Comparative Analysis of Various Seed Sludges for Biohydrogen Production from Alkaline Pretreated Rice Straw

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ABSTRACT

The present work studied the effects of alkali pretreatment on the cellulosic biomass of rice straw. The improvement in the cellulose content and reduction in the lignin and hemicellulose percentage was observed with alkali pretreatment. Fourier transformation infrared spectroscopy (FTIR) and Scanning electron microscopy (SEM) analysis confirm the modification in the surface structure of alkali rice straw. Further, the study investigated the potential of different types of seed sludge as inoculum sources for dark fermentative biohydrogen production. In comparison to other sludge samples (beverage industry, food industry, and sewage treatment plant sludge), the mixed culture of sewage treatment plant sludge had the highest cumulative volume of biohydrogen (90.52 mL), as well as the highest hydrogen production yield (0.75 moleH_2/mole) with the substrate utilization of 86.72%. The results provide information on the best sludge source for enhancing biohydrogen production in the dark fermentation method.

INTRODUCTION

Hydrogen is regarded as one of the most promising fuels of the future owing to its significant properties, such as high energy content (142 kJ.g^{-1}) in comparison to hydrocarbon fuels. Presently, most of the hydrogen (50%) is produced by conventional thermo-chemical techniques like thermal or steam reforming of natural gas or petroleum fractions (Sgobbi et al. 2016, Rani et al. 2022, Ram et al. 2023). To promote a more environmentally sustainable fuel, hydrogen production methods should strive to prevent or reduce CO_2 emissions. In recent years, biological hydrogen production has gained a lot of attention among the many hydrogen production techniques (Trchounian et al. 2017). Dark fermentation has become more attractive due to the high hydrogen production rate, no required light source, the use of a variety of potential substrates, and simplicity in construction (Lukajtis et al. 2018, Mohammed et al. 2018). In the dark fermentation process, different types of biomass/ substrates such as agricultural waste, fuelwoods, energy crops, livestock residues, algal feedstocks, food waste,

lignocellulosic biomass, municipal solid waste (MSW), dairy waste, industrial waste, etc., could be utilized as a carbon source to the microorganisms (Nasirian et al. 2011). Lignocellulosic biomass is a better option for biohydrogen production due to its massive potential of availability on earth, low price, and enrich in high carbohydrate contents (Kumar et al. 2015, Rani et al. 2023). Rice straw is found to be one of the plentiful, renewable agricultural residues accessible in Haryana, Punjab, Madhya Pradesh, and Uttar Pradesh, India. Rice straw, being an agricultural waste, could be utilized as a raw material for biohydrogen production. The primary components of lignocellulosic biomass are cellulose, hemicellulose, and lignin. The complicated structure of lignocellulosic biomass makes it challenging to use as a feedstock for biofuel production (Sarangi & Nanda 2020, Akubude et al. 2021). Therefore, pretreatment is necessary to simplify the complex structures of this biomass, which further affects the biohydrogen efficiency generation technology. The pretreatment process can partially remove lignin and hemicellulose, reduce cellulose crystallinity, and increase porosity (Saratale et al. 2013, Soares et al. 2020,

Ram et al. 2023). Alkali pretreatment with sodium hydroxide is the most well-known and cost-effective, and it has been frequently used to generate bioenergy from agricultural waste. Alkalis usually attack the lignin-hemicellulose bonds to solubilize the hemicellulose, leading to high delignification and exposing the cellulose to hydrolysis (Zheng et al. 2018, Yadav et al. 2020).

It has been studied that H_2 yield not only upon the type of pretreatment/hydrolysis process but also on the composition and quality of the substrates, type of micro-organisms, either consortium (e.g., anaerobic digested sludge) or pure cultures of mesophilic (e.g., *Clostridium butyricum*) and thermophilic bacteria (e.g., *Caldicellulosiruptor saccharolyticus*), physiological conditions, type of reactor (Rai & Singh 2016). The observation demonstrates that mixed consortia are preferable over pure consortia since they do not require a specific aseptic environment, resulting in simple handling and broad applicability. Further, these cultures are less impacted by the alteration in substrate type and compositions (Łukajtis et al. 2018).

The present research work aims to develop an alternative renewable, sustainable, and cost-effective green approach for biohydrogen production involving the process that utilizes low-cost rice straw waste. The main objective of the study is to investigate the effects of alkali pretreatment on the chemical composition and surface structure of rice straw. Furthermore, the current work aims to compare different sludge types to identify the most suitable one for optimizing biohydrogen production from alkaline-pretreated rice straw.

MATERIALS AND METHODS

Feedstock and Alkaline Pretreatment

Rice straw samples were collected from the rural areas of Fatehabad district (Haryana). The collected samples were washed, dried, and cut into small pieces before grinding and sieved with 80 mm mesh size. The powder samples were oven-dried at 50°C and stored in an airtight bottle for further experiments and subsequent studies.

Alkaline pretreatment was employed to delignify the biomass. Dried rice straw and dilute sodium hydroxide (2% NaOH) were applied to the rice straw. A solid-to-liquid ratio of 5:100 (w/v) was used to make sure that the rice straw powder was completely immersed in the solution. Kept the mixture in the autoclave at 121°C and 15 psi for 60 min. After this, it was allowed to cool. Filter the pretreated rice straw sample from the mixture with muslin cloth and, neutralize the residue by washing it with distilled water, and continue washing till the pH becomes neutral or 7.0.

The pretreated solid was completely dried at a temperature of 50°C.

Seed Sludge

Various sludge samples were collected from different selected sites, such as beverage industry sludge (BS), food industry sludge (FS), and sewage treatment plant sludge (STP), which contains anaerobic bacteria. The samples from different sites were collected in Ziplock polybags, which were sealed and stored at 4°C temperature for the isolation procedure. For the test study, to improve the fermentative microbial flora and get rid of methanogenic and other hydrogen-consuming bacteria that reduce the effectiveness of hydrogen-producing bacteria, the seed sludge was heated at 90°C for 30 min. The following inorganic supplements $(g.L^{-1})$ were present in the feed medium in appropriate amounts: $NaHCO_3$ 6.72, NH_4HCO_3 5.24, $MgCl_2.6H_2O$ 0.1, K_2HPO_4 0.125, $MnSO_4.6H_2O$ 0.015, $FeSO_4.7H_2O$ 0.025, $CuSO_4.5H_2O$ 0.005, and $CoCl_2.5H_2O$ 0.000125 (Endo et al. 1982).

Enrichment of hydrogen-producing mixed cultures was carried out in 125 mL serum vials with a 50 mL working volume following a method described elsewhere (Sivagurunathan et al. 2014). The sterile pre-produced peptone-yeast-glucose (PYG) medium contained the following nutrients (in $g.L^{-1}$): 10, peptone, 10, yeast extract, 0.001, resazurin, 0.5, L-cysteine-HCl, 10, glucose. The pH was adjusted to 7.0 using either 1 N HCl or NaOH before autoclaving. The media was autoclaved at 121°C and 15 psi pressure for sterilization. The PYG medium with inoculum was incubated at 70°C for 10 min for the activation of spores into vegetative cells. After the activation of vegetative cells, serum vials were kept under shaking conditions at 110 rpm at 37° C temperature for 4 days, and anaerobic conditions were maintained to observe the growth of consortia. Freshly grown enriched mixed cultures were used as the inoculum for all the fermentation experiments.

Experimental Design

Batch-mode reactor: The batch tests for biohydrogen production were conducted in 125 mL serum vials with a working capacity of 60 mL containing seed sludge (25 mL), supplement solution (10 mL), and substrate (25 mL). To maintain an anaerobic condition, the nitrogen was first flushed through the vials for five min. The batch reactors were sealed with an airtight stopper with an aluminum cap and incubated at a temperature of 37°C while maintaining an agitation speed of 120 RPM. The initial cultivation pH was adjusted to 7.0 at a temperature of 37 °C. All experiment sets were carried out in triplicate to ensure reproducibility,

and the data were reported as mean values. A 25-mL syringe was used to measure the total gas volume using the syringe displacement method. Every 24 hours, the syringe was reset to zero. A needle was injected into the stopper of the syringe to assess the total amount of gas. However, it was observed that the gas pressure within the container could potentially displace the syringe plunger, leading to a quantifiable change in the syringe plunger's volume, which is then recorded as the generated gas volume. A gas chromatograph equipped with a thermal conductivity detector (TCD) was used to measure the hydrogen content.

Analytical Methods

The characteristics of raw rice straw and alkali-pretreated rice straw were tested in the laboratory. The chemical constituents of raw and alkali-pretreated rice straw samples, such as cellulose, hemicellulose, and lignin, were analyzed by the standard method described (Goering & Soest 1970). SEM (Zeiss Sigma500) was used to examine morphological changes in the untreated and alkali-pretreated rice straw. Oven-dried and fine-powdered samples were used to determine the morphology of rice straw. Samples were mounted on the aluminum stubs with the gold coating in a sputter before image analysis. Fourier Transformation Infrared Spectroscopy (FTIR) investigated the various functional groups of the native and alkali-pretreated rice straw samples. FTIR spectra were taken on a Perkin-Elmer Spectrum. The instruments with an absorption wave range between 400 - 4000 cm^{-1} were used for all samples. A trace amount of fine ground sample was mixed with potassium bromide (KBr) to analyze the various spectral lines. A gas chromatograph (5800, Centurion scientific instrument, capillary column, TCD detector) was used to analyze the composition of the biohydrogen gas produced in the reactor. The temperature settings for the column, injector, and detector were 80, 150, and 220°C, respectively. The nitrogen gas was used as a carrier gas, and the flow rate of nitrogen gas was maintained at 20 ml min-1. A pressure-lock gas syringe with a capacity of 1 mL was used for the gas injection. The cumulative biohydrogen production was determined using a mass balance equation (1) (Logan et al. 2002)

$$V_{Hi} = V_{Hi-1} + C_{Hi}(V_{Gi} - V_{Gi-1}) + V_W(C_{Hi} - C_{Hi-1}) \quad …(1)$$

where V_{Hi} and V_{Hi-1} are cumulative volumes of biohydrogen at the current and previous times. V_{Gi} and V_{Gi-1} are the total volumes of biogas at the current and previous times,

C_{Hi} and C_{Hi-1} are the fractions of biohydrogen (%) in the headspace of the bottle at the current and previous time

VW is the cumulative headspace volume within the bottle.

A gas chromatograph (Nucon, 5765) equipped with an FID (flame-ionized detector) was used to measure the volatile fatty acid concentrations (VFAs) at the end of the fermentation process. The temperature settings for the column, injector, and detector were 145, 175, and 185°C, respectively. The initial oven temperature was 95°C and held for 5 min, and further, the temperature was raised to 145°C at 10°C.min^{-1}. Nitrogen was employed as the carrier gas, with a flow rate of 6.0 mL.min^{-1}.

RESULTS AND DISCUSSION

Chemical and Structural Characterization

Table 1 illustrates the percentage of the three major components: cellulose, hemicellulose, and lignin. It was found that the relative proportion of cellulose was increased after alkaline pretreatment as compared to the native rice straw sample. Alkali pretreatment increased the cellulose content from 35.5% in raw rice straw samples to 60.48% by removing amorphous materials from the biomass. Furthermore, alkali pretreatment enhances the accessibility of cellulose, leading to the maximal production of fermentable sugars during enzymatic hydrolysis. The hemicellulose and lignin content were reduced from 24.19% and 13.81% in raw rice straw to 12.28% and 3.38% in alkaline pretreated rice straw, respectively. Alkali pretreatment with sodium hydroxide (NaOH) causes biomass to swell up, which leads to an increase in internal surface area and thereby enhances enzymatic accessibility. Simultaneously, it also decreases the degree of crystallinity of cellulose and disruption of ester bonds between hemicelluloses and lignin. This disruption promotes the solubilization of both lignin and hemicellulose components. Numerous research related to this study also reported similar results (Barman et al. 2012, Zheng et al. 2018).

After sodium hydroxide pretreatment, the surface morphology of rice straw was influenced by the reaction of sodium hydroxide with ester bonds, which led to the removal of lignin and the release of cellulose. The raw rice straw has a smooth, compact surface structure with tightly packed fibers grouped in bundles, as shown by the SEM images (Fig. 1A). The smooth surface indicated that the fibers were covered in lignin. Alkali pretreatment revealed significant changes in the surface morphology of the rice straw sample, which represents the damage to the structure and composition of biomass. The hemicellulose and lignin in the pretreated straw samples were partly removed, fractured, or became loose, exposing interior structures (Kshirsagar et al. 2015). The alkali-pretreated rice straw showed a loose, dispersed, and fragmented fibrous structure containing holes and pits (Fig. 1B). Additionally, it facilitates the breaking up of fiber bundles and movement of

Table 1: Chemical composition of raw rice straw and alkali pretreated rice straw.

Components	Raw rice straw	Alkali pretreated rice straw
Cellulose [%]	35.5	60.48
Hemicellulose [%]	24.19	12.28
Lignin [%]	13.81	3.38

fibrous bundles from one site to another, reducing their cell contents and eliminating other chemical components. The disruption of rice straw components because of dilute alkali pretreatment was also reported by several researchers (Singh et al. 2014, Hartati et al. 2021).

The changes in chemical composition and functional group before and after alkali pretreatment were examined by FTIR. Fig. 2 (A, B) represents the functional bands corresponding to their functional group present in the raw and alkali pretreated rice straw biomass in which the X-axis (cm^{-1}) symbolizes wavenumber whereas on Y-axis symbolizes transmittance (%T). The FTIR spectrum of rice straw was observed in the region of 500-4000 cm^{-1}.

Table 2 represents the spectrum band and functional group present in raw and alkali-pretreated rice straw. The predominate peaks at 3430.57, 2923.31, 1637.19, 1420.95, 1365.41, 1103.17 in raw rice straw, whereas peaks at 3412.06, 2922.77, 1637.32, 1431.7, 1376.25, 1035.41, 897.1 were observed in alkali pretreated rice straw. It was found that the alkali pretreatment resulted in disappearing of certain peaks and others shifting locations. Furthermore, the alkali pretreatment caused a reduction in the hydroxyl group concentration in the biomass compared to the raw sample, as evidenced by a decrease in the intensity of the O-H absorption band. Table 2 shows peaks and bands that correspond to different functional groups found in raw and alkali-pretreated rice straws.
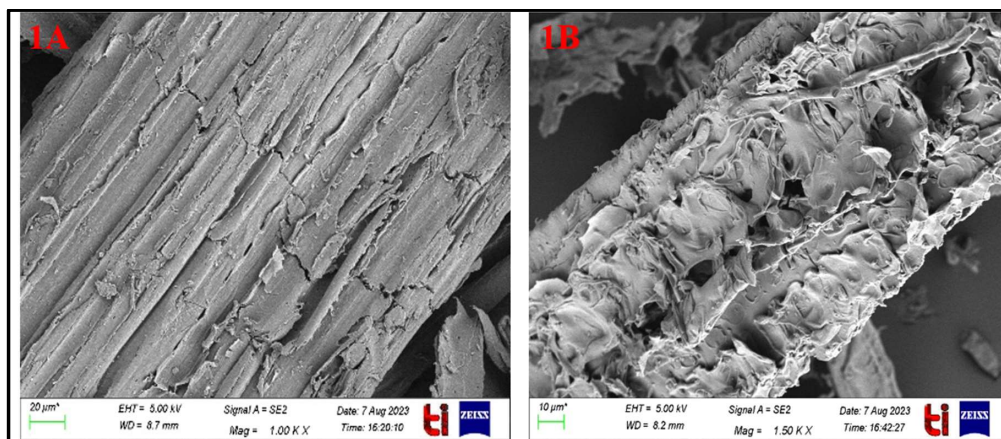

Fig. 1A: SEM images of raw rice straw, 1B: SEM image of alkali pretreated rice straw.
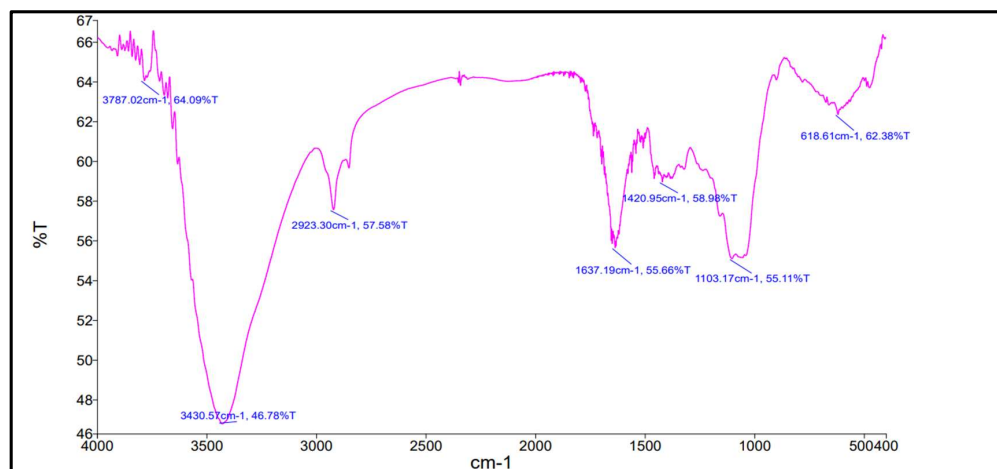

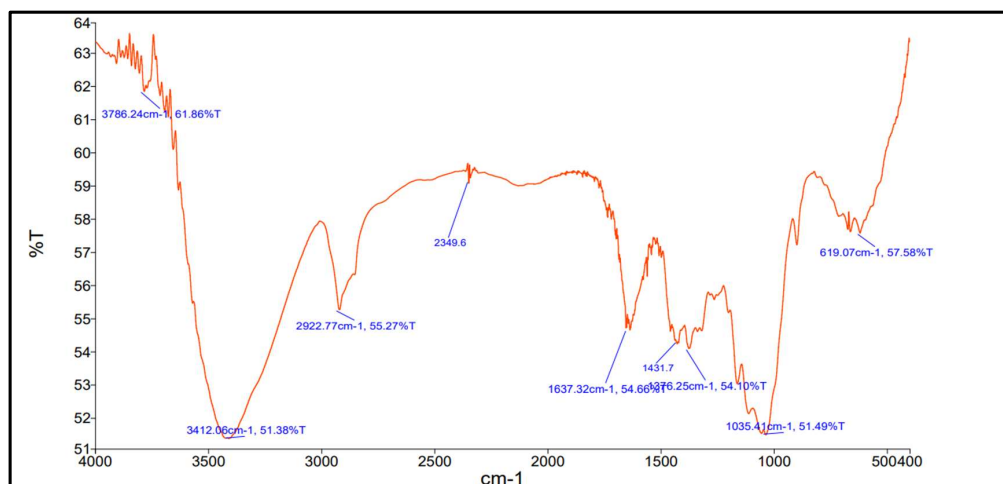Fig. 2A: FTIR spectrum of raw rice straw.

Fig. 2B: FTIR spectrum of alkali pretreated rice straw.

Table 2: FTIR spectrum band and functional groups present in raw and alkaline rice straw.

Frequency (cm^{-1})	Functional group	Raw RS	Alkali RS	Band assignment
4000-3000	O-H stretching	3787.02	3786.24	Lignin
		3430.57	3412.06	
2923-2900	C-H stretch	2923.31	2922.77	Cellulose
1650-1630	C=C stretch	1637.19	1637.32	Lignin
1440-1400	C=H bend	1420.95	1431.7	Lignin, Cellulose
1380-1360	C-H deformation	1365.41	1376.25	Cellulose
1332-1330	CH_2 Wagging	ND	ND	Cellulose
1110-1050	C-O-C& C-O stretch	1103.17	1035.41	Cellulose
898-880	C-H deformation	ND	897.1	Cellulose

Isolation of Bacterial Growth

Sludge samples from different selected sites were collected to isolate the bacterial consortia, which have the maximum capability to degrade the lignocellulosic residue. The sludge samples, such as beverage industry sludge (BS), food industry sludge (FS), and sewage treatment plant sludge (STP), were collected from Haryana. Heat treatment was applied to seed sludge (90°C for 30 min) to obtain dominant microbes for hydrogen production, and hinder the activity of methanogenic bacteria to consume hydrogen. Bacterial isolation was done in two assays i.e., plate as well as liquid assay. For the plate method assay, an agar medium was used to culture the isolates. Liquid method assay, isolates were inoculated into broth medium and kept in an incubator at 110 rpm at 37°C for 4 days.

To observe the growth of mixed consortia, the optical density of each seed sample has been analyzed using a UV-VIS spectrophotometer at the wavelength of 520nm, starting from zero hour to 120 h. Fig. 3 represents the bacterial growth phase of three different seed isolates. It has been observed that the bacterial growth starts increasing from 10 h of inoculation and reaches a maximum at 48 h. After that, it gradually decreases. The exponential growth of bacteria when the cells are dividing by binary fission and doubling their numbers after each generation time is known as the Exponential or log phase (10-48 h). From zero to 10 h represents the lag phase where the bacteria are metabolically active but not dividing. After the log phase, the stationary phase (48-120 h) is achieved, in which the growth of bacterial cells reaches a plateau as the number of dying cells equals the number of dividing cells. As nutrients become less available and waste products increase, the number of dying cells continues to rise. As a result, bacterial population growth experiences a sharp decline.

From Fig. 3 It has been found that the STP sludge's inoculation showed maximum bacterial growth among the three taken seed sludge.

The Influence of Various Microbial Cultures on Biohydrogen Production

To achieve efficient dark fermentative biohydrogen

production from renewable feedstocks, it is crucial to identify promising mixed microbial communities capable of effectively breaking down and fermenting the biomass to produce biohydrogen. Thus, it is crucial to evaluate seed sludges from various sources that contain microorganisms of various types and activities (Chen et al. 2012). The ability of LCB to produce biohydrogen utilizing various sources of inoculums or sludge was reviewed by Ren et al. (2009). Sivagurunathan et al. (2014) performed research in a threefold combination of mixed cultures comprising cow dung, pig slurry, and sewage sludge to enhance biohydrogen production when utilizing glucose as the substrate. The findings revealed a significant improvement in both hydrogen yield and production rate when using the mixed cultures of pig slurry and sewage sludge, with values of 2.34 moles of H_2 per mole of glucose and 6.76 L per day. This contrasted with the performance of the single culture of pig slurry, which yielded 1.59 moles of H_2 per mole of glucose and 4.43 L per day. Another study reported by Amekan et al. (2018) investigated to assess the impact of different sources of inoculum (fruit waste digester (FW), cow dung digester (CD), and tofu waste digester (TW)), as well as their combinations (FW-CD, CD-TW, FW-TW, and FW-CD-TW), on the production of hydrogen from melon waste. The research demonstrated that the highest cumulative hydrogen production, reaching 743 mL (with a yield of 207.56 mL per gram of volatile solids), was observed with the FW inoculum. However, an even more impressive result was obtained with

the combination of FW-CD-TW, which yielded 1,132 mL of hydrogen (at a rate of 231.02 mL per gram of volatile solids). These findings indicate the significance of microbial diversity and interactions in influencing both the yield and the rate of hydrogen production from melon waste in batch fermenters.

To establish a stable hydrogen production process, it is important to understand the impact of enhanced interactions between microbial diversity and the biohydrogen production process (Sivagurunathan et al. 2014). Hence, in the current work, we investigated the impact of different seed sludge, such as beverage industry sludge (BF), food industry sludge (FS), and sewage treatment plant sludge (STP), on the performance of biohydrogen production, and soluble metabolites accumulation using alkali pretreated RS in the batch test. The data obtained from these three types of seed sludges tested during fermentation of alkali RS (10 $g.L^{-1}$) into biohydrogen production and fermentation end products are summarised in Table 3.

It was observed from Fig. 4 that the heat-pretreated STP mixed consortia had the highest cumulative volume of biohydrogen (90.52 mL) and hydrogen production yield (0.75 $moleH_2$/mole, substrate) among the three mixed cultures. No methane production was observed in any of the three seed sludges after undergoing heat treatment. Earlier research revealed that most of the anaerobic sludge might be improved by thermal enrichment to promote hydrogen generation. Heat
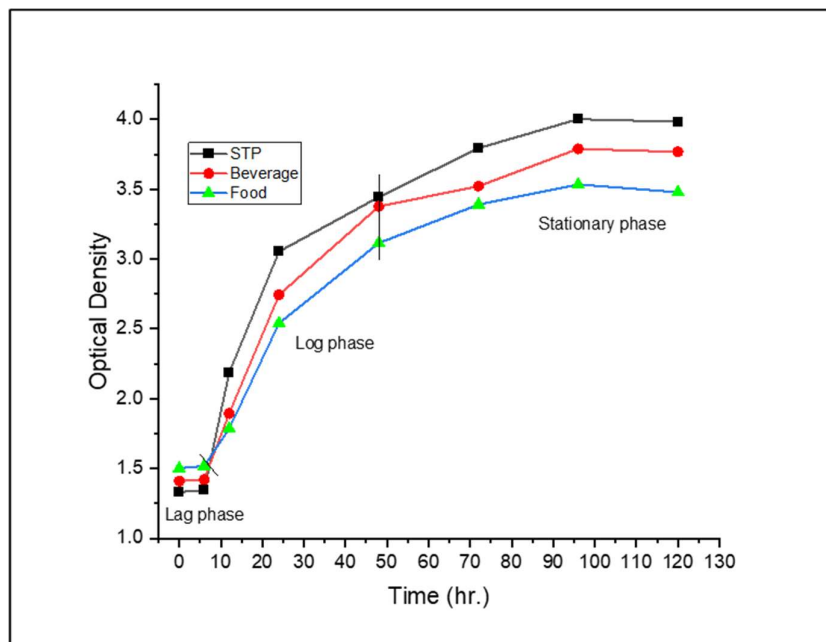

Fig. 3: Bacterial growth phases of three different isolated sludge.

treatment can enrich anaerobic microbes that can produce hydrogen at high temperatures and inhibit other bacteria (Lin et al. 2006, Li & Fang 2007). The maximum biohydrogen was reported at 96th h of fermentation in STP mixed consortia with substrate utilization of 86.72% (Fig. 4). Biohydrogen production ceased after 96 h of fermentation, potentially due to the inhibitory effects of compounds formed during hydrolysis (Srivastava et al. 2017).

Fig. 4 illustrates that the lag phase time (120th h) was prolonged in FS and BS due to decreased bacterial activity of mixed consortia, which resulted in a lower hydrogen yield and substrate utilization rate as compared to STP bacterial consortia. Yang and Wang (2019) studied biohydrogen production by co-fermentation of sewage sludge and grass residue. It was found the efficiency of hydrogen fermentation improved at optimized substrate concentration (10 g.L^{-1}), and the maximum hydrogen yield and VS removal were 45.6 mL/g-VS$_{added}$ and 13.7%, respectively.

Some soluble metabolites, including ethanol and organic acids (acetate, propionate, butyrate), are frequently produced during the hydrogen fermentation process along with hydrogen (Maru et al. 2016). On the one hand, these metabolites are useful indicators for characterizing the performance of the fermentation process. On the other hand, these soluble metabolites can also cause the inhibition of hydrogen production. Table 3 illustrates soluble metabolites end product profiles. As shown in Table 3, the VFAs composition constituted mainly acetate and butyrate with little production of ethanol. However, the amount of acetate, butyrate, and ethanol varies depending on the type of inoculum employed. In comparison to FS and BS, it was observed that STP microbial consortia comprised maximum VFAs as acetate (1025.71 mg.L^{-1}), butyrate (453.41 mg.L^{-1}), and ethanol (80.19 mg.L^{-1}).

The maximum bacterial growth was observed in STP's sludge inoculation which represents that the STP mixed consortia have maximum capability to degrade the lignocellulosic residue as compared to FS and BS. Further, biohydrogen production potential was also reported maximum in STP bacterial inoculation.

CONCLUSIONS

The current study emphasizes the practical value of utilizing rice straw as a feasible raw material for biohydrogen production through the dark fermentation process. The efficiency of the alkali pretreatment method to break down the lignocellulosic complex structure was confirmed through chemical characterization and SEM and FTIR analysis. Mixed microbial culture plays a significant role in biohydrogen production due to their ability to interact. Mixed cultures can utilize a wider range of substrates, which can increase the efficiency of biohydrogen production. Each microorganism can contribute its unique set of enzymes and metabolic pathways to convert different substrates into hydrogen. Furthermore, maximum cumulative biohydrogen production and high hydrogen yield were observed in
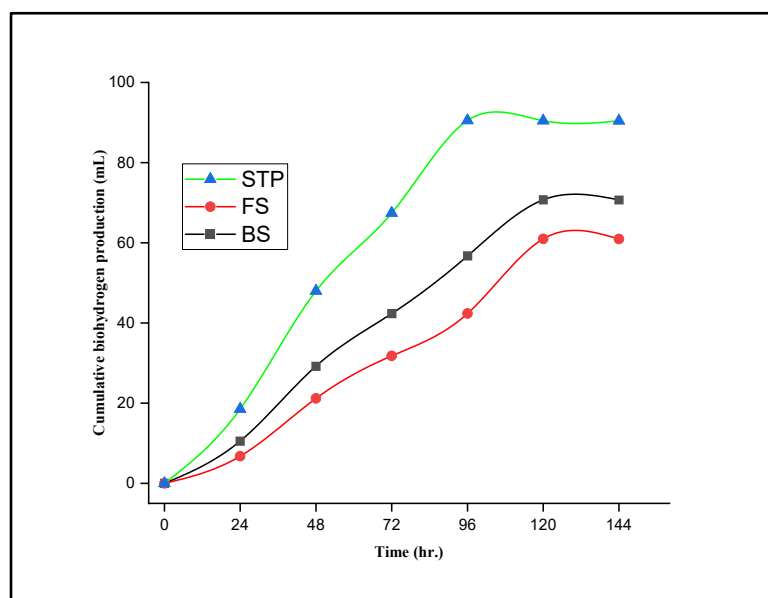

Fig. 4: Cumulative biohydrogen production during fermentation of alkali RS by three seed sludge (STP, FS, BS).

Table 3: Biohydrogen production performance and fermentation end products from alkali pretreated RS (10 g.L^{-1}) using various mixed consortia as seed at initial pH 7.0 and temperature of 37°C.

Seed sludge	Final pH	Cumulative H$_2$ [mL]	Hydrogen Yield (mole H$_2$/mole)	Substrate Utilization [%]	Acetate [mg.L^{-1}]	Butyrate [mg.L^{-1}]	Ethanol [mg.L^{-1}]
STP	5.42	90.52	0.75	86.72	1025.71	453.41	80.19
BS	5.67	70.74	0.67	82.07	796.83	348.73	64.39
FS	5.82	60.98	0.56	79.28	614.88	292.81	53.75

sewage treatment plant sludge inoculation as compared with beverage and food sludge. This study showed that mesophilic dark fermentation of alkali-pretreated rice straw with mixed cultures could increase hydrogen yield, hence improving the possibility of biohydrogen production from lignified agricultural residues.

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