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ORIGINAL ARTICLE

Bio-Hydrogen Production Using Cassava Processing Wastewater Disclosed by 16s rRNA Sequences and SEM

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ABSTRACT

The highest H_2 yield of 3131 ml/L.d achieved at optimal mesophilic condition with an OLR of 23.34 kg COD/(m^3·d) and optimal pH of 5 -6 resulted from starch processing wastewater. H_2 yield of 2007 ml/L.d with a COD removal of 59% was obtained at OLR of 28.86 kg COD/ (m^3·d). The H_2production obtained at mesophilic temperature was higher than thermophilic temperature. Immobilization of cells by addition of Bio-film in the CSTR reactor. Further in addition the reactor environment was exposed to SEM and 16S rRNA sequencing. The rod shaped microorgnisms species of Bacillus cereus was mostly identified and also presence of Bacillus thuringiensis, which stated as efficient starch utilizing hydrogen producers was abandoned in the system.

Keywords: *Starch processing wastewater; CSTR; Bio-hydrogen; Bacillus cereus.*

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INTRODUCTION

The low cost energy sources is being researched, due to corresponding shortage and dependence on fossil fuels. Now a day's electricity generation in commercial and transport of renewable energy sources fuel have enlarged in developing countries creating considerable social, environmental and financial gains[1].Due to this kind of transformation energy, H_2 is reflected to be an ecofriendly fuel as well as hopeful vector, which releases zero carbon dioxide during its combustion and generate electricity by using microbial fuel cell. In contrast with hydrocarbon fuels, hydrogen has the highest energy value (122 kJg^{-1}).Hydrogen is produced during high temperature and its energy intensive process like non-catalytic fossil fuels includes oxidizing methane and hydrocarbon renovation[2]. As a result, bio-H_2 production, photo and DF, and direct and indirect photolysis received special attention. The promising technology in production of bio-H_2 is identified as dark fermentation. In this concern the feed stocks usage made higher rates of H_2 production (100 – 400 ml $H_2L^{-1}h^{-1}$) than any new technologies in biological methods and moreover, the simplicity of reactor is relatively parallel to developed anaerobic digestion technology[3].The higher potential for improving bio-hydrogen, through dark fermentation is carried by using chemical waste. The major key reasons for effective process of bio-hydrogen is the good utilization of raw material, cheaper and widely available[3].Cassava processing starch wastewater is rich in carbohydrate and it is promising substratum used for fermentation and sustainability in maintain methods. This application has been helped heavily to convert polluted wastewater into renewable energy supply[4].The wastewater from starch processing industry contains nitrate, phosphorus and rich in carbohydrates[5]. The results from[6], fermentation method of hydrogen production technologies

advancement, the substrates discovery for possible industrial scale application had become a priority mission. H_2 production using food processing waste using mixed anaerobic microbial sludge's are an enticing alternate to pure/co-cultural microbial sludge[7-8]. In the intention of reducing environmental stresses, including restriction of nutrients, Temperature increases and pH, and the acidogenic reactor inoculated with mixed cultures instead of pure cultures.The anaerobic microflora sludge comprised of microorganisms that consume and produce H_2[9]. The pre-treatment is extensively utilized to enrich the sludge with bacteria that produce hydrogen and destroy hydrogen consuming microorganisms.The best suitable general method of pre-treatment is sludge heat treatment[10],[9],[11].Anaerobic batch reactor having pH 5.5, for base treatment helps to reduce methanogens activity and it is optimally enhancing the hydrogen production rate[12].pH 5-6 is optimal for production of H_2 and the range of pH reduced and increased, which enhance the metabolic change with VFAs[13]. Therefore, in the optimal environment identification, this research was concentrated for anaerobic DF of starch-processing wastewater (initial influent starch processing wastewater concentration, Temperature and pH) intended in Bio-H_2production. Isolate and morphology of microbial population responsible for vital role in H_2 producer's population using SEM and 16S rRNA amplification analysis and sequence.

MATERIAL AND METHODS

Influent and seed sludge preparation

Wastewater from a cassava flour plant in Tamil Nadu that was used to manufacture starch from cassava was collected. The effluent was incubated at a temperature was about lower than 4°C to prevent microbial biodegradation. The anaerobic mixed sludge considered as reactor feed was procured from pilot scale treating cassava processing starch wastewater. The pre-heat treatment of sludge was through heating itat 95°C for 15 minutes[14] to reduce the hydrogen consuming bacteria activities. The reactor was injected with anaerobic sludge of 4L and biomass concentration of 4.5 g/l and remaining was inoculated with cassava processing starch wastewater.

Experimental Setup

As illustrated in Fig. 1, DF was carried out utilizing a CSTR equipped with four automated units: a feeding tank, the main body of the reactor, an auto-gas measurement sensor unit, and an automated temperature control system. It was made of stainless steel. Temperatures of 35°C and 55°C have been frequently maintained with agitation at 120 rpm. For flow rate control, the influent feeding rate in the variable speed pump and HRT was maintained by a speed variation pump. The feed tank holds 10 litres of feed, while the overall capacity of the reactor is 21.78 litres. 5L is for the gas collecting chamber at the top of the reactor, and 16.34 L is the working capacity for the bioconversion process. The bioreactor has 0.215 m of diameter and 0.6 m of height. The complete mechanism's bioconversion took place in four segments of the CSTR: anaerobic seed sludge, influent supply at the bottom, substrate consumption rate in the centre, bio-film in the middle of the reactor, and the gas collecting chamber at the top.

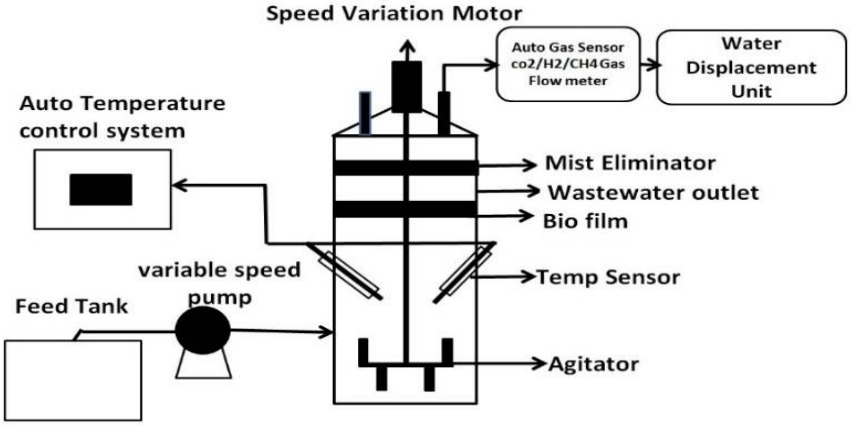

Fig .1 Schematic representation of CSTR

Analytical methods

The Auto Gas Measuring Sensor Unit, which consists of three sensors, was used to identify biogas generated by the CSTR reactor (H_2, CO_2, and CH_4). It aids in sensing the quantity of biogas produced by the reactor, with values presented in ppm on the LED display. When the biogas passes through the sensor unit, it is sent to the water displacement unit, which calculates the overall quantity of biogas generated. It was injected into the GC using a syringe for biogas composition confirmation, and the biogas composition was evaluated using GAS Chromatography (GC 7410) having Thermal Conductivity Detector (TCD) and a

column of stainless filled with nitrogen gas as the carrier gas for biogas analysis. The temperature of the injector column was maintained at 80 degrees Celsius. Both the influent and effluent were subjected to chemical analysis. COD, BOD, TS, VS, and SS were determined according to standard methods (APHA, 2005) [15].

Cell morphology, 16S rRNA amplification and sequence analysis

The sewage granules were taken out from the reactor, to examine the microbial morphology and 16S rRNA sequence analysis. The anaerobic sludge samples taken out from the effluent port during the period of highest H_2 content in the biogas production occurs. The following procedure were adopted for SEM as well as 16s rRNA sequence analysis. The sludge samples were first stabilized through soaking similar amount of 6 per centglutaraldehyde around 2 hours, centrifuged and drained continuously in a Phosphate Buffer solution for three time's minimum following fixation and held at 4°C overnight. Consecutively the specimens dehydrated through concentration increasing ethanol solutions: 10%, 15%, 30%, 50%, 75%, 90%, and ultimately twice subjected to 100% ethanol wash. Every specimen dried for 2 days at approximately 37°C. It was placed on the SEM sample holder and coated in a sputter coating package with gold. Such samples were further examined at varying magnifications bySEM and the related SEM images were recorded.

Isolation of microorganisms from acidogenic sample Approximately 1 ml of CSTR effluent sample was diluted 6 and 7 times and then plated on sterile nutrient agar plate. Following a 24-48-hour incubation period in a bacteriological incubator, the sample was examined for microbial growth. The bacteria colonies were enumerated, and the most prevalent culture was streaked onto a fresh sterile nutrient agar plate. Phylogenetic analysis gene sequencing was used to identify bacteria in the dominant culture.Using the QIAGEN DNA isolation kit (Qiagen), genomic DNA was isolated from overnight grown cultures of chosen bacterial isolates, suspended in 100l of elution buffer (10mM/L Tris HCl, pH 8.5), and analysed by measuring the OD at 260nm. For PCR amplification, a 50L reaction mixture including 100 ng template DNA, 20 mol 16S rRNA primers, 200 M dNTPs, 1.5 mM MgCl2, 1U Taq DNA polymerase (MBI Fermentas), and 10 L 10x Taq polymerase buffer was employed. The 16s rRNA primer sequences used are listed below.

27f: (5' – AGAGTTTGATCCTGGCTCAG-3')
1522r (5′-AAGGAGGTGATCCANCCRCA-3')

A thermal cycler used to execute amplification, which included an initial temperature at 95°C for 5 minutes, 35 denaturation cycles at 94°C for 45 seconds, 56°C for 45 seconds for annealing and 72°C for 1 minute for first extension, and finally at 72°C for 5 minutes for final extension (iCycler; BioRad Laboratories, CA). A 1 percent agarose gel 16S rRNA amplicons in a 1x TBE buffer at 100V was used to examine PCR results. Applied bio-systems sequencing the amplified product (ABI PRISM 3730 Genetic Analyzer).

RESULTS AND DISCUSSION

Temperature and pHin optimal conditions

Initial usage of OLR 2.08 and 2.29 kg COD / m^3d, bacteria should use organic source primarily utilized in growth of biomass, not for H_2 production which results in reduction in HPR [16]. The steady state HPR were 3039, 3102, 3131, 3201 and 2991 ml/d on 49, 50, 51, 52 and 53rd day for 35^{o}C, 1939 ml/d on 50th day, 1988 ml/d on 53rd day, 1990 ml/d on 53rd day, 1963 ml/d on 54th and 1971 ml/d on 55th dayobtained for 55^{o}C represented in figure.2.

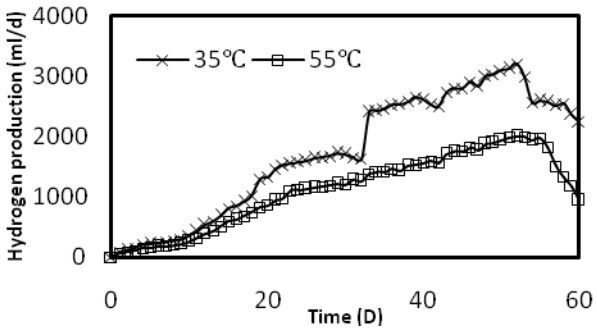

Fig .2 Bio-H_2 production at 35^{o}C and 55^{o}C

Hydrogen was produced from organic matter degradation using AD, so the rate of hydrogen production was in tandem with OLR. Therefore, the increase in OLR from 2.08 and 2.29 Kg COD/m^3.d to 30.87 and 28.86 Kg COD/m^3.d, which enhance the H_2production rate. H_2 production of maximum achieved at 35^{o}C

was 3131 ml/d; it is comparatively greater than 2007 ml/d obtained at 55°C. The variation in hydrogen production rates can be attributed to differences in the bacterial numbers and organic substrate influent rate [17]. The pH of the medium fluctuates during DF, which can have a significant impact on bio-H_2 production. As a result, a difference between the initial and functional pH must be carried out in a continuous process with pH control. For both temperature instances, the pH 5 to 6 reached in this investigation was the final pH 5 to 6. This value was quite similar to the best described for various wastewaters from industry, which include wastewater from rice winery [18], wastewater from food industry[19] and wastewater from dairy industry [20] as shown in table.1.

Table 1. Bio-H_2 production using different types of wastewater

Substrates	**COD (g/l)**	**T (°C)**	**pH**	**H_2 (ml-H_2/g COD)**	**Reference**
Tequila vinasses	27	35/55	5.5	73.4/62.4	[23]
RWW	34	55	5.5	234	[18]
Rice slurry	5.5	37	4.5	326	[24]
Dairy wastewater	15.3	37	5.5	303	[20]
Brewery wastewater	6	35.9	5.9	149.6	[25]
Food industry wastewater	40	35	5.5	165	[19]
Cassava starch	24	37	6.0	179	[21]
Cassava starch wastewater	2.08-30.87/2.29-28.86	35/55	5-6	107.3/73.5	This study

At 35°C and 55°C, bio-H_2 was produced utilizing wastewater (ghee whey). Bio-H_2 at 36°C was greater (206 mL-H_2 $gCOD^{-1}$) than at 55°C (206 mL-H_2 $gCOD^{-1}$) (178 mL-H_2 $gCOD^{-1}$) [21] and [22] suggested that for bio-H_2 synthesis using cassava processing starch effluent, a temperature of 37°C is preferable to 55°C. Finally, mesophilic (35°C) H_2 generation using Cassava processing starch effluent was shown to be more effective than thermophilic (55°C) in anaerobic CSTR.

Optimal substrate concentration in mesophilic and thermophilic H_2 production

The steady state COD removal efficiencies obtained at 35°C were 85% on 51st to 52nd day, 88% was obtained on 59th to 60th day respectively. Whereas, the maximum of 59% COD removal obtained on 52nd to 56th day and 61% was achieved on 57th to 60th for 55°C were presented in figure.3.

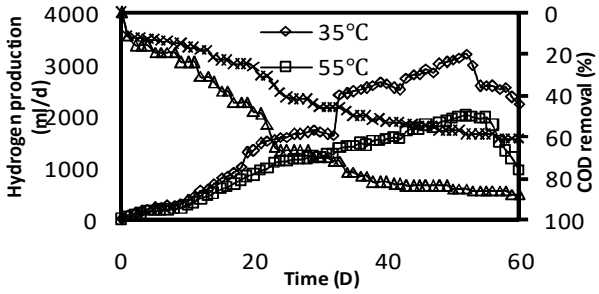

Fig .3 COD removal vs Bio-hydrogen production at 35°C and 55°C

The lowest efficiency in COD removal in both mesophilic and thermophilic temperature studies may be attributed to the minimal acclimatization period available[26]. The VSS concentration increased by utilizing the substratum with the maximum COD removal of 88% achieved on 60th day for 35°C. The efficiency in COD removal was higher at 35°C, than 55°C. Gradual growth in the OLR made a different surroundings for micro-organism adaptation, resulting in a variation earlier achieving stable efficiency in COD removal[27].

Metabolic pathways and biomass concentration

The figure shows the VFA concentrations in the acidogenic reactor. The majority of the volatile acids accumulated inside the reactor are 5, acetic, and propionic acids[28]. The maximum VFA concentration was found at HRT of 16 hours with the hydrogen production of 1726 ml/d and minimum VFA concentration at 24h HRTwith H_2 of 84 ml/d at 35°C. However, the VFA production of 1526 to 2805 mg/l with H_2 of 1203 ml/d occurs at VFA concentrations of 2805 mg/l at 55°C. VFA concentration defines rate of H_2 production accordance with reactor pH as illustrated in figure.4.

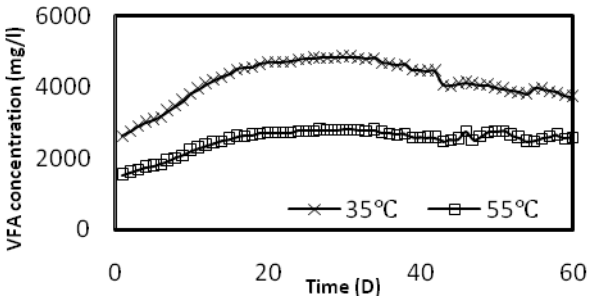

Fig .4 VFA concentration in a CSTR at 35°C and 55°C

To maintain the reactor in acidic condition and production rate of H_2, VFA plays an important role. H_2 production through acidogenic bacterial communities is continuously determined by VFA production. Hence the micro-organisms metabolism changes is due to the VFA changes. In addition the pH was identified as an essential factor inducing the H_2 producing bacteria, since it affects the metabolic pathways through hydrogenase activity[11]. Alkalinity in the reactor determines the reactor stability, hence the alkalinity of the reactor was continuously monitored. It was clearly state that, the alkalinity of the acidogenic reactor vary between 1322 to 9861 mg/l at 35°C and 918 to 6848 mg/l at 55°C was attained. The maximum hydrogen production of 2007 ml/d gained at an alkalinity concentration of 6022 mg/l at 35°C. The figure.5 shows the VFA/Alkalinity ratio for acidogenic reactor.

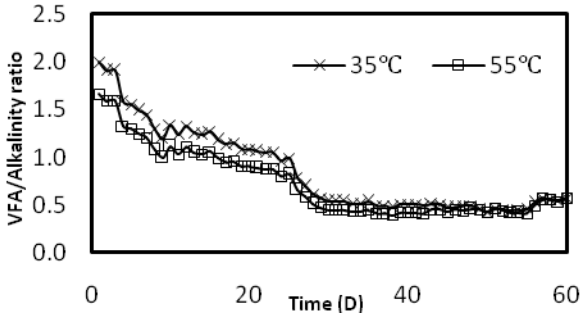

Fig. 5 VFA/Alkalinity ratio in CSTR at 35°C and 55°C

It our study the VFA/Alkalinity ranges between 2-0.4 and 1.7-0.4. Hence, the effluent VFA/Alkalinity ratio attained more than 2 or less than 2, which demonstrate the proper functioning of the anaerobic process. Simpson 1960, [29]Stated that the VFA/Alk ratio essentially low in range for stable anaerobic digester. It relates that the overall period during experimental, the reactor in stable condition due to the control of VFA was low in proportion to available alkalinity in the reactor. Figure.6 depicts that the biomass concentrations after the H_2 generation process at various substrate concentrations.

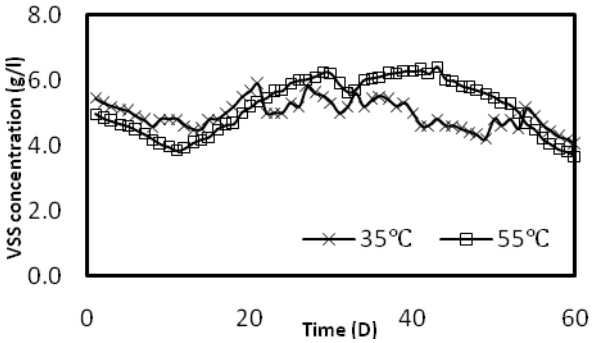

Fig .6 Biomass concentration at 35°C and 55°C

The findings showed that when the COD concentration increase, the total biomass level concentration also increased.Initial biomass of 5.45 g/l was continuously increasing and decreasing in the biomass concentration and finally reduced in the VSS concentration. While the biomass of 5.84 g/l, the rate of H_2 was maximum at 35°C and 5.24 g/l at 55°C, with constant pH maintained between 5 and 6 experimented by [30]. With a larger COD concentration, however, a reduced pH was achieved. It shows that hydrogen-producing bacteria exploited the removed cassava processing wastewater for both their development and the synthesis of organic acid. This outcome was very similar to that of [31].

Population in microbial analysis and morphology

A scanning electron microscopy was identified and observed the sample under different magnifications. Utmost the research concentratedin the distribution populatin in micrbial communitea in CSTR and the findings showed partial disparity of the distribution over the micrbial population under different experimental conditions[32]**.** The anaerobic sludge from the CSTR was analyzed for SEM, which is shown in the Figure.7.

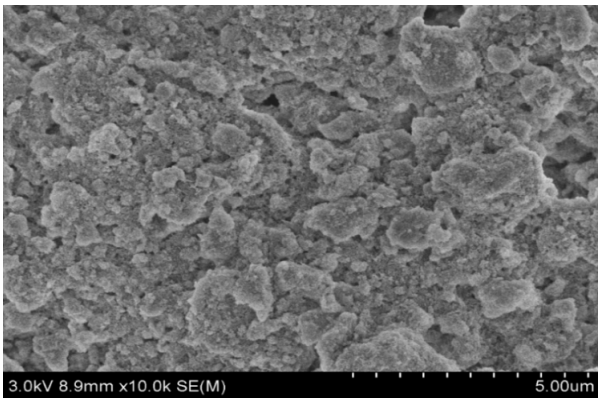

Figure .7 SEM Image of the Acidogenic effluent

Cell structure and Morphology

The rod shaped*Bacillus cereus* is a Gram-positive, motile, facultatively anaerobic, beta-hemolytic and spore-forming bacterium. B. cereus comes under the bacteria group of facultative anaerobes as well as genus like *Bacillus thuringiensis*, can yield endospores. The colonies on agar range from non-pigmented to grayish-white, as shown in Figure.4.

Figure .8 Isolated acidogenic bacteria on Nutrient agar plate

Analysis of Phylogenetic Trees

BLASTN software was used to connect the sequences of these 16S rRNA genes to sequences retrieved from Gene Bankand [33]then software CLUSTAL Wused for alignment[34]. Kimura's two-parameter adjustment was used to measure distances [35]A neighbor method was used to create phylogenetic trees [36]. Bootstrap analysis was conducted on the basis of 1000 replications. The MEGA4 kit[37]is used. Genomic DNA of given Acidogenic bacterial isolate Figure 9(a). The PCR amplification profiles of Acidogenic bacterial isolate Figure 9(b).

Figure .9(a) Genomic DNA of given Acidogenic bacterial isolate

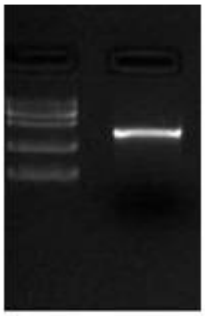

Figure .9(b) Acidogenic bacterial isolate PCR amplification profile
Conditions: Agarose electrophoresis gel, 1.5 percent (Lane a: 1kb DNA Ladder; b: Sample) 5000, 4000, 3000, 2000, 1000, 1 KB DNA Ladder (bp).

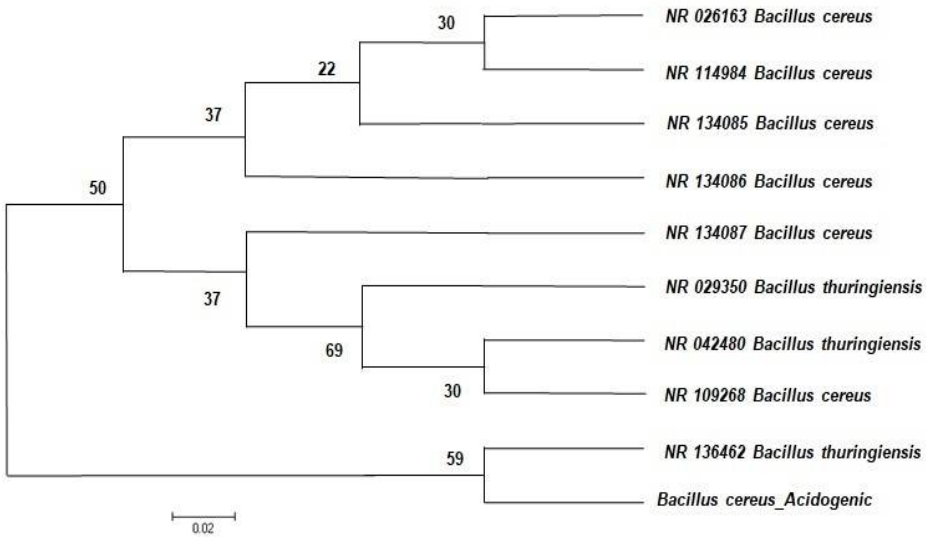

Figure .11 Phylogeny Tree Analysis of the Acidogenic Bacteria

Acidogenic bacteria is analyzed by using phylogeny tree analysis are illustrated in Figure.10. Evolutionary history was indicated by Neighbor Joining process[34]**.** The length of the branch = 0.08628581 existence in the optimum tree is presented. The duplicate percentage of the trees respected to the bootstrap clustered together is revealed in the following branches[38]**.** When the branch length in the same measurement the tree is scaled to a scale as the evolutionary range used the method of the Kimura 2 parameter[33]as well as substitutions per site in units corresponding to the base number. The encoding positions used were 1^{st}+2^{nd}+3^{rd}+Noncoding. The pairwise deletion option and only pairwise comparisons have eliminated all position containing alignment gap with data missing. 1352 was the final total number places in the dataset. MEGA4 was used to perform the phylogenetic analysis [39-40]**.** Based on the NCBI's BLAST analysis, RDB taxonomic analysis, and tree of phylogeny, it was determined that the provided sample belonged to the Bacillus cereus taxon.

CONCLUSION

The research concluded with an initial pH 5 to 6,mesophilicrange, and maximum organic loading rate in KgCOD/m^3.d as 23.34 was identified as the best acidogenic fermentation environments for producing bio-hydrogen from starch processing effluent. However, under certain circumstances, the ideal operational value should be considered since different characteristics, such as substrate source, microbial seed source, dominating species in cultures, and so on, may favour contrasting environmental conditions. Aside from harming bacteria and lowering hydrogen generation, a high influent OLR is harmful to the environment. In addition, cell immobilization using biofilm increase hydrogen yield. It was usually identified rod-shaped, size range of 1-5 mm, *Bacillus thuringiensis,* Firmicutes phylum *Bacillus cereus*are highest number in population of all species detected on treated anaerobic mixed sludge in the optimal concentration and fermentation condition. This species has also been found to be very efficient in synthesis of hydrogen from starch.

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