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Enhancement of Bio-Hydrogen Production from Sewage Sludge and Fallen Trees Leaves via Anaerobic Bioreactor

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ABSTRACT

Egypt faces significant challenges in managing sewage sludge (SS), which is generated in large quantities. This study investigates the feasibility of utilizing SS as a renewable resource for biohydrogen production through anaerobic digestion (AD). As a sustainable energy source with minimal use of hydrocarbons and a high energy yield, it is a promising alternative to fossil fuels. The utilization of a specialized pretreatment technique, in conjunction with the combination of fallen Trees leaves (FL) as a carbon-rich source and SS as a substrate with high nitrogen content, demonstrates significant potential for optimizing the microbial degradation process and maximizing biohydrogen production from SS combined with FL. Four treatment conditions for the SS were used in the present experiments: control phase (CP), thermal pretreatment phase (TP), co-fermentation phase with 0.25 g of volatile solids (VS) of FL/1.0 g SS (TPFL-1), and 0.5 g of VS of FL/1.0 g SS (TPFL-2). The results proved that the hydrogen production from TP exceeds that of CP by a factor of 232. When mixing FL with SS, it can lead to efficient biohydrogen production. According to our results, the optimum mixing ratio of SS to FL was 0.5 g VS of FL/1.0 g SS, and by this ratio, biohydrogen production was higher than the other ratio by 20%. The removal of COD and VS has increased by 27% and 21%, respectively, compared to other ratios. Concisely, the deployment of the prepared FL with SS led to improved biohydrogen production and enhanced sludge treatment efficiency.

KEYWORDS: Bio-Hydrogen; Anaerobic Digestion; Co- fermentation; Fallen trees leaves; Sewage Sludge.

تعزيز إنتاج الهيدروجين الحيوي من حمأة الصرف الصحي وأوراق الأشجار المتساقطة عبر المفاعل الحيوي اللاهوائي

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الملخص

تواجه مصر تحديات كبيرة في إدارة حمأة الصرف الصحي (SS) والتي تنتج بكميات كبيرة. من خلال ذلك فان هذه الدراسة تلقي الضوء علي جدوى استخدام حمأة الصرف الصحي كمورد متجدد لإنتاج الهيدروجين الحيوي من خلال عملية الهضم

اللاهوائي(AD) .يعتبر الهيدروجين مصدر للطاقة المستدامة ذات الانتاجية العالية مع الحد الأدنى من استخدام الهيدروكربونات وبذلك يعتبر الهيدروجين بديل واعد للوقود الأحفوري الغير متجدد. لدراسة انتاجية الهيدروجين من حمأة الصرف الصحي وتحسين عملية التحلل الميكروبي من الهاضم اللاهوائي، تم اجراء اربعة تجارب مختلفة وبيانها كالاتي:اولا تجربة التحكم CP وذلك باجراء عملية الهضم اللاهوائي للحمأة بدون اي معالجات مسبقه او اضافات. ثانيا تجربة المعالجة الحرارية للحمأة (TP) وذلك بتعريض الحمأة الي صدمه حرارية كمعالجة مسبقة لها.ثالثا تجربة التخمر المشترك الحراري (TPFL -1) وذلك بتعريض الحمأة الي صدمه حرارية كمعالجة مسبقة لها ثم خلطها مع ورق الشجركمصدر للنتروجين لزيادة نسبة النتروجين الي الكربون بنسبة 0.25 جرام (مواد الصلبة متطايرة) ورق شجر : 1.0 جرام حمأة (مواد الصلبة متطايرة). رابعا تجربة التخمر المشترك الحراري (TPFL -1) وذلك بتعريض الحمأة الي صدمه حرارية كمعالجة مسبقة لها ثم خلطها مع ورق الشجركمصدر للنتروجين لزيادة نسبة النتروجين الي الكربون بنسبة 0.50 جرام (مواد الصلبة متطايرة) ورق شجر : 1.0 جرام حمأة (مواد الصلبة متطايرة). ووفقا للنتائج للتجارب الاربعة تبين الاتي: إنتاج الهيدروجين من تجربة المعالجة الحرارية يتجاوز إنتاجه من تجربة التحكم بحاولي 232 مره. بالاضافة الي انه وعند خلط الحمأة المعالجة حراريا مع اوراق الاشجاركمصدر للنتروجين، ادي ذلك الي زيادة انتاجية الهيدروجين بصورة كبيره وملحوظه، ومن خلال النتائج كانت نسبة 0.50 جرام (مواد الصلبة متطايرة) ورق شجر : 1.0 جرام حمأة هي نسبة الخلط المثالية، حيث ان هذه النسبة كان إنتاج الهيدروجين الحيوي أكثر من النسب الأخرى بنسبة 20٪، كما تمت زيادة إزالة الاكسجين الكميائي المستهلك COD والمواد الصلبة المتطايره VS بنسبة 27٪ و 21٪ علي التلاتيب مقارنة بالنسب الأخرى. الخلاصة فان عملية خلط معالجة حمأة الصرف الصحي حراريا بالاضافة الي خلطها مع ورق الشجر كمصدر للنتروجين ادي إلى تحسين إنتاج الهيدروجين الحيوي وتعزيز كفاءة معالجتها.

الكلمات المفتاحية: الهيدروجين الحيوي، المخمر اللاهوائي، التخمير المشترك، ورق الاشجار المتساقط، حمأة الصرف الصحي.

1. INTRODUCTION

According to data from the Egyptian Central Agency for Public Mobilization and Statistics, Egypt's population exceeded 100 million in 2021, positioning it as one of the most populous countries in both Africa and the Middle East. This substantial population size results in high water consumption, consequently leading to a significant volume of wastewater being generated. To address this issue, wastewater treatment plants are utilized to treat the wastewater, resulting in the production of SS as a by-product [1,2]. The situation in Egypt regarding SS is a matter of concern. Recent reports, as cited by Hassan et al. In 2022 [3] and Hassan et al. In 2023 [4], indicate that Egypt produced nearly 2.1 million tons of sludge in the year 2018 from just three governors: Cairo, Giza, and Alexandria. Recognizing the challenges posed by the large volume of sludge generated, efforts are being made to develop effective treatment methods that can address environmental concerns and promote sustainable practices. By focusing on innovative approaches to SS treatment, researchers aim to ensure the long-term viability and environmental integrity of the sanitation sector in Egypt [5].

On the other hand, paramount challenges confronting humanity in the present century encompass environmental pollution and energy insecurity [6]. The escalating concern of global warming, attributed to the emission of greenhouse gases, particularly carbon dioxide (CO_2), and the depletion of fossil fuel reserves, propels the exploration of alternative, sustainable energy resources [6]. Biomass (especially sewage sludge) stands out as a preeminent choice for renewable and viable energy, capable of substituting a substantial proportion of conventional fossil fuels, alongside solar and wind energy. Simultaneously, the utilization of bioenergy holds the potential to augment the quality of life in developed nations, emerging as a formidable source for biofuels [8–10]. Simultaneous achievement of pollution control and bioenergy production can be realized through diverse conversion processes employed in the transformation of biomass into bioenergy.

Anaerobic digestion stands out as one of the most efficacious technologies for the conversion of biomass into biofuel [8].

Anaerobic Digestion (AD) is a widely used method for the digestion of SS, often in combination with various substrates [11–14]. AD is a biological process that takes place in the absence of oxygen. During this process, microorganisms decompose organic matter present in the sludge, transforming it into biogas and a digestate rich in nutrients, this process not only helps to reduce the volume of SS but also produces renewable energy in the form of biogas, which can be used for the generation of electricity or for purposes of heating [15]. Additionally, the resulting digestate can be further treated and used as a nutrient-rich fertilizer [13]. AD offers an efficient and sustainable approach to managing SS while harnessing its potential for energy recovery and resource recycling [12].

Hydrogen as one of the produced biogases considered a valuable energy source. Hydrogen, known for its superior energy density and environmentally friendly nature [16], stands out as a clean fuel option. Due to its clean combustion byproduct (H_2O) and high energy yield (141.9 J/kg), hydrogen holds great promise as an energy source for sustainable development [1]. However, it is important to note that the primary technologies used for hydrogen production, such as oil reforming, coal gasification, and water electrolysis, are currently associated with sustainability challenges, high energy consumption, and significant costs.[17-19].

In the context of renewable hydrogen production, biological processes such as dark fermentation and photo fermentation are highly favored [20]. Among these two processes, dark fermentation appears to be more viable in practical terms due to its numerous advantages, including stable hydrogen yield, rapid hydrogen production rate, straightforward operational conditions, low energy requirements, and cost-effectiveness [20,21].

Based on recent research, it has been revealed that the utilization of dark fermentation, particularly during the hydrolysis and acidogenesis stages, is an effective method for generating bio-hydrogen from high-organic-content wastewater [16]. Moreover, these studies have shown that SS exhibits a lower potential for hydrogen production compared to substrates rich in carbohydrates. The yield of hydrogen from SS is below 1 mmol H_2/g volatile solids (VS) [22,23].

The dark fermentation of SS faces several challenges, including the scarcity of readily fermentable substrates [1,22], low bio-hydrogen yields, and difficulties in scaling up fermentative bioreactors [24]. In addition, the utilization of single organic waste in dark fermentation (known as mono-fermentation) is often hindered by the limitations associated with substrate properties, such as nutrient imbalances and the presence of inhibitory compounds [24]. To address these challenges, the concept of co-fermentation, which involves the simultaneous utilization of multiple substrates with complementary characteristics, has emerged as a promising strategy to enhance hydrogen conversion efficiency and promote effective waste recycling [25,26].

To address these challenges, various methods have been explored to overcome the limitations. These include strategies focused on optimizing the substrate composition or modifying the processing methods [27]. For example, it has been reported that the dark fermentation for hydrogen production improved by retreating the substrate or inoculum that helps in decreasing the activity of the bacteria that produce methane and increasing the activity of the bacteria that produce hydrogen [28]. Pretreatment methods, including thermal, chemical, or enzymatic techniques, have been employed to improve the accessibility of fermentable substrates for microorganisms. These methods involve the disruption of cell structures and liberation of the substrates, thereby enhancing their availability [28]. As a result, these pretreatment methods contribute to an overall improvement in hydrogen production during the process of dark fermentation [22,29]. Numerous pre-treatment processes, including mechanical [30], thermal [31], chemical [32], and irradiation [33] methods, have been suggested to enhance the fermentability of SS. Thermal and chemical pretreatments have been extensively studied in previous research [1, 34–36]. Thermal pre-treatment, plays a crucial role in promoting the growth of biohydrogen-producing bacteria. By suppressing the activity of methanogens, thermal pre-treatment selectively inhibits their growth, creating a favorable environment for the proliferation of biohydrogen-producing bacteria [1,37].

Dark fermentation, a biochemical process, is subject to the influence of pH and temperature throughout the fermentation phase [38]. The optimal pH and temperature conditions for hydrogen production in SS are distinct [38,39]. In order to promote the proliferation of hydrogen-producing microorganisms, dark fermentation is typically carried out under mildly acidic conditions (pH < 6), utilizing carbohydrate-rich substrates [40]. Additionally, these microorganisms possess the capability to inhibit competing microorganisms such as methanogenic archaea [41].

To overcome these inherent limitations, can explore alternative approaches to modify the processing methods [42]. For instance, a potential solution involves implementing the co-fermentation of SS alongside a diverse range of organic wastes. This innovative approach aims to enhance the efficiency and effectiveness of the processing methods. This approach aims to improve process efficiency by adjusting the C/N ratio, increasing the availability of bioavailable carbohydrates, and diluting the inhibitors [14, 42]. Previous studies have delved into the realm of co-fermentation, specifically focusing on the combination of SS with a diverse array of organic wastes. These organic wastes, characterized by their high C/N ratios and ample carbohydrate content, encompass a wide range of materials such as crop residue, crude glycerol, FL residue, food waste, tofu residue, FL ower waste, and molasses wastewater [43]. Through the co-fermentation processes involving SS and various organic wastes, researchers have consistently observed higher levels of biohydrogen productivity compared to the sole fermentation of SS [44]. This indicates that the combination of SS with organic wastes possessing high C/N ratios and abundant carbohydrate content enhances the biohydrogen production process. The synergistic effect of co-fermentation leads to increased biohydrogen yields, making it a promising approach for efficient and sustainable bioenergy production.

Besides the previously mentioned co-substrates, leaves, particularly fallen leaves, also hold significant potential as co-substrates for biohydrogen fermentation. Fallen tree leaves, which are abundant in forestry waste and form a major component of yard waste, exhibit a high C/N ratio (exceeding 30) and contain substantial quantities of biodegradable carbohydrates like cellulose and hemicellulose [45]. This makes them an attractive option for enhancing the biohydrogen production process.

In Egypt, the burning or disposal of tree leaves is a common practice that has detrimental effects on the environment [46]. However, if these leaves are fermented with SS to produce hydrogen synergistically, it has the potential to yield significant amounts of energy and offer substantial environmental benefits [47]. Despite these promising advantages, there is limited information available regarding the co-fermentation process of fallen tree leaves with sludge for biohydrogen production. Only a few studies have explored this specific area, leaving a gap in our understanding. Furthermore, while some investigations have examined the performance of SS co-fermentation processes mentioned earlier, most of these studies have primarily focused on aspects such as biohydrogen yield, organic degradation, and metabolic product formation [47-49].

The aim of this study was to explore the potential of biohydrogen production from SS under thermophilic fermentation and co-fermentation conditions in a 100 L bioreactor. The specific objectives were as follows: (1) to investigate the feasibility of biohydrogen production directly from raw SS, (2) to examine the impact of heat-treated SS on biohydrogen production, and (3) to evaluate the performance of the co-fermentation process for biohydrogen production using a combination of sludge and FL at various mixing ratios. To the best of our knowledge, such a study of the effects of different pretreatments and co-fermentation on hydrogen production from sewage sludge using an up-scale of 100 L has not been conducted before. Bio-energy yields produced from this scale have also been assessed in this study, with analysis of the microbiological examination in the ideal case.

2. Materials and Methods

2.1. Properties of raw SS and Fallen trees leaves.

The SS used as the feedstock in this study was obtained from the El-Gabal El-Asfar wastewater treatment plant, located in the Qalyubia government of Egypt. Prior to use, the sludge underwent a sieving process to remove debris and unwanted materials. The sieved sludge was then directly utilized for the experiments. Fallen leaves from trees were collected on the premises of the wastewater treatment plant. To prepare the fallen leaves for use as feedstock, they were first dried and commented to a size of approximately 18-mesh. Prior to being utilized, the committed leaves underwent pretreatment with 1.0% HCl (w/w) at a temperature of 100 °C for a duration of 15 minutes [1,50]. This pretreatment process was employed to enhance the bioavailability of the fallen leaves. For further reference, you can find the key characteristics of both the sludge and FL in **Table 1**.

Table 1. Physicochemical properties of the municipal SS and Fallen trees leaves

Parameter	SS	FL
PH	7.62	–
EC (ds m^-1)	1.82	2.63
Moisture content (%)	60	8.35
Total organic carbon (%)	36.7	47.81
Total N (% of D.W)	2.73	0.612
C/N ratio	13.44	78
TS (g/L)	26.55	89.6
VS (g/L)	17.92	80.19
VS/TS (%)	67	89.5

2.2. Batch experiments

In order to investigate the production of bio-hydrogen from a 100L scale using SS, various pretreatment methods were applied to the sludge mixture, including the addition of FL as a bulking agent. The control (C) experiment involved no pretreatment of the sludge. The thermal pretreatment (TP) experiment subjected the sludge mixture to heat treatment at 75°C for 15 minutes [37]. The treated sludge by thermal pretreatment (TPFL -1) experiment included co-fermentation with a ratio of 0.25 g FL to 1.0 g SS. Another experiment, TPFL -2, also involved treated sludge by thermal pretreatment (75°C for 15 minutes) and co-fermentation with a ratio of 0.50 g FL to 1.0 g SS. The mixtures for different experiments were prepared according to the details provided in **Table 2**.

Table 2. Mixtures for different experiments

No. Of Run	Mixture name	Pretreatment method	FL (g) (V.S)	SS (g) (V.S)
Run 01	C	No pretreatment	-	-
Run 02	TP	Thermal	-	-
Run 03	TP/G1	Thermal	0.25	1.0
Run 04	TP/G2	Thermal	0.50	1.0

Characteristics of Mixtures for different co-fermentation experiments as described in **Table 3**.

Table 3. Mixtures for different co-fermentation experiments

Parameters	pH	COD $_{tot}$ (g/L)	VFAs (g/L)	TS (g/L)	VS (g/L)
C	7.62	32.43	0.82	26.55	17.92
TP	6.40	39.70	0.74	21.00	14.20
TPFL -1	6.21	38.50	0.95	37.12	21.69
TPFL -2	6.30	39.10	0.97	46.50	27.01

2.3. CSTR setup and operation

To produce bio-hydrogen from mixed SS, the pilot-scale CSTR with a total volume of 100L and working volume of 70 L was utilized (amount of effluent digested). The area of the reactor had three sampling or feeding ports on the side and a bottom port for sludge removal. Four holes on the upper side of the reactor were used for gas collection, temperature and pH monitoring, and manual titration. Mechanical mixing of the CSTR content with external pumped recirculation was carried out. The temperature was maintained by using electrical heaters. The resulting gas was collected into 25-liter steel, and after that, the gas was received by using gas bags as shown in **Fig. 1**. The CSTR was fed with mixed SS that had been pre-treated and operated in batch mode. Samples were taken on days 2, 4, 6, 8, and 10.

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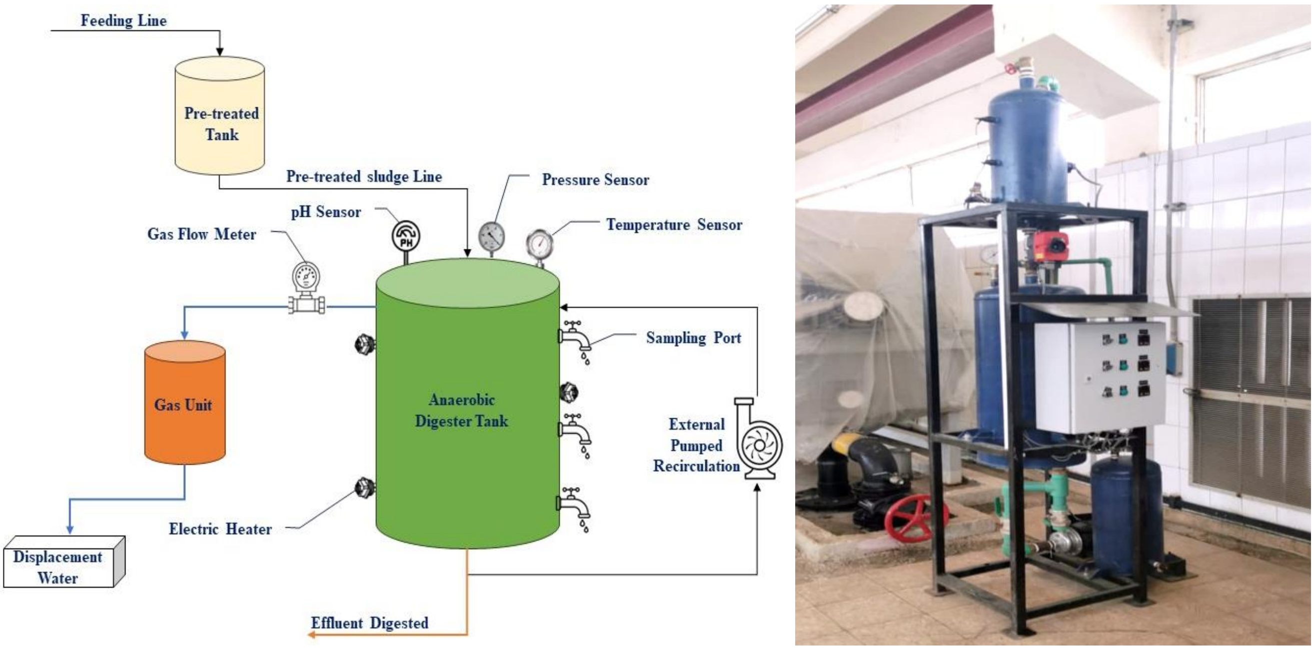


Fig. 1. Schematic diagram of CSTR producing hydrogen from mixed SS

2.4. Analysis and Calculations

The gas production in continuous stirred tank (CSTR) reactors was assessed using the water displacement technique for each operating day. The pH value was measured using a JENWAY 3510 device, while the chemical oxygen demand (COD) was measured using the HACH method [11]. The measurement of volatile fatty acids (VFAs), ammonia nitrogen (NH4-N), total Kjeldahl nitrogen (TKN), total solids (TS), and volatile solids (VS) was conducted following the standard methods for the examination of water and wastewater [29]. VFAs and ammonia nitrogen were measured using steam distillation with Behr S-1 equipment from Germany. Total solids were determined by drying the samples at 105 °C for 24 hours, and volatile solids were calculated from the loss on ignition after ashing the dried residue at 550 °C for 4 hours. The biogas composition, including H_2, CH4, H_2S, CO_2, and O_2 was determined using portable biogas 5000 gas analyzer (Geotech, Geotechnical Instruments (UK) Ltd, England).

2.5. The analysis of fallen tree leaves

Atomic force microscopy (AFM) is a powerful imaging technique that can be utilized for the analysis of fallen tree leaves at the nanoscale level. AFM allows for high-resolution imaging of the leaf surface, providing valuable insights into its topography, structure, and physical properties. Furthermore, AFM can be employed to investigate the interactions between fallen tree leaves and other materials or surfaces. This includes studying adhesion forces, frictional properties, and surface interactions with water or other liquids. Such investigations can contribute to our understanding of the leaf's surface chemistry and its role in ecological processes. **Fig. 2** is shown the results of AFM for fallen tree leaves which were used in our study.

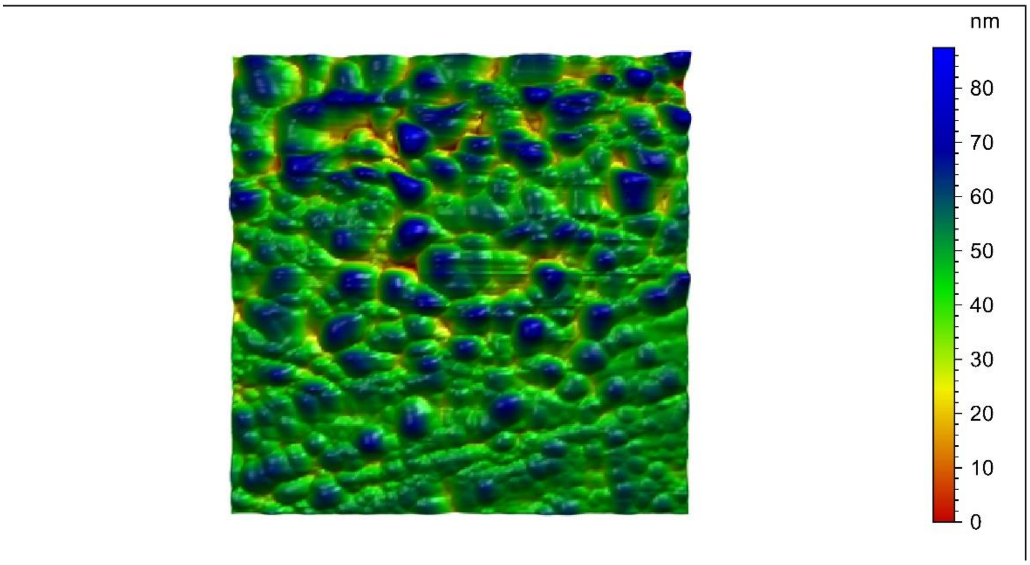


Fig. 2 AFM for fallen tree leaves

Transmission Electron Microscopy (TEM). TEAM is a powerful imaging technique that allows for high-resolution visualization of the internal structures of biological samples at the nanoscale.TEM works by passing a beam of electrons through a thin section of the leaf sample. TEM analysis played a vital role in our research by providing detailed information about the internal structures of leaves at a nanoscale level. It allowed us to explore and analyze various cellular components and their interactions, leading to a deeper understanding of leaf biology. The insights gained from this analysis have significant implications for fields such as plant physiology, biochemistry, and biotechnology. **Fig. 3** is shown the results of TEM for fallen tree leaves which were used in our study.

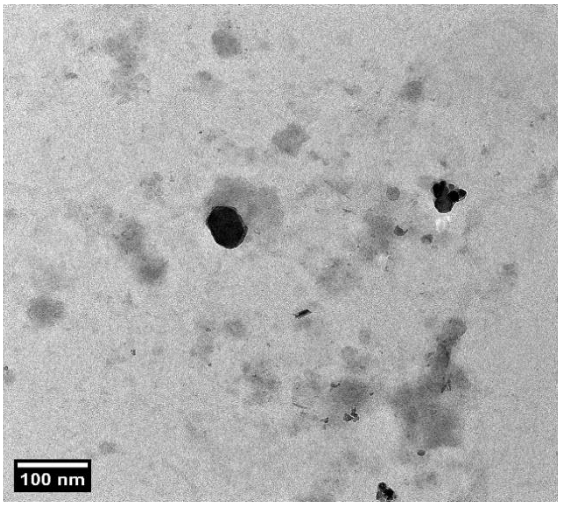


Fig. 3 TEM for fallen tree leaves

2.6. Bacterial Isolation From Reactors

A series of steps were followed to isolate the prevalent bacteria in the reactor under operation conditions. The selected colonies were determined by the system of Biolog GEN III

(BIOLOGY, USA). This system employs a Micro Plate™ test panel that utilizes 94 biochemical tests to profile and identify a wide range of Gram-positive bacteria and Gram-negative. The systems software for microbial was utilized to determine the bacterial isolates based on their phenotypic patterns on the GEN III Micro Plate.

After identification, suspected colonies were streaked onto Trypticase Soya Agar (TSA) plates from Sigma-Aldrich, UK. These plates were then incubated for 24 hours at 37 °C overnight, with the incubation temperature maintained [51]. This step allowed for further growth and observation of the bacterial isolates.

By following these steps, researchers were able to isolate and identify the prevalent microorganisms present in the reactor under operation conditions. This information is crucial for understanding the microbial community dynamics and their potential contributions to biohydrogen production.

3. Results and Discussion

3.1.Bio-hydrogen Production from 100 L SS bioreactor at different conditions.

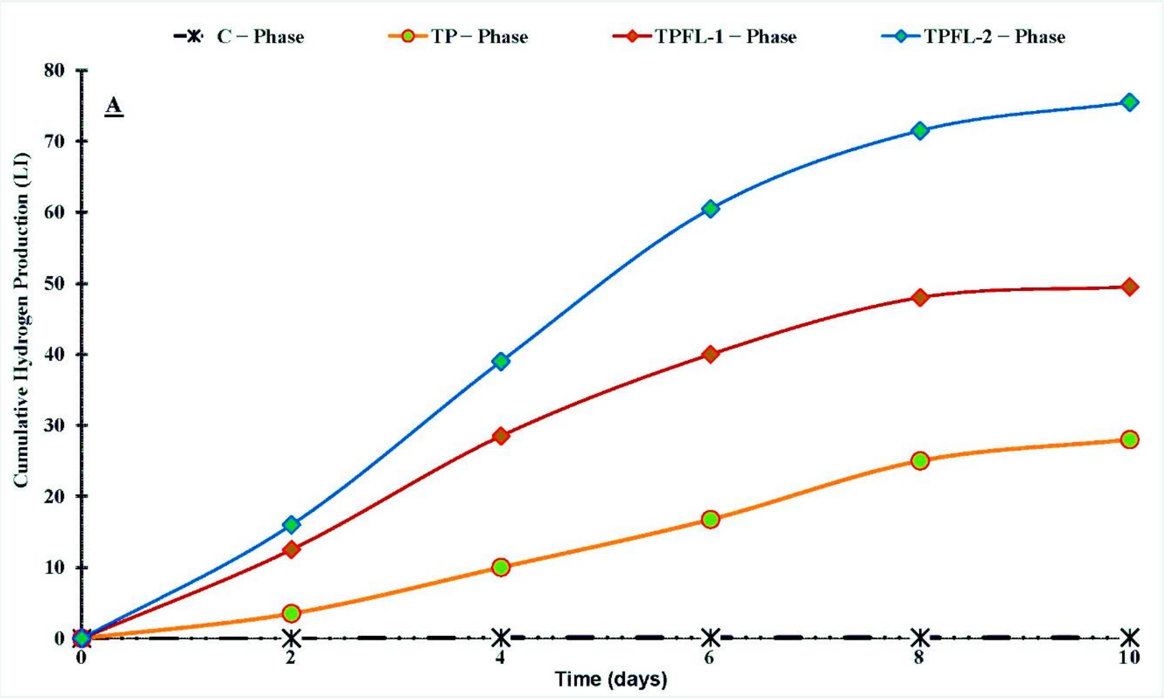
The potential for bio hydrogen production was evaluated through batch assays on mixed SS at different conditions as has been namely before (C, TP, TPFL -1, and TPFL -2). The hydrogen yields of non-prorated and retreated sludge in fermentation and co- fermentation anaerobic conditions are shown in **Fig. 4**. As per the results of the batch experiment, the pretreatment method used in the mixed SS leads to the production of different bio-hydrogen gas and consequently the production of different hydrogen yields.

The hydrogen yield parameter is often used to assess the efficiency of hydrogen fermentation. The sludge without pre-treatment was used as a control to the whole experiment with producing hydrogen gas and following hydrogen yield as shown in **Fig. 4**. The results showed that the little amount of hydrogen gas production was indeed possible from raw SS, where the maximum biohydrogen yield achieved was 0.03 L H_2/g VS (**Table 4**).

The second objective of this study was to examine the impact of heat-treated SS on biohydrogen production. The heat treatment was conducted at 75°C for 15 min (TP) to simulate AD conditions. Compared to raw SS, heat-treated sludge exhibited a higher biohydrogen production rate and yield. **Fig. 4**, showed that heat treatment significantly improved the biohydrogen production from SS. The maximum biohydrogen yield achieved after heat treatment was 6.48 L H_2/g VS. These findings indicate Temperature can improve the metabolic activity of fermentative microorganisms, which can affect the hydrogen production rate. Hydrogen usually produced as a byproduct by fermentative microorganisms in their metabolism, which involves breaking down organic matter in the SS in the absence of oxygen. This microorganism metabolic activity can be influenced by several factors, including temperature [52]. The findings align with previous studies that have documented the beneficial effect of heat treatment on biohydrogen production from SS [53-56]. The experimental results indicate that all pre-treatments can enhance hydrogen production from SS through anaerobic fermentation, albeit at varying rates. Among the pre-treatments, heat pretreatment demonstrated the highest efficacy and practical feasibility for real-world applications.

To evaluate the performance of the co-fermentation process for biohydrogen production using a combination of heat-treated SS with FL at various mixing ratios. Different mixing ratios of pretreated sludge and fallen tree leaves (1.0 : 0.25,and 1.0 : 0.50, (volatile solids)) were tested. **Fig. 4**, also shows that the addition of fallen tree leaves significantly improved the biohydrogen production compared to using sludge alone. The maximum biohydrogen yield achieved was 8.64 L H_2/g VS for the 1.0: 0.50 ratio (**Table 4**), which was 34% higher compared to using pretreated sludge alone. These results are in line with studies that have reported the advantages of co-fermentation for biohydrogen production from mixed substrates [57-59].

The biohydrogen yields obtained in this study were compared with previous research on biohydrogen production using different feedstocks. The results showed that the biohydrogen yields obtained from raw SS, heat-treated SS, and the co-fermentation process were comparable or higher than those reported in the literature. This indicates that the methods employed in this study have the potential to be efficient and effective in biohydrogen production. Further optimization of process parameters and scaling up of the bioreactor could potentially enhance the biohydrogen production potential even further.



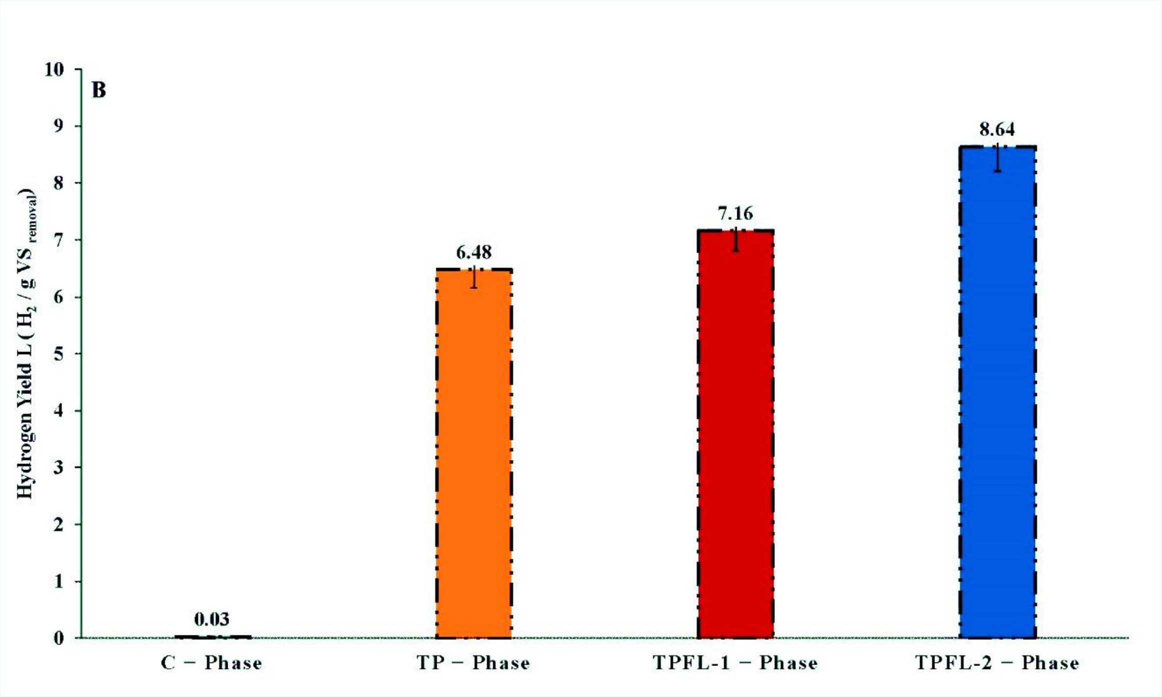


Fig. 4. Effects of various conditions on Cumulative Hydrogen Production (A) and Hydrogen yield (B) for the co-fermentation process.

3.2.Effect of pre-treatment and co- fermentation on the characteristics of SS

To evaluate the synergistic effect of pre-treatment and co-fermentation on the sludge composition and characteristics, the experiments of thermal pre-treatment and co-fermentation were conducted. The results obtained from these experiments showed significant changes in the sludge composition and characteristics compared to the controls where no pre-treatment or co-fermentation was employed. Because the mechanisms of methods are different, the impacts of these pretreatments on the sludge were also different [60]. **Table 4**, summarizes the effects of the four experiments on the sludge after anaerobic fermentation and because these experiments can break part of the bacteria structure and the microbial cells of sludge, the biogases have been produced and detoxification of the waste has been happening as recorded from many researchers [61].

The fermentation or co-fermentation process of SS typically involves three stages: hydrolysis, acidification, and methanogenesis. The production of hydrogen takes place during the initial stage, where the pH value plays a crucial role in biohydrogen fermentation. Generally, it is believed that the optimal initial pH of biohydrogen fermentation falls within the range of 5.0 to 6.0 [62,63].

Hydrogen was produced at the acidic pH and alkaline pH as the lysis of the cell strengthened along with the acidic and alkaline pH not neutral as compatible with the results of many previous studies [63,64]. Up to date, some studies have demonstrated that at alkaline conditions, the VFA production was improved significantly, and the yield was even over 3 or 4 times that at pH 5.0 or under uncontrolled neutral pH conditions [32,65], and this is consistent with the results of our study since the hydrogen productivity in alkaline pretreatment is almost twice as high as in acidic (**Table. 4**). This can be used to explain why the combination of solubilized protein from sludge and the formation of VFAs and ammonia during bio-hydrogen fermentation occurs.

The removal of the volatile solids (V.S) is commonly employed as a means to indicate the effectiveness of waste reduction in the process of fermentative biohydrogen production. To illustrate the VS removal efficiency in the experiments of thermal pre-treatment and co-fermentation of various mixing ratios, **Fig. 5A** is presented. Based on the findings presented in **Fig. 5A**, it can be observed that all the experiments of thermal pre-treatment and co-fermentation exhibited significantly higher efficiency in removing volatile solids (VS) compared to row SS. The row SS resulted in a mere 21.99% removal of VS, as shown in **Fig. 5A**, indicating the limited degradability of the sludge's organic matter. This phenomenon can potentially be attributed to factors such as nutrient limitations, specifically an unsuitable carbon-to-nitrogen (C/N) ratio, as well as the intricate composition of the SS, which hampers the microbial degradation of its organic constituents [66]. The most significant decrease was achieved through thermal pre-treatment (TP), resulting in a VS reduction from 14.20 to 9.88 g/L (30.44%) (1.1 times higher than the raw sludge), as indicated in **Table 4**. After co-fermenting with fallen trees leaves, the VS removal was improved to 31.68%, and 32.55% at the mixing ratios of 0.25:1.0, and 0.50:1.0, respectively (**Fig. 5A**). The maximum volatile solids (VS) removal observed in this study exhibited a notable increase when juxtaposed with the fermentation of sludge and organic fraction of municipal solid waste (OFMSW), which resulted in a removal rate of 12% [67]. Similarly, the co-fermentation of sludge with rice straw exhibited a maximum removal rate of 13% [68].

Chemical Oxygen Demand (COD tot) reduction is a significant parameter for measuring the productivity performance of bio-hydrogen along the study [69]. The COD tot reduction was monitored throughout the anaerobic process period. As shown in **Fig. 5B**, each thermal pre-treatment and co-fermentation process enhanced the reduction of total chemical oxygen demand, as indicated in **Table 4**. Thermal Pretreatment (TP) achieved the most significant decrease, resulting in a COD total reduction from 39.70 to 27.60 g/L (2.15 times higher than the raw sludge), however, the decrease of COD tot was from 38.50 g/L to 24.88 g/L in TPFL -1 phase and the reduction was from 39.50 g/L to 20.85 in TPFL -2 phase. A decrease in COD indicates an efficient utilization of organic matter by microorganisms, leading to enhanced hydrogen production [70–72].

Total Solids (TS) play an important role in generating biohydrogen from SS via anaerobic fermentation. To increase bio hydrogen yield and improve overall process performance, it is essential to optimize the TS content in an appropriate range. Anaerobic fermentation converts complex organic compounds in waste material to simpler compounds, such as volatile fatty acids and carbon dioxide. The production of biohydrogen results from organic matter A process after which, the amount of solids (more precisely, VS) reduces accordingly. As shown in **Fig. 5C**, each distinct thermal pre-treatment and co-fermentation procedure contributed to an augmentation of the reduction for (TS) by a factor ranging from 11 % to 33.87 %. The most significant decrease was achieved through (TPFL -2), resulting in a TS reduction from 46.50 to 30.69 g/L, however, the results of total solids redaction through the different pretreatments were variable.

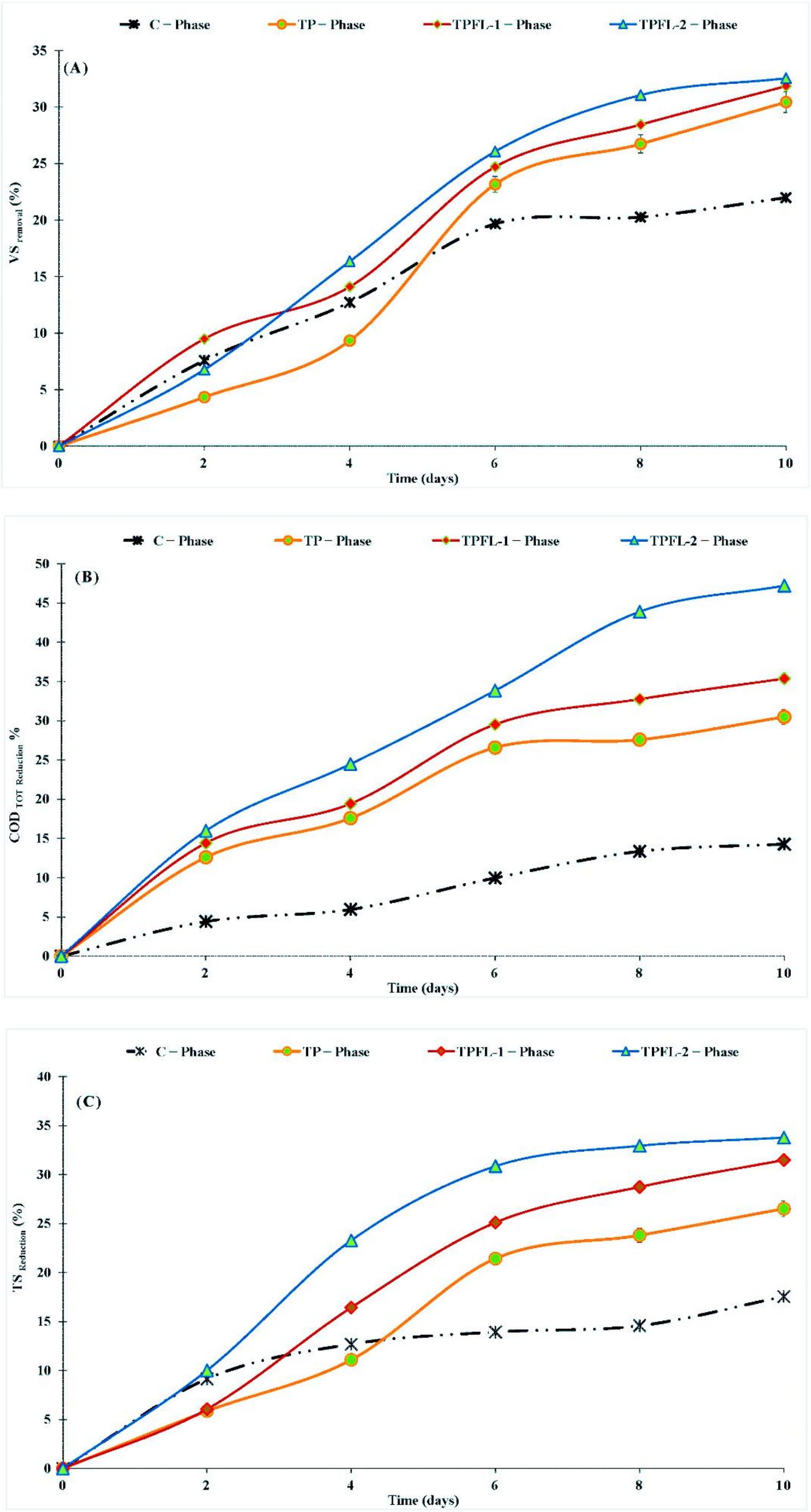


Fig. 5. The effects of Effects of various conditions on (A) VS, (B) COD and (C) TS reductions from CSTR reactor (100 L) for the co-fermentation process.

Table 4. Effect of various experiments on mixed SS characteristic

Item	Control (C)		Thermal (TP)		Thermal with co-fermentation (TPFL -1)		Thermal with co-fermentation (TPFL -2)	
	R*	T**	R*	T**	R*	T**	R*	T**
pH	7.62	7.45	6.40	6.50	6.21	6.55	6.30	6.52
COD tot (g/L)	32.43	27.80	39.70	27.60	38.50	24.88	39.50	20.85
TS (g/L)	26.55	21.89	21.00	15.43	37.12	25.43	41.00	34.00
VS (g/L)	17.92	13.98	14.20	9.88	21.69	14.78	26.85	18.11
Max H_2 yield (L H_2 / g VS removal)	0.00	0.03	0.00	6.48	0.00	7.16	0.00	8.64

R* = Raw sludge T** = Digested Sludge

3.3.Volatile Fatty Acids (VFAs) production at various experiments from CSTR reactor (100 L).

During anaerobic fermentation, the breakdown of organic matter by microorganisms in the absence of oxygen leads to the production of various byproducts. One such byproduct is volatile fatty acids (VFAs), which are organic acids with a relatively low molecular weight. VFAs are generated through the degradation of complex organic compounds like carbohydrates and fats. The utilization of bio hydrogen in anaerobic fermentation can have a significant impact on the production and concentration of VFAs. Several factors influence the production of VFAs, including the type of organic matter being fermented, pH and temperature conditions within the fermentation environment, and the composition of the microbial community involved in the fermentation process [73].

Fig. 6 illustrates the concentrations of VFAs and confirms that the production of VFAs reached its maximum value in the TPFL -2 phase, with a recorded value of 3.65 g VFAs/g VS. Conversely, this value decreased to 3.43 g VFAs/g VS in the case of TPFL -1, which was comparable to the TP-phase where the recorded value was 2.90 g VFAs/g VS. In the CSTRs, the hydrogen yield exhibited variability and did not exhibit a direct correlation with the concentration of VFAs during the fermentation process. This observation suggests the occurrence of homoacetogenesis process, similar to what was observed in [74]. These findings align with those reported by Wan et al. in 2016 [36], who obtained a hydrogen yield of 0.85 mmol H_2/g VSS from SS in a thermophilic batch process at 55 °C. The study also highlighted that co-fermentation with the mixmum ratios were optimal for hydrogen production from anaerobic fermenters.

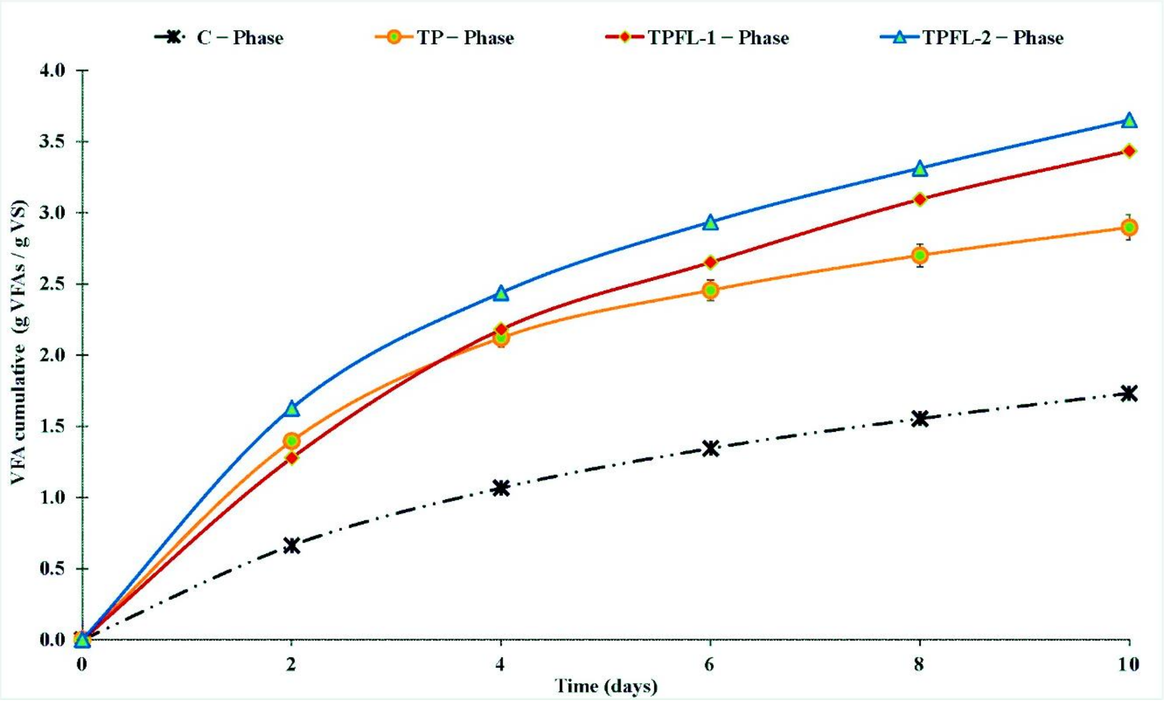


Fig. 6. The effects of various conditions on VFAs yields from self-fermented CSTR reactor.

3.4 Microbiological analysis of the sludge during the optimum phase.

Among the various methods for biohydrogen production, the utilization of biohydrogen-producing microbes has emerged as one of the most sustainable and effective approaches. In the examination of the microbial community extracted from the TPFL phase, by Biolog GEN III, it was confirmed that two of bacterial species were present: licheniformis and pseudomycoides Bacillus. These findings align with a study conducted by Shah et al. [75], who observed that there are two strains of Bacillus sp., specifically F2.5 and F2.8, exhibited biohydrogen yield through the fermentation of food waste. These specific bacterial strains demonstrated the capability to produce up to 61 mL H_2/g VS and are considered promising candidates for the development of biohydrogen-producing inoculants. These results are consistent with another study by Hassan et al. [24], which also identified Bacillus licheniformis as a species capable of enhancing biohydrogen production through the fermentation process.

3.5. Calculation of the total energy yields from 100 L SS self-fermented bioreactor at various experiments

The calculation of the total energy yields from a 100 L SS self-fermented bioreactor can be performed by comparing the energy outputs obtained from at various experiments. These experiments aim to enhance the efficiency of the fermentation process and maximize energy production. By quantifying the energy yields, researchers can determine the most effective experiments for SS fermentation.

Table 5 presents the energy yield values of the self-fermented anaerobic CSTR, utilizing the heating values of 120 kJ/g VS fermenter for biohydrogen gas [76]. The energy yields obtained from the hydrogen fermenter were 86,400 kJ/g VS fermenter, while for the TPFL -2 and control phases, they were only 300 kJ/g VS fermenter, respectively. These findings confirm that the co-fermentation process can significantly enhance energy yields compared to the control phase. The utilization of pre-treatment methods or co-fermentation processes in preceding hydrogen production resulted in an increase in energy yield. In a recent study by Hassan et al. (2022) [13], a novel technology was employed to further enhance energy yields from the hydrogen fermenter. The authors suggested that thermal pretreatment could maximize energy yields from food waste. Additionally, by implementing technologies such as electrodialysis, VFAs concentrations could be increased. However, it is important to note that this study focused on a 100 L fermenter, unlike previous studies which utilized a smaller 5-liter scale.

Table 5. Energy yields from 100 L SS self-fermented bioreactor at various experiments

phase	Hydrogen yield (mL / g VS)	Energy yield (K j / g VS)
C	30	300
TP	6,480	64,800
TPFL -01	7,160	71,600
TPFL -02	8,640	86,400

4. Conclusions

In this groundbreaking pilot-scale investigation, we systematically delved into the repercussions of thermal pretreatment and Co-fermentation on biohydrogen production from mixed sewage sludge, operating at a 100 L scale. This pioneering study marks a significant leap in our understanding of the intricate dynamics influencing biohydrogen production under the influence of thermal pretreatment and Co-fermentation at a pilot-scale hitherto unexplored. The outcomes of this study conclusively demonstrate that thermal pretreatment of sewage sludge leads to a remarkable increase in biohydrogen production yield, surpassing the untreated sewage sludge by an impressive 232-fold. Additionally, the co-fermentation of sewage sludge and Fallen Trees Leaves manifests a synergistic effect, significantly influencing and enhancing biohydrogen production. Methodically conducted experiments led to the identification of an optimal mixing ratio of 1.0:0.5 (sewage sludge to fallen tree leaves). At this precise proportion, the biohydrogen yield achieved an outstanding 84.6 L/g-VS, surpassing yields obtained from mono-fermentation of raw sludge, thermal pretreatment, and a 0.25 ratio of fallen leaves by 310%, 33%, and 20%, respectively. Furthermore, the removal rates of Chemical Oxygen Demand (COD) and Volatile Solids (VS) exhibited notable increments of 27% and 21%, respectively, when compared to other ratios. The implications of these findings underscore the potential for optimizing sewage sludge pretreatment and co-fermentation strategies, thereby opening avenues to enhance biohydrogen production. This not only contributes to the advancement of sustainable energy generation but also signifies a promising stride toward harnessing energy from wastewater resources in an environmentally conscious manner.

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Ethical approval:

Not applicable. This manuscript does not contain any research on humans or animals.

Availability of data and materials

The datasets used and/or analyzed during study are available from the corresponding author upon reasonable request.

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