It was reported by researchers that a short hydraulic retention time could wash

**Table 3** Organic acid analysis in completely stirred-tank reactor

| pH–Day | Acetic acid (mg/mL) | Propionic acid (mg/mL) | Butyric acid (mg/mL) |
|---|---|---|---|
| 4.0–38 | 1.048 ± 0.032 | 0.008 ± 0.001 | 0.153 ± 0.062 |
| 4.0–52 | 0.497 ± 0.016 | 0.006 ± 0 | 0.166 ± 0.001 |
| 5.0–67 | 2.649 ± 0.001 | 0.070 ± 0.001 | 1.152 ± 0.001 |
| 6.0–82 | 0.916 ± 0.011 | 0.512 ± 0.711 | 0.485 ± 0.001 |
| 6.0–85 | 0.834 ± 0.018 | 0.056 ± 0.001 | 0.387 ± 0.003 |
| 6.0–97 | 1.196 ± 0.003 | 0.074 ± 0.011 | 0.637 ± 0.002 |

out hydrogen-producing microorganisms. For this reason, it was decided to work with the 24-h hydraulic retention time in the following periods, considering the continuous batch feeding of the CSTR as mentioned in the material and method section, and not stirring while feeding (approximately 3 h), and then, continuing the stirring upon completion of the feeding. In order to determine an average concentration of COD inlet concentration in the CSTR, the average inlet concentration was determined by taking samples from the hose connection from the CSTR to the additional reactor at certain time intervals. The sample was taken from the mentioned Sect. 10 min before the end of the feeding time as the COD inlet samples. With this sampling timing, it was aimed that the feeding (tap water + trace elements) was about to end, so the average inlet concentration of the reactor medium was determined. COD outlet concentration was determined by taking a sample from the outlet tank and measuring it. The CSTR was operated at an average inlet organic loading rate of 197 mg COD/L in the 1st period, and the outlet concentration was found to be 101 mg COD/L (36–40 days) and 104.5 mg COD/L (41–46 days).

Operating conditions for the second period were kept the same as the conditions for the first period (6.66 g.black cumin/L (on average 198 mg COD/L) and pH 4.0), changing the hydraulic retention period to 24 h only. Biohydrogen production studies that used mixed culture reported that the reduction of HRT had a decreasing effect on propionic acid production (Hussy et al., 2003; Cha & Noike, 1997). In this period when hydrogen production started with the HRT change, the maximum hydrogen production was measured as 8 mL $H_2$/day (on day 52). In the second period, the average outlet COD concentration was found to be 107.6 mg COD/L.

The third period operating conditions were changed to organic loading ratio of 6.66 g. black cumin/L (on average 207.5 mg COD/L), hydraulic retention time to 24 h and pH to 5.0. The fact that only pH was changed in the operating conditions for the third period compared to the second period (Fig. 5) caused a significant increase in gas production performance as given in Fig. 4. At the beginning of this period (day 59), gas production with the effect of pH change was found to be 5.5 mL $H_2$/day. Depending on the acclimation of bacteria to the pH of the environment, an average gas production of 10 mL $H_2$/day in the following days and an average hydrogen gas production of 20.4 mL $H_2$/day between days 69 and 79 were determined. In the third period, the maximum hydrogen production was found to be 25.2 mL $H_2$/day on day 78. The average COD outlet concentration in this period was found to be 105.8 mg COD/L. Operating conditions for the fourth period were changed to organic loading ratio of 6.66 g.black cumin/L (on average 198.3 mg COD/L), hydraulic retention time to 24 h, and pH to 6.0. Hydrogen production with pH change at the beginning of this period was found to be 12.8 mL $H_2$/day, and the amount of gas produced as of day 89 was found to be 31.6 mL $H_2$/day (Fig. 4). After that day (between days

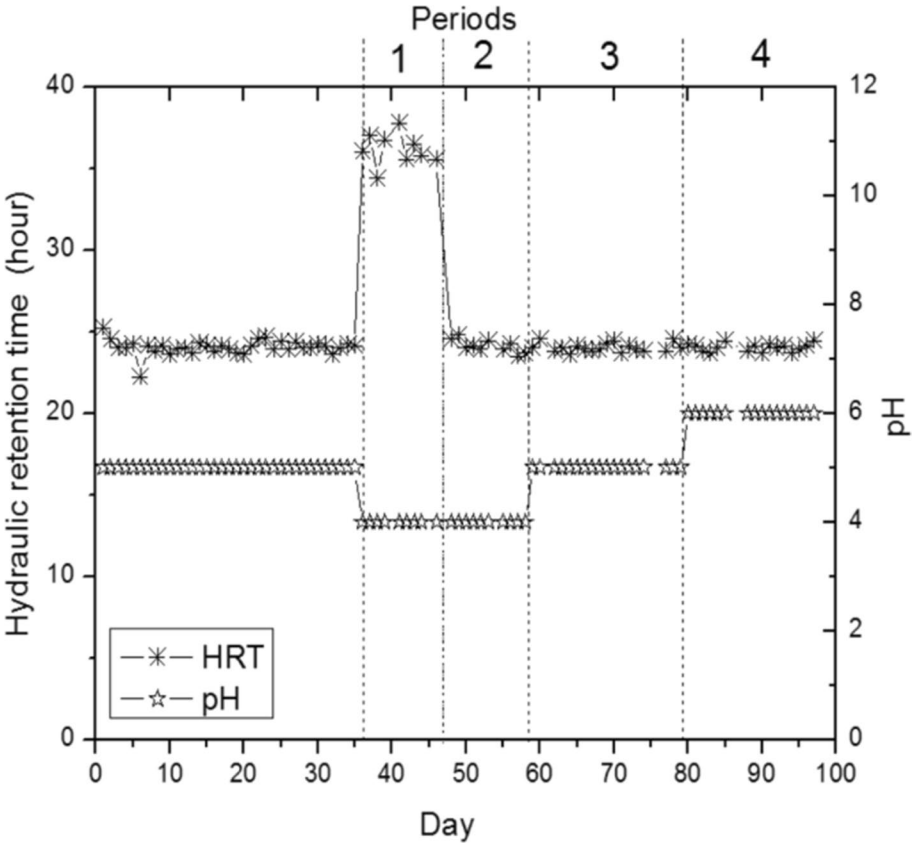


**Fig. 5** HRT and pH operating conditions in completely stirred-tank reactor

90 and 97), an average of 25 mL $H_2$/days hydrogen was produced. In the fourth period, on day 90, the maximum hydrogen production was found to be 32.1 mL $H_2$/day. The average COD outlet concentration in this period was found to be 109.2 mg COD/L. In the literature, it is reported that as the hydrogen production performance increases, the need for further treatment at the bioreactor outlet arises (Kothari et al., 2012; Khanna & Das, 2013; Bičáková & Straka, 2012). The study carried out by Kyazze et al. (2008) is similar to this research in terms of heat treatment of sludge and direct use of organic matter. In the study conducted by Kyazze et al. (2008), anaerobic sludge heat-treated at 110 °C for 10 min was used with various types of biomass to determine the hydrogen yield in a continuous stirred-tank reactor (CSTR). The hydrogen yield of $62.4 \pm 6.1$ mL $H_2$/g dry matter was obtained when 20 g/L dry matter of fodder maize slurry was studied at pH 5.3, the hydrogen yield of $75.6 \pm 8.8$ mL $H_2$/g dry matter was determined when 20 g/L dry matter of wilted perennial ryegrass was studied at pH 5.3, and the hydrogen yield was found to be $21.8 \pm 8$ mL $H_2$/g dry matter when 10 g/L dry matter of fresh perennial ryegrass was studied at pH 5.3. Another study is similar to the present research in terms of reactor type and grinding of the organic matter. In the aforementioned study, which is similar to this research and carried out by Li and Liu (2012), cornstalk was dried at 60 °C and brought to 1 mm in size using a herb mill. A continuous stirred-tank reactor (CSTR) with a working volume of 8

L was used, and this reactor was placed on a magnetic stirrer to ensure sufficient mixing. The reactor was flushed using nitrogen gas for 30 min before inoculation with *Clostridium thermocellum* cultures. Then, 24 h after this application, 100 mL of *C. thermosaccharolyticum* culture was inoculated into the *C. thermocellum* monoculture to initiate the co-culture process. The reactor was operated at 55 °C by adding 10 g/L of the raw cornstalk. The hydrogen yield was found to be 74.9 ml/g cornstalk. These findings reveal that the type of organic matter used, pH, specific bacteria, or mixed culture (heat treatment applied to sludge) affect hydrogen production.

### 3.2 The effect of pH and hydraulic retention time on gas production in fluidized bed reactor (FBR)

The reactor was operated for approximately 100 days with the acclimation phase and different operating (1–4 period) conditions. In the study, the acclimation phase of the bacteria to the environment lasted 35 days. Gas production was not observed in the first 20 days, and un-balanced gas production was observed after then. In this acclimation phase, the reactor was operated with an organic loading ratio of 4.44 g.black cumin/L (192.6 mg COD/L), hydraulic retention time of 24 h and pH 5.0. On day 35, it was determined that there was continuous hydrogen fermentation in gas production under steady state conditions. Besides, batch reactor evaluations were also carried out for the fluidized bed reactor in order to predict possible effects in continuous batch fed reactors. In batch reactors, non-pretreated waste was tested at different pH (4.0, 5.0 and 6.0) and organic loading rates (2.22, 4.44 and 6.66 g.black cumin/L), and the optimum hydrogen production was found in reactors with the organic loading ratio of 6.66 g.black cumin/L among all reactors operated at different pH values. Accordingly, it was decided to operate FBR at an organic loading rate of 6.66 g.black cumin/L in all periods. As in CSTR, the following operating conditions were applied in the first period: Organic loading ratio of 6.66 g.black cumin/L (average 132.8 mg COD/L), hydraulic retention time of 36 h and pH 4.0. With this change, it was aimed to accelerate the gas production in the process. However, it was found that this change stopped gas production (Fig. 6). Furthermore, during this period, due to a problem in the return pump on day 46, return could not be performed and methane production of 0.62 mL.$CH_4$/day was determined. Kumar et al. (2016) reported that propionic acid production and HRT limited hydrogen production. In the study by Hussy et al. (2003), which was conducted using wheat starch as substrate, a significant decrease in propionic acid production was observed with the reduction of HRT to 12 h. The study by Cha & Noike (1997), which was conducted using starch as substrate with continuous fermentation, found that propionic acid production was present with HRT of 24–48 h, but not with HRT of 12 h. Other studies report that propionic acid concentration shows no significant difference in the HRT range of 4–24 h (Yu et al., 2003; Chen & Lin, 2003), and it decreases with increasing HRT (Dinopoulou et al., 1988). In all of the aforementioned studies, the reasons for the differences in propionic acid production depending on HRT can be listed as nutrients, primarily the type of organic material used, and sludge (inoculum) and heat pretreatment (temperature and time) applied to the sludge. Given that microorganisms may be washed out as the hydraulic retention time decreases, this study considered that the unstable hydrogen production in FBR in the first period was associated with 36-h HRT and it was appropriate to operate again at 24-h HRT (Fig. 6). Table 4 shows the organic acid analyses performed at certain time intervals in the fluidized bed reactor. As mentioned in the material and method section of the FBR, it was operated in the HRT of 24 h in all periods,

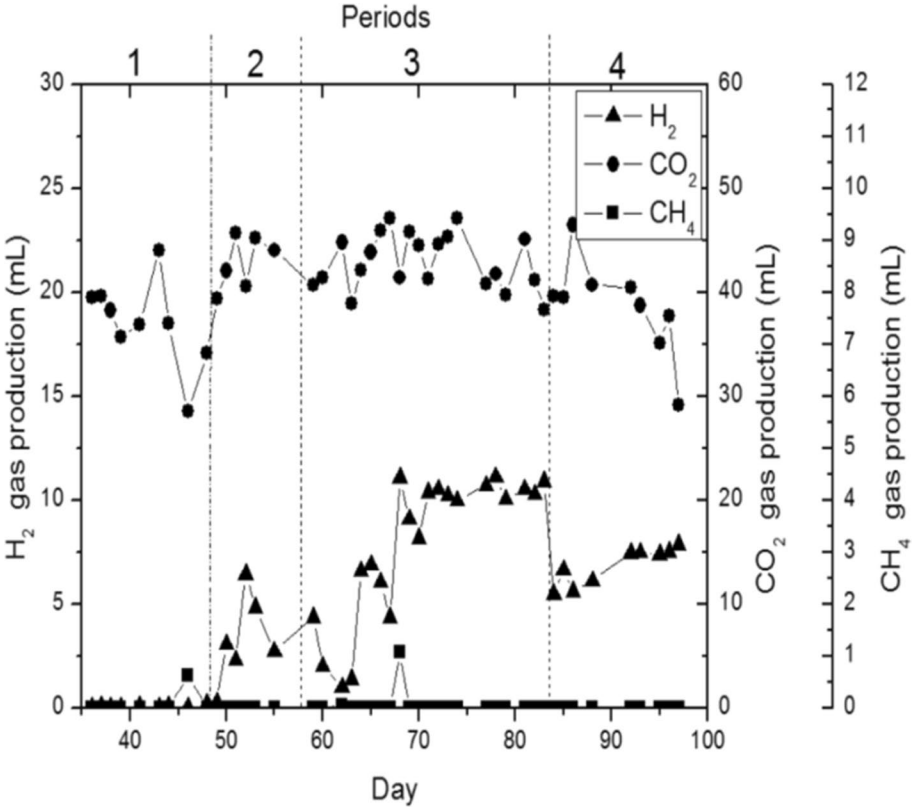


**Fig. 6** Hydrogen gas generation in fluidized bed reactor in different operating periods

**Table 4** Organic acid analysis in fluidized bed reactor

| pH–Day | Acetic acid (mg/mL) | Propionic acid (mg/mL) | Butyric acid (mg/mL) |
|---|---|---|---|
| 4.0–50 | 1.393 ± 0.008 | 0.066 ± 0.003 | 0.317 ± 0.003 |
| 4.0–52 | 0.172 ± 0 | 0.019 ± 0 | 0.314 ± 0.001 |
| 5.0–67 | 1.896 ± 0.010 | 0.043 ± 0.001 | 1.002 ± 0.001 |
| 5.0–83 | 2.650 ± 0.011 | 0.071 ± 0.004 | 1.146 ± 0.005 |
| 6.0–85 | 0.942 ± 0.004 | 0.034 ± 0.001 | 0.217 ± 0.004 |
| 6.0–97 | 1.379 ± 0.009 | 0.047 ± 0.002 | 0.634 ± 0.004 |

taking into account the continuous batch feeding and not performing the return while feeding (approximately 1 h), and resuming the return upon completion of the feeding. In the fluidized bed reactor (FBR), the average COD inlet concentration was determined by sampling from the return section. The sample was taken from the mentioned Sect. 5 min before the end of the feeding time as the COD inlet samples. With this sampling timing, it was aimed that the feeding (tap water + trace elements) was about to finish, so that an average value could be obtained in the inlet concentration in the reactor environment. COD outlet

concentration was determined by taking sample from the outlet tank and testing the sample. Fluidized bed reactor (FBR) was operated at an average inlet organic loading rate of 132.8 mg COD/L in the first period, and the outlet concentration was found to be 93 mg COD/L (36–43 days) and 77.4 mg COD/L (44–48 days).

Operating conditions for the second period were kept the same as the conditions for the first period (6.66 g.black cumin/L (on average 143.2 mg COD/L) and pH 4.0), changing the hydraulic retention period to 24 h only. Biohydrogen production studies that used mixed culture report that the reduction of HRT has a decreasing effect on propionic acid production (Hussy et al., 2003; Cha & Noike, 1997). In this period when hydrogen production started with the HRT change, the maximum hydrogen production was found to be 6.4 mL $H_2$/day (on day 52) (Fig. 6). In the second period, the average outlet COD concentration was found to be 74.7 mg COD/L. The operating conditions for the third period were changed to the organic loading ratio of 6.66 g.black cumin/L (on average 136.3 mg COD/L), and hydraulic retention time to 24 h and pH to 5.0 as given in Fig. 7. The fact that only pH was changed in the operating conditions for the third period compared to the second period caused a significant increase in gas production performance as given in Fig. 6. At the beginning of this period (day 59), gas production by pH change was found to be 4.4 mL $H_2$/day. Depending on the acclimation of bacteria to the pH of the environment,

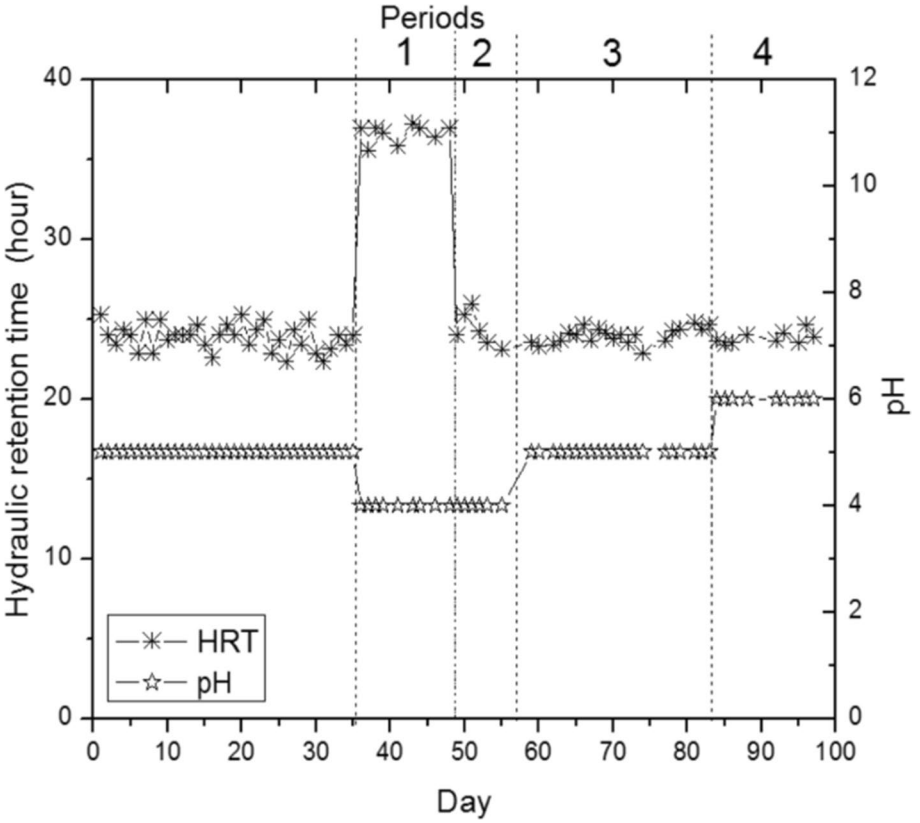


**Fig. 7** HRT and pH operating conditions in fluidized bed reactor

an average gas production of 4.1 mL $H_2$/day in the following days and an average hydrogen gas production of 10.2 mL $H_2$/day between days 68 and 83 were determined. Furthermore, since there was a pump problem (no return) on day 68 during this period, methane production of 1.0 mL.$CH_4$/day was determined. In the third period, maximum hydrogen production was found to be 11.1 mL $H_2$/day on day 78, and the average outlet COD concentration was 79 mg COD/L. The operating conditions for the fourth period were changed to the organic loading ratio of 6.66 g.black cumin/L (on average 129.4 mg COD/L), and hydraulic retention time to 24 h and pH to 6.0 as given in Fig. 7. As shown in Fig. 6, hydrogen production by pH change at the beginning of this period was found to be about 5.5 mL of $H_2$/day, and gas production of approximately 7.6 mL $H_2$/day was determined between days 92 and 97. In the fourth period, on day 97, the maximum hydrogen production was found to be 7.9 mL $H_2$/day. The average outlet COD concentration in this period was found to be 69.7 mg COD/L.

### 3.3 Batch reactors

Mostly, batch reactors were used in biohydrogen production studies due to ease of use. The research conducted by Alemahdi et al. (2015), which is similar to the present study in terms of pretreatment of mixed culture and using untreated organic matter, examined the raw rice straw. In this study, co-digestion of raw rice straw and wastewater treatment plant sludge was examined under mesophilic conditions in batch serum bottles with a total volume of 150 mL with a maximum working volume of 120 mL. The inoculum was heat-treated for 30, 45 and 60 min at temperatures of 80 °C, 90 °C and 100 °C, respectively, before dark fermentation to inhibit methanogen activity and achieve maximum biological hydrogen yield. Harvested rice straw was divided into pieces of 15 g, and they were mixed using a domestic mixer for 10 min. The sizes of fine rice straw (250–500 mm), medium rice straw (500 mm-2 mm) and large rice straw (2–20 mm) were obtained using a sieve. Thus, it was determined whether size reduction has an effect on biohydrogen production in terms of determining the optimum size. The optimum hydrogen yield was found to be $14.54 \pm 0.29$ Nml $H_2$/g VS by pretreatment applied to the sludge at 100 °C for 60 min. It was also reported that the large-size rice straw (2 mm-20 mm) supported high biohydrogen yield. In the present study, heat-treated anaerobic sludge was used with untreated black cumin in various organic loading rates (2.22 g black cumin/L, 4.44 g black cumin/L, 6.66 g black cumin/L) at pH 4.0, pH 5.0 and pH 6.0 to biological hydrogen production. Non-pretreated waste black cumin was used at organic loading rates of 2.22 g.black cumin/L, 4.44 g.black cumin/L and 6.66 g.black cumin/L, and the hydrogen production potential of waste black cumin was researched operating at pH 5.0 (Table 5). In the reactors with 2.22 g.black cumin /L and 4.44 g.black cumin /L, the maximum hydrogen production was found to be $22.10^{-4}$ mL and $133.10^{-4}$ mL at hour 18, respectively. After the maximum hydrogen production was determined, hydrogen production decreased due to the decrease in the amount of organic matter and the change in pH. In the reactor with 6.66 g.black cumin /L, gas production occurred about $500.10^{-4}$ mL between the hours 8 and 25. With a sharp increase after the 25th hour, maximum hydrogen production of $2182.10^{-4}$ mL was found at the 29th hour. After the 29th hour, hydrogen production decreased ($129.10^{-4}$ mL) until the 72nd hour. As of the 72nd hour, hydrogen production slightly increased and it was found to be $693.10^{-4}$ mL at the 82nd hour. The reason for the re-increasing after that decrease may be the fact that the pH change affected the activity of the dominant species. Since pH significantly affects the activity of dominant species in a mixed culture, it is

**Table 5** Biohydrogen production at pH 5.0 with the use of non-pretreated waste black cumin at different organic loading rates

| Organic loading rate | Measurement time (hour) | $H_2$ ($10^{-4}$ mL) | $CO_2$ ($10^{-2}$ mL) | $CH_4$ ($10^{-4}$ mL) |
|---|---|---|---|---|
| 2.22 g black cumin/L | 2 | 0 | 65 | 0 |
| | 15 | 18 | 65 | 0 |
| | 18 | 22 | 65 | 0 |
| | 40 | 15 | 65 | 0 |
| | 64 | 17 | 64 | 8 |
| 4.44 g black cumin/L | 2 | 0 | 65 | 0 |
| | 18 | 133 | 65 | 0 |
| | 40 | 52 | 65 | 0 |
| | 64 | 4 | 65 | 0 |
| | 89 | 15 | 65 | 20 |
| | 111 | 11 | 64 | 173 |
| 6.66 g black cumin/L | 2 | 0 | 65 | 0 |
| | 8 | 483 | 65 | 0 |
| | 25 | 505 | 65 | 0 |
| | 29 | 2182 | 64 | 0 |
| | 45 | 1257 | 65 | 0 |
| | 48 | 1161 | 65 | 0 |
| | 53 | 1069 | 65 | 0 |
| | 72 | 129 | 54 | 0 |
| | 82 | 693 | 65 | 0 |
| | 97 | 560 | 65 | 3 |

considered as an important factor affecting the result of $H_2$ fermentation (Sivagurunathan et al., 2016).

Non-pretreated waste black cumin was used at organic loading rates of 2.22 g.black cumin/L, 4.44 g.black cumin/L and 6.66 g.black cumin/L, and the hydrogen production potential of waste black cumin was researched operating at pH 4.0 (Table 6). In the reactor with 2.22 g.black cumin/L, the maximum hydrogen production was found to be $70.10^{-4}$ mL at the 19th hour; in the reactor with 4.44 g.black cumin /L, the maximum hydrogen production was found to be $121.10^{-4}$ mL at the 19th hour; and in the reactor with 6.66 g.black cumin /L, the maximum hydrogen production was found to be $383.10^{-4}$ mL at the 24th hour. After the maximum hydrogen production was determined, hydrogen production decreased due to the decrease in the amount of organic matter and the change in pH.

Non-pretreated waste black cumin was used at organic loading rates of 2.22 g.black cumin/L, 4.44 g.black cumin/L, 6.66 g.black cumin/L, and the hydrogen production potential of waste black cumin was researched at pH 6.0 (Table 7). In the reactor with 2.22 g. black cumin/L, the maximum hydrogen production was found to be $66.10^{-4}$ mL at the 20th hour; in the reactor with 4.44 g.black cumin/L, the maximum hydrogen production was found to be $243.10^{-4}$ mL at the 20th hour; and in the reactor with 6.66 g.black cumin/L, the maximum hydrogen production was found to be $498.10^{-4}$ mL at the 17th hour. After the maximum hydrogen production was determined, hydrogen production decreased due to the decrease in the amount of organic matter and the change in pH.

**Table 6** Biohydrogen production at pH 4.0 with the use of non-pretreated waste black cumin at different organic loading rates

| Organic loading rate | Measurement time (hour) | $H_2$ ($10^{-4}$ mL) | $CO_2$ ($10^{-2}$ mL) | $CH_4$ ($10^{-4}$ mL) |
|---|---|---|---|---|
| 2.22 g black cumin/L | 2 | 0 | 65 | 0 |
| | 19 | 70 | 65 | 0 |
| | 42 | 40 | 65 | 0 |
| | 64 | 18 | 65 | 0 |
| | 89 | 11 | 64 | 80 |
| 4.44 g black cumin/L | 2 | 0 | 63 | 0 |
| | 15 | 114 | 65 | 0 |
| | 19 | 121 | 65 | 0 |
| | 43 | 85 | 65 | 0 |
| | 65 | 11 | 65 | 32 |
| 6.66 g black cumin/L | 2 | 0 | 65 | 0 |
| | 8 | 33 | 65 | 0 |
| | 24 | 383 | 65 | 0 |
| | 29 | 343 | 65 | 0 |
| | 46 | 258 | 65 | 0 |
| | 54 | 217 | 65 | 0 |
| | 72 | 133 | 65 | 0 |
| | 82 | 107 | 65 | 0 |
| | 108 | 88 | 65 | 3 |

In completely stirred-tank reactor, the dominant species determined at pH 4.0 and a DGGE band density of 0.03 include *Thiofractor thiocaminus (NR_113568.1)*, *Sulfurospirillum alkalitolerans (NR_108632.1)*, *Sulfurospirillum cavolei (NR_041392.1)*, *Sulfurospirillum carboxydovorans (NR_115313.1)* and *Hydrogenimonas thermophila (NR_024811.1)*. The dominant species determined at pH 5.0 and a DGGE band density of 0.04 include *Enterobacteria*, *Salmonella bongori (NR_116124.1)*, *CFB group bacteria*, *Brenneria goodwinii (NR_118273.1)* and *Erwinia amylovora (NR_114520.1)*. The dominant species determined at pH 6.0 and a DGGE band density of 0.007 include *Streptomyces kurssanovii (NR_115777.1)*, *Streptomyces lavendulocolor (NR_043825.1)*, *Streptomyces libani (NR_115779.1)*, *Colwellia rossensis (NR_025957.1)*, *Streptomyces phaeofaciens (NR_115789.1)* and *Colwellia psychrotropica (NR_026055.1)*. In fluidized bed reactor, dominant species were determined at pH 4.0 and a DGGE band density of 0.04, at pH 5.0 and a DGGE band density of 0.03 and at pH 6.0 and a DGGE band density of 0.04. It was determined that the dominant species determined in the fluidized bed reactor at the relevant DGGE band densities were the same as the completely stirred-tank reactor. In their study, Chen et al. (2012) used argon gas to create anaerobic conditions in batch serum bottles (150 mL) with a working volume of 60 mL. They examined the biohydrogen production potential of untreated rice straw with a size of < 0.297 mm with anaerobic sludge heat-treated at 95 °C for 40 min. In the research, which was studied at 55 °C in the pH range of 3.5–8.0, maximum hydrogen production was determined to be 733 ml using 90 g TS/L rice straw concentration at optimum pH 6.5. The results of the PCR-DGGE analysis of anaerobic sludge revealed that *Clostridium pasteurianum*,

**Table 7** Biohydrogen production at pH 6.0 with the use of non-pretreated waste black cumin at different organic loading rates

| Organic loading rate | Measurement time (hour) | $H_2$ ($10^{-4}$ mL) | $CO_2$ ($10^{-2}$ mL) | $CH_4$ ($10^{-4}$ mL) |
|---|---|---|---|---|
| 2.22 g black cumin/L | 2 | 0 | 63 | 0 |
| | 20 | 66 | 65 | 0 |
| | 43 | 18 | 65 | 0 |
| | 66 | 18 | 64 | 0 |
| | 90 | 15 | 65 | 7 |
| 4.44 g black cumin/L | 2 | 0 | 64 | 0 |
| | 20 | 243 | 65 | 0 |
| | 43 | 70 | 65 | 0 |
| | 66 | 73 | 65 | 0 |
| | 90 | 41 | 65 | 7 |
| 6.66 g black cumin/L | 2 | 0 | 65 | 0 |
| | 8 | 96 | 65 | 0 |
| | 17 | 498 | 65 | 0 |
| | 21 | 450 | 65 | 0 |
| | 37 | 343 | 65 | 0 |
| | 44 | 299 | 65 | 0 |
| | 73 | 192 | 65 | 0 |
| | 98 | 144 | 65 | 0 |
| | 104 | 125 | 65 | 0 |
| | 123 | 107 | 65 | 12 |

*Clostridium stercorarium* and *Thermoanaerobacterium saccharolyticum* bacteria supported hydrogen production. As stated in the present study and the study of Chen et al. (2012), the temperature, as well as the organic material source used in the reactor, affects the diversity of the species of bacterium that produce biohydrogen.

## 4 Conclusion

Nigella sativa waste, which is residue from black cumin oil industry, was studied in continuous batch fed and batch reactors for biological hydrogen production by dark fermentation. The use of non-sterilized waste without pretreatment offers a great advantage industrially as it brings no extra cost to the process. It was found that biological hydrogen production occurred under all pH conditions in reactors operated under different operating conditions. Experimental results showed that reduction of HRT increased the hydrogen production. In completely stirred-tank reactor and fluidized bed reactor, at pH 5.0, maximum hydrogen was found to be 25.3 and 11.1 mL/day, respectively. The dominant microbial population determined at pH 5.0 included *Enterobacteria*, *Salmonella bongori*, *CFB group bacteria*, *Brenneria goodwinii*, *Erwinia amylovora*, *Thiofractor thiocaminus*, *Sulfurospirillum sp.* and *Hydrogenimonas thermophila*. Each of these dominant species can be used as specific bacteria for biological hydrogen production. According to the results of the present study, it was determined that mechanically crushed black cumin waste, which is a residue of the

black cumin oil industry, can be successfully used in biohydrogen production without any pretreatment process. This result indicates that the oil industries are global promising candidates for sustainable biohydrogen production in terms of using their various organic wastes generated after production.

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