Whole Cell of Pure Clostridium Butyricum CBT-1 from Anaerobic Bioreactor Effectively Hydrolyze Agro-Food Waste into Biohydrogen

TAWAF ALI SHAH (tawafbiotech@yahoo.com)
Shandong University of Technology - West Campus: Shandong University of Technology
https://orcid.org/0000-0002-5030-4729

Li Zhihe
Shandong University of Technology - West Campus: Shandong University of Technology

Li Zhiyu
Shandong University of Technology - West Campus: Shandong University of Technology

Andong Zhang
Shandong University of Technology - West Campus: Shandong University of Technology

Di Lu
Shandong University of Technology - West Campus: Shandong University of Technology

Wang Fang
Shandong University of Technology - West Campus: Shandong University of Technology

Han Xuan
Shandong University of Technology - West Campus: Shandong University of Technology

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Abstract

Recycling organic waste and converting them into renewable energy is a promising route for cleaning environment and effective industrial reactions. This study targeted to isolate a pure anaerobic culture with potential to hydrolyze different biomass and bio-H_2 production. For this, a sample of full-scale anaerobic digester, fed with a mixture of biomass was inoculated on Reinforced Clostridial Medium (RCM) in strict anaerobic conditions. An anaerobic *Clostridium Butyricum* CBT-1 strain was isolated, identified from morphological and 16S rRNA sequence. The strain expressed amylase, cellulase and peroxidases activities. CBT-1 efficiently showed fast growth rate of 3.10 OD/600nm after 72 hours incubation time on Azure B and crystal violet dyes. The strain exhibited 82.4% and 78.5% decolorization of Azure B and crystal violet dyes respectively. In batch fermentation experiment, the CBT-1 produced highest of 3.06, 2.67 and 2.46 mol/mole H_2 yield from glucose, starch and cellulose respectively. Whereas, the CBT-1 showed low 0.43 mol/mole from untreated rice straw, and comparatively high H_2 yield of 1.91 and 2.01 mol/mole rice straw hydrolysate and kitchen waste respectively. The cumulative volumetric yield of H_2 was 358.15, 300.8 and 294.5NmL/gSub from glucose, starch and cellulose respectively. Similarly, the cumulative H_2 volume was 76.7, 184.4, 237.2 NmL/gVS from untreated rice straw, rice straw hydrolysate and kitchen food waste. This study emphasizes the prospects to find similar robust anaerobic culture for hydrolyzing complex biomass. Such strains could be used as standard co-inoculum for bio-H_2 and biocatalyst for commercial scale applications.

1. Introduction

Energy is the basic human need for daily activities. Currently, the world relying on energy of petroleum based fuels generated from fossil sources. The problems with petroleum fuels are releasing of carbon dioxide and increasing of global warming. Further, the fossil sources are depleting quite rapidly and the cost of petroleum based fuels are increasing globally (Roy and Das 2016). To control the price hike and environmental pollution, alternative energy sources have been put on the target (Shah 2017). Renewable energy from cheap carbon sources, robust product specific strains and new bioprocess technological are the possible options (Shah et al. 2018a, Shehbaz 2018). The agricultural waste biomass (grasses, straw) and kitchen waste could be utilize (Wong et al. 2016). The kitchen waste contain mostly, fruits and vegetables, which are easily digestible for the microorganisms (Rodríguez-Valderrama et al. 2020). However, grasses and waste straw needs some pretreatment either chemical, physical or biological to for easy fermentation process (Shah 2018). The reason of pretreatment is to remove the lignin, from waste straw, which acts as a barrier in fermentation process. Lignin removal speed up fermentation and increase the yield from the substrate per gram (Ali et al. 2020). Ideally, this treatment should be nominal and mild to lower the cost and effective for the depolymerization for complex biomass (Shah et al. 2018a). Thus dilute chemicals treatment (H_2SO_4 acid, NaOH alkali) process can be choose as an inexpensive pretreatment approach (Shah and Tabassum 2018, Shah et al. 2019).

Nature is full of wonderful microorganisms (Shah et al. 2018b, Shah 2019), it only needs to be explore for the screening of target specific bioproduct (Shah et al. 2016b, Aimen et al. 2020). Microorganisms are sources for production of various value added metabolites. In fact, a microbial cell is functioning as a factory, which synthesized different kinds of useful secondary materials. Bacterial species are predominant among other microorganisms with enormous potential of biomolecules production. Previously, we reported pure *Bacillus sp.* strains capable of conversion of Municipal Organic Food Waste into biohydrogen.

This study further emphasized to screen out more anaerobic fermentative bacteria, which can convert multiple waste biomass into fuels. Anaerobic digestion and anaerobic granular sludge are reported with a wide range of archaea, bacteria and fungi producing hydrogen and methane gas. In the list of these anaerobic microorganisms, *Bacillus sp.* strains and *Clostridium sp.* strains are the significant culture inside anaerobic digestion (Collet et al. 2004, Winter 2005). Other culture like enterobacters, *Aeromonas, Pseudomonas*, *Streptomyces* and several more isolates are reported (Oh et al. 2003). However, these cultures are either low for H_2 potential or non H_2 producing. Additionally, these microbes either unable to grow even on simple carbon beside complex biomasses. That does not determine them ideal for H_2 production and high scale processing. Any strain capable of converting multiple substrates like starch, cellulose, straw and food waste simultaneously, could be the best culture for commercial applications (Kothari et al. 2010, Hay et al. 2013).

Clostridium butyricum is gram positive, spore forming and one of the highest anaerobic bacterium existing in anaerobic digestion processes. *Clostridium butyricum* is reported with capabilities to ferment various polysaccharides (Cai et al. 2011, Cai et al. 2013). It can produce a variety of products like acetic acids, butyric acids, solvents like glycerol, butanol and gases (H_2). However, many *Clostridium* strains have been isolated, but only few of them are found effective in fermentation of complex polysaccharides carbohydrates (Cai et al. 2011, Cai et al. 2013). This study focused on bio-H_2 production from a pure anaerobic strain capable of hydrolyzing rice straw hydrolysate and kitchen food waste. To get this objective, a granular sludge was collected from an anaerobic reactor actively operational on straw and food waste biomasses. The sample of this anaerobic reactor was screened to isolate the most active and vital *Clostridium* strains that have the capacity to hydrolyze various biomass including rice straw hydrolysate and kitchen waste. Successfully, *Clostridium butyricum* CBT-1 strain is purified and demonstrated capabilities of waste biomass hydrolysis. This is our first effort towards purification of *Clostridium butyricum* CBT-1 for bio-H_2 production. It can be stretched to explore more industrial biochemicals of *Clostridium butyricum* strains. The study emphasize for improvement in batch fermentation process and development of standardized co-inoculum of pure anaerobic *Clostridium* strains capable of bio-H_2 gas production. The experiments were designed as showed in graphical figure below.

2. Material And Method

2.1 Material Preparation

Most of the chemicals tested in this study were of Sigma Aldrich (Germany). An overnight dried rice straw was grounded by grinding machine to a fine powder of < 0.5 cm size. The rice straw was measured as 40.0% C, 0.95% N, and 4.1% H. The VS and TS composition was 85.3% and 81.5% of the rice straw respectively, representing a high organic matter. The kitchen organic food waste was collected from the University Cafeteria. The unwanted materials like plastic bags, papers, glass and mud were removed. A homogeneous mixture is prepared and the kitchen organic waste samples is stored at 4^{o}C. Rice straw hydrolysate (RSH) was prepared with 1% NaOH at 121°C for 15 min autoclaving. After alkali treatment, the rice straw was subjected to another pretreatment step, this time with 1% H_2SO_4 at 121°C for 15 min autoclaving. The RSH was filtered, washed with water and dried at room temperature (Minu et al. 2012). The untreated rice straw and rice straw hydrolysate samples were bound with gold palladium on black carbon tape. These samples of untreated rice straw and rice straw hydrolysate were photographed by scanning electron microscope (SEM). SEM images were taken with magnification resolution of 1000 um to see the disrupted fibrous structure of untreated rice straw and rice straw hydrolysate after alkali and acid treatment (Qu et al. 2017).

2.2 Isolation Process, Reinforced Clostridial Medium (RCM)

Anaerobic granular sludge sample was collected in a bucket from an anaerobic reactor fed with organic municipal waste and straw residue after proper mixing to get a homogenized sample. From this bucket, 100 mL samples in triplicates were taken and sealed tightly in 250 mL anaerobic bottles. The 250 mL bottles were kept at 80°C for 3 hr. A 100 mL of Reinforced Clostridial Medium (RCM) compose of meat extract 0.1g/100 mL, starch 0.1g/100 mL, yeast extract 0.3g/100 mL, glucose 0.5g/100 mL, peptones 0.1g/100 mL, sodium acetate 0.3g/100 mL, sodium chloride 0.5g/100 mL, L-cysteinium chloride 0.3g/100 mL, and agar 0.5g/100 mL was sterilized in autoclaved at 121°C for 15 minutes. The RCM bottles were inoculated with 20 mL volume from each granular sludge sample after heat treatment. The RCM bottles were flushed with nitrogen gas for 4 minutes and incubated in the thermal anaerobic chamber at 37°C temperature. After 72hr of growth, fresh sterile RCM broth of 100 mL volume was inoculated with 5 mL from each pregrown RCM bottles. The RCM bottles were incubated again in the thermal anaerobic chamber at 37°C temperature. This time, after 72 hr, the grown culture in RCM bottles was serially diluted in normal saline and 100 uL of culture inoculum from each dilution was spreaded in to sterile RCM agar plates. RCM plates were incubated in strict anaerobic conditions (placed inside an anaerobic chamber equipped with 10% H_2, 5% CO_2 and 85% N_2 gas mixture) at temperature of 37°C. After incubation time for 48 hr culture growth was checked. From the growth culture, a pure single colony was streaked in to fresh nutrient agar plate. RCM media was used for long-term culture growth and spore generation. The pure culture cells was stored at refrigerator temperature (4°C) in sterile semisolid RCM broth tubes and glycerol tubes separately.

2.3 Colony Morphology and Enzyme Qualitative Assay

Spores were picked from the RCM agar plate and activated at 60°C for 15 minutes. Slide smear was prepared. Gram staining was performed to check the morphology of the pure culture under microscope. Also on fresh RCM and nutrient agar plates media, the macroscopic morphology like color, shape, and texture of the colony was observed. Further identification was done by performing biochemical tests using procedure of Bergey's Manual of Systematic Bacteriology. The pure culture was subjected to qualitative enzymatic study for cellulase and amylase using standard plate method. Pure culture was grown in RCM broth at 37°C, 120 rpm for 24 hr. A 100 µL of cells volume from RCM broth was diluted in 0.9% NaCL. A 3 µL sample of diluted cell suspension was freckled onto media plates in triplicates. For cellulase 5 g/L carboxymethylcellulose (CMC), and for amylase 20 g/L starch was added respectively. The agar plates of CMC and starch were incubated at 37°C for 72 h. The enzyme activity was checked by flooding gram iodine solution for amylase test and 0.1% Congo red solution for cellulase test. The plates were left on room temperature for 30 minutes followed by washing with 1 M NaCl. The zone of hydrolysis on both starch and CMC plates were recorded (Shah et al. 2016b).

2.4 Growth and Degradation Potential of Dyes

CBT-1 pure culture was grown in RCM broth at 37°C, 120 rpm for 24 hr. A 100 µL of freshly grown CBT-1 cells volume from RCM broth was added into sterilized 50 mL bottles of mineral salt media (MSM). The MSM was supplemented with 0.5g/L Azure B and 0.5g/L crystal violet dye in separate bottles as defined previously (Picart et al. 2016, Ravi et al. 2017). Uninoculated media bottles of Azure B dye and Crystal violet dye were used as control media samples. The bottles were kept in shaking incubator conditions of 37°C and 150 rpm for seven days. Daily 2 mL sample was taken to check absorbance at 620nm under UV-visible spectrophotometer for growth observation. Similarly, 5 mL sample before start of experiment and 5 mL samples after completion of seven days experiment were taken. The samples were centrifuged at 10000 rmp for 10 minutes for each sample bottle. The absorbance of supernatant was measured at 651nm for Azure B dye and 592nm for crystal violet dye decolorization, respectively (Picart et al. 2016). The amount of decolorization for both dyes was calculated using the following equation as:

$$\text{Decolorization Percentage} = \frac{X_i - X_f}{X_i} \times 100 \qquad \text{(Eq.1)}$$

Where X_i = initial absorbance at first day

X_f = final absorbance at last day

2.5 Polymerase Chain Reaction (PCR) of 16S rRNA Gene

A pure colony was picked from RCM agar plate and was grown in sterile Luria Broth (LB Oxiod pH 0.6) at 37°C, 120 rpm for 24 hr. A 2 mL of cells volume from LB broth was centrifuged at 10000rpm for 15 minutes and the supernatant was discarded from the cells pellet. The cells pellet was treated with 300 µL TE Buffer (Tris HCl 10 mM, EDTA 1mM, 1 M NaCl, pH 8.0). A 100 µL of 10% SDS was added to the tubes. The cells pellet was heated at 80°C for 30 minutes. Proteinase K buffer in 1M Tris HCl of 200 µL was

added and kept in water bath at 50°C for 60 minutes. A 50µL of 20g RNase was added at room temperature for 60 minutes. Then 200 µL of 6M-NaCl solution and chilled absolute ethanol was added and centrifuged at 10,000rpm for 10 minutes. The supernatant was transferred to other eppendorfs and chilled 1000 µL phenol-chloroform-iso amylalcohol was added again centrifuged at 10000rpm for 10 minutes. The upper most supernatant was wash with 70% chilled ethanol. The cells pellet is dissolved in 100 µL TE buffer. A 1% agarose gel was prepared and the DNA was loaded in 0.5x solution of TBE. The agarose was heated in microwave oven for 1 minute and cooled up to 45°C. A 0.3µg/mL ethidium bromide (Roche, Germany) was added for staining. Electrophoresis was carried out for 1 hour at 80 V. The DNA bands were visualized in UV Transilluminator (UVItec, EEC) and digital photograph was taken. The extracted DNA was then kept at -20 ºC for PCR reaction. The 16S rRNA region was amplified with forward primer FD1 (5′CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG3′) and reverse primer RD1 primer (5′CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC3′). Taq DNA polymerase (Fermentas, USA) of 1.5 µL, 1.5µL of 50ng genomic DNA, 15µL 10x PCR Super mix, 1.5µL from (25ng/µL) forward primer and reverse primer were added. PCR buffer water of 11.5µL were added to PCR tubes and gently vortexed for 5 seconds. PCR conditions were set as such : initial denaturation of one time at 94°C for 2 min, 94°C for 1 minute in each cycle, extension at 72°C for 1 minute and annealing at 55°C for 1 minute is processed for 30 PCR cycles. The PCR product was cleaned with QIAquick PCR Purification Kit (Qiagen, MD, USA). The amplified PCR sample was sequenced. The raw sequence of PCR was filtered through the sequence analysis package (DNA-Star). Fasta sequence of 16S rRNA was searched through NCBI Basic Local Alignment Search Tool (BLAST) for genetically similar species strains. Phylogenetic tree was constructed using MEGA7 software(Shah et al. 2019).

2.6 Batch Fermentation for Biohydrogen Potential (BFBP) from Glucose, Starch and Cellulose

Glucose, starch and cellulose 15 g/L was added into sodium phosphate buffer (SPB) of pH 6.5 supplemented with K_2HPO_4 (2.5 g/L), (2.5 g/L) $NaHCO_3$ solution, 2 mL of vitamin solution in 250 mL Pyrex bottles. The volume of media was kept to 100 mL equally in all Pyrex bottle. The pH was balanced at 6.5 for each Pyrex bottle. Then media was autoclaved for 15 min at 121° C. Pure colony of CBT-1 was anaerobically grown in sterilized LB broth at 37°C overnight. The media Pyrex bottles containing 15 g/L glucose, starch and cellulose separately were inoculated. A starting value of CBT-1 with 0.3nm optical density (600 nm) was equally added into all triplicates bottles. Control (uninoculated) media Pyrex bottles in triplicates containing 15 g/L glucose, starch and cellulose without culture inoculation were run in parallel at the same conditions. All media Pyrex bottles were closed using a silicon plug. Anaerobic conditions were adjusted by N_2 gas flushing for 4 minutes in all experimental bottles. The BFBP experimental Pyrex bottles were incubated at 37°C in a thermostatic chamber at static condition for 14 days. The daily volume of biohydrogen produced was measured using water displacement process. A 25% acidified (pH < 3) NaCL solution of 0.5 Liter flask was prepared to record the volume of daily gas released from the headspace of each Pyrex bottle. The amount of water move in graduated cylinder is

correspondingly equal to the amount of gas released from the headspace of each Pyrex bottle. The hydrogen, carbon dioxide composition were measured by Gas chromatography (micro-GC Varian 490GC).

2.7 BFBP from Organic Food Waste and Rice Straw Hydrolysate

After simple substrates (cellulose, glucose and starch) BFBP confirmation experiments, the culture of CBT-1 was assessed for capability of bioH_2 production from complex substrates. The CBT-1 was freshly grown in 100 mL Pyrex bottle of LB medium at 37°C, for 48hr in a thermostatic incubator. Sodium phosphate buffer (SPB) of pH 6.0 supplemented with K_2HPO_4 (2.5 g/L), (2.5 g/L) $NaHCO_3$ solution, 2 mL of vitamin solution, and 15 g/L rice straw hydrlysate, 15 g/L untreated rice straw and 15 g/L VS of kitchen food waste were prepared for media. A total of 100 mL media volume was kept in each 250 mL Pyrex bottle. Control Pyrex bottles added with untreated rice straw, rice straw hydrolysate and kitchen food waste were run in parallel. All Pyrex bottles were autoclaved for 15 min at 121°C. A 0.3nm optical density of CBT-1 was inoculated in the rice straw hydrlysate, untreated rice straw and kitchen food waste Pyrex bottles. The control Pyrex bottles were left uninoculated. Each Pyrex bottle was flushed for 5 min with N_2 gas. All the samples were managed in triplicate. The batch fermentation was run for 14 days at static condition by incubating in 37°C thermostatic chamber. The daily volume of biohydrogen produced was measured using water displacement process as described above in 2.6 section.

2.8 Kinetic Calculations

The kinetic for hydrogen production rate and yield was measured as did earlier in our study (Shah et al. 2016a).The volume of gas produced in the control Pyrex bottle was subtracted from the gas volume in inoculated Pyrex bottle to calculate actual gas yield. The Microsoft Excel program was used to calculate volume of hydrogen (H_2), volume of headspace, concentration of H_2 at time t and t-1. Whereas, concentration (X) of total H_2 volume at time t and the specific H_2 concentration at time t and t-1. The daily H_2 volume raw data was normalized at standard temperature and pressure (STP). The cumulative H_2 volume of each substrate in mL/gVS and mol/mol yield was mathematically calculated using the equation Eq-2 from the daily (H_2) volume and concentrations.

$$H2\,Vol.\,t = XH2,\,GH2\,V,\,t + HSV.\,(XH2,\,t - XH2,\,t-1) \quad \text{(Eq-2)}$$

Total volume of H_2 in mL = $H_{2\,Vol}$, t,

Concentration of H_2 = XH_2

Gas volume of H_2 each time = G H_2V, t

Headspace volume = HSV

The H_2 yield was calculated as shown in the equation Eq-3.

$$H2\ (Y) = \frac{Cumulative\ H2}{Weight\ of\ substrate}$$ (Eq-3)

Where H_2 yield (Y) = measured as mL/gVS as substrate load was based on VS to each Pyrex bottle in BHFP.

The H_2 yield (Y) mol H_2/g glucose was measured by equation Eq. 4.

$$H2(y) = \frac{Cumulative\ H2}{\frac{22.4}{\frac{Weight\ of\ glucose}{180}}}$$ (Eq-4)

where ,

1 mole of ideal gas at standard temperature and pressure (STP) = 22.4 L/mol volume

1 mole of glucose = 180 g/mol

The estimated kinetic of total hydrogen potential was measure by modified Gompertz Model (Eq. 5) by Statistical software (IBM SPSS Statistic 23) through nonlinear regression model for each sample separately and the values of P, R and L were calculated.

$$Y(t) = P \times exp\left\{ - exp\left[\frac{R_* e}{p(\Delta - t)} \right] (L - t) + 1 \right\}$$ (Eq. 5)

Where Y (t) is total yield of hydrogen (mL) in total time of incubation (t), P is the hydrogen production (mL), R is highest rate of production (mL/d), and L is the lag phase time in days (d), e is equal to 2.718282.

2.9 Analytical methods

Rice straw and kitchen food waste was analyzed for carbohydrate composition. Total solids (TS), volatile solids (VS), ash, moisture, carbon content, nitrogen, lignin, glucan, xylose, galactose, mannose and arabinose were measured as described in the standard laboratory analytical procedure (LAP) (Sluiter et al. 2013). The TS measurement was calculated by taking 1 gram of rice straw and kitchen food waste, oven dried at 105°C overnight in a crucible. The weight of oven dried rice straw and kitchen food waste was measured again and TS value was calculated using this Eq. 6:

$$TS\ (\%) = \frac{WSD - WD}{DS - WD}$$ (Eq. 6)

Where, WSD = Weight of dried residue + dish, WD = Weight of dish, DS = Dish + substrate.

The VS of rice straw and kitchen food waste was calculated by burning the oven dried rice straw and kitchen food waste samples at 550°C for 30 minutes. The samples of rice straw and kitchen food waste

were cooled down in a desiccator at room temperature. The difference in measured weight was found using Eq. 7:

$$VS\ (\%) = \frac{WDR - WA}{DS - WA} \quad \text{(Eq. 7)}$$

Where: *WDR* = Weight of dried residue + dish, WA = Weight of ash, DS = Dish + substrate.

Rice straw sample was acid treated using the National Renewable Energy Laboratory (NREL)'s analytical method. Carbohydrate monomers (glucan, xylose, galactose, mannose and arabinose) were measured by HPLC (Shimadzu, SPD-MZ0A). Samples before fermentation and post-fermentation experiments of glucose, cellulose, starch, rice straw, rice straw hydrolysate and kitchen food waste were collected for VFA analysis. For carbohydrates detection, a standard solution of (H_20 and methanol) were run as carrier at a speed reaction of 0.6 mL/min and 80°C. The filtered samples before and after completion of batch fermentation assay, were treated with phosphoric acid (H_3PO_4). The samples were run in HPLC (C18 column, mobile phase 1:1) parallel to standard concentrations of ethanol, methanol, *n*-butyric, propionic, acetic, and valaric acid (Shah et al. 2016a).

3. Results And Discussion

3.1 Selection of Targeted Strain

A sample from anaerobic reactor was grown on RCM to enriched only anaerobic culture. Out of four different culture isolates, only one colony was picked that showed qualitative expression for peroxidases, amylase and cellulase potential. The DNA was extracted from pure colony of this isolate named as CBT-1. Primers FD1 and RD1 were used to amplify the 16S rRNA gene by PCR. From PCR sequence and NCBI Blast result, 16S rRNA sequence of the pure CBT-1 culture showed 99.1% homology with *Clostridium butyricum* strains. The sequence of CBT-1 has been assigned with accession number OM698377 in NCBI Gene submission programme. The similarity pattern of this strain was made through MEGA 7.0 by neighbor joining method as showed in phylogenetic tree (Fig-1). The CBT-1 strain demonstrated its homology with other anaerobic *Clostridium* strains as well.

3.2 Characterization of the Strain

A total of four different pure microbial colonies were picked from the RCM agar plates based on morphology differences. These colonies were screened for peroxidases, amylases, and cellulases activities by qualitative hydrolysis of Azure B dye, Crystal Violet dye, starch, and cellulose as sole carbon source respectively. Out of four cell colonies, only the CBT-1 isolate showed growth potential and decolorization of Azure B and Crystal violet dyes. CBT-1 maximum growth was observed at 4th day of incubation time. The growth-started decline after 6th day as showed in (Fig. 2). The growth pattern of CBT-1 is similar to previously reported studies of bacterial culture on similar dyes (Chandra et al. 2007, Abd-Elsalam and El-Hanafy 2009).

In Fig. 3, (A,B) images it was found that the CBT-1 strain is a Gram positive, rod shaped, spore forming, and mesophilic anaerobic culture. Colony of the CBT-1 was convex, lobate and off white on LB agar medium at 37°C for 48 hr. In the gram staining (Fig. 3, A) slide smear, its cells appeared as straight rods occurring in single or pairs (Fig. 3). The CBT-1 also showed zone of hydrolysis on both CMC and starch plate, indicated positive for its enzyme activities (amylases and cellulases) as shown in the Fig-3. In blue medium (C) in Fig. 3, a zone around colonies showed starch hydrolyzing activity. Similarly, in reddish medium (D) in Fig. 3, a zone around colonies showed existence of cellulolytic activity. The ability of the CBT-1 strain to grow on starch and CMC as a carbon source and formation of zone around colonies confirmed that they could metabolize it into product. Also can hydrolyse other biomass containing cellulase and starch composition. The CBT-1 growth ability confirmed its degradation efficiency of both dyes. It was found 82.4% decolorization of Azure B dye and 78.5% of crystal violet dye after seven days of incubation respectively (Fig. 3). Previously, we also observed decolorization of Azure B dye using pure culture of *Bacillus* sp. strains (Shah 2014). These pure cultures of *Bacillus* sp. strains were producing peroxidase (ligninases, laccase). The investigation of dyes degradation potential was for the validation of enzymes system and expression of ligninases, laccase, and plate assay of CMC for cellulases, and starch for amylase. This strategy helps in confirmation of lignocellulosic and kitchen food waste digestion for bio H_2 production. This strain was selected for further analysis.

3.3 Substrate Composition

Rice straw and kitchen waste was analyzed for its compositional analysis. The rice straw was containing 20.2% lignin, total organic carbon 51.5%, total nitrogen 0.55%, total solid 92.5% and volatile solid 85%. Whereas, the kitchen waste was containing 5.2% lignin, total organic carbon 45.7%, total nitrogen 1.5%, total solid 16.5% and volatile solid 78.6%. The detail composition of substrates shown in the (Table 1). The compositions of rice straw is consisted with earlier described results (Shah et al. 2019).

Table 1
Composition of Substrate in Percentage

Parameters	Rice Straw	Rice Straw Hydrolysate	Kitchen Food Waste
Moister	5.5	6.2	70.5
Ash	9.3	5.5	12.3
Glucan	41.5	38.6	8.1
Xylan	21.2	17.5	6.4
Arabinan	2.1	1.0	0.3
Galactan	1.3	0.5	NP
Manan	NP	NP	NP
Lignin	20.2	4.5	5.2
Total solid	92.5	83.0	16.5
Volatile solid	85.0	80.4	78.6
TOC	51.5	49.2	45.7
TN	0.55	0.35	1.5
(NP = not present, TN = total nitrogen, TOC = total organic carbon)			

Further, the rice straw was treated with acid and NaOH. The effect of treatment was checked for the untreated rice straw, NaOH treated rice straw and after both treatment of 1% NaOH and 1%H_2SO_4 acid. The SEM images make it obvious that the untreated rice straw micrograph was smooth, flat, and very compact structure (Fig-4-A). After the NaOH hydrolysis, the compact structure of rice straw was degraded (Fig-4-B). The impact was even more severe after both NaOH and H_2SO_4 acid treatment. This could be due the removal of basic barrier element lignin from the surface of rice straw along with hemicellulose component, showed in (Fig-4-C). The SEM images of Fig-4 clearly revealed breaks in the silicon waxy morphology of the rice straw. Therefore, the rice straw hydrolysate was containing less lignin then as rice straw. Similar changes in surface destruction of the lignocellulosic biomass due to pretreatment is also reported (Zeng et al. 2011).

3.4 Daily and Cumulative Hydrogen Yield

The *Clostridium* species consist of huge number of extracellular enzymatic system (cellulosomes) necessary for hydrolysis of complex biomass. Several *Clostridium* species are reported for utilization of pure carbohydrates (i.e., sucrose, glucose, and galactose), polysaccharides enriched municipal waste and lignocellulosic biomass comprise of hemicelluloses and celluloses. The efficient utilization of

lignocellulosic biomass require a pretreatment step to convert the substrate into sugar units for easy uptake and fast fermentation process (Latifi et al. 2019). This study first confirm the extracellular enzymes (peroxidase, cellulases, and amylases) and then tested for biohydrogen production from pure carbohydrates. Rice straw hydrolysate was included, which could be an encouraging step towards the use of plant biomass for the synthesis of bioH_2 and other valuable products.

As per objective of the study, the culture of *Clostridium Butyricum* CBT-1 was first evaluated for H_2 production from glucose, cellulose and starch. It was found that the *Clostridium Butyricum* CBT-1 released H_2 with similar yields from starch and cellulose, whereas slightly different in case of glucose as carbon source. Glucose gives comparatively higher yield than cellulose and starch. The *Clostridium Butyricum* CBT-1 produced H_2 yield of 3.06, 2.67 and 2.46 mol/mole of consumed glucose, cellulose and starch respectively. Likewise, *Clostridium Butyricum* CBT-1 produced H_2 yield of 0.43, 1.91, 2.01 mol-H_2/mol of consumed untreated straw, rice straw hydrolysate and KFWS respectively. Similarly, the daily volumetric rate of H_2 production from glucose to starch and cellulose was noted. The daily highest H_2 volumetric rate was 48 mL H_2/d from glucose, 43.3 mL H_2/d from starch and 41.5 mL H_2/d from cellulose respectively. Similarly, the cumulative volume of H_2 potential was 358.15 NmlH_2/substrate from glucose, 300.8 NmlH_2/substrate from starch and 294.5 NmlH_2/substrate from cellulose respectively as showed in (Fig. 5). The hydrogen yield of this study is in line with previous reported H_2 yield from similar substrates like glucose, starch, and cellulose (Xing et al. 2009). Recombinant *C. thermocellum* DSM 1313 is reported for H_2 production from cellulose and hemicellulose substrates, which suggested that pure culture, can use plant waste straw for value added products (Xiong et al. 2018). A co-culture of *E. aerogenes* and *C. butyricum* showed a 2mol H2/mol glucose production rate from starch waste and corn (Yokoi et al. 1998). Whereas, the highest rate H2 yield of 3-3.8 mol H2/mol hexose, have been observed from *Pyrococcus furiosus, Thermotoga spp*, and *Thermoanaerobacterium spp* (Verhaart et al. 2010).

The (Fig. 6) illustrates the daily and cumulative H_2 production from untreated rice straw, rice straw hydrolysate and kitchen food waste. The daily highest H_2 volume was 11 NmL/gVS from untreated rice straw, 30.2 NmL/gVS from rice straw hydrolysate and 34.1 NmL/gVS from kitchen food waste. The maximum cumulative volume of H_2 produced was only 76.7 NmL/gVS from untreated rice straw, 184.4 NmL/gVS from rice straw hydrolysate and 237.2 NmL/gVS from kitchen food waste respectively.

No methane gas was found from the batch fermentation of all the substrates (glucose, starch, cellulose, rice straw and kitchen food waste fermentation experiments. The concentration of H_2 detected in GC was in the range 25–40% for the tested substrates. The outcomes of the present study are comparable to previously reported cumulative H_2 yield from similar biomass and carbon carbohydrate saccharides (Xing et al. 2009, Shah et al. 2016b).

3.5 Advantages and Comparison of Pure culture for Hydrogen Production

In this study, the CBT-1 strain total hydrogen yield was compared as showed in (Fig. 7). It was found that he highest H_2 yield was exhibited 393.18 NmL/gVS from glucose. Whereas, the cellulose and starch comparatively produced similar H_2 yield was exhibited 314.18 and 325.62 NmL/gVS respectively. The untreated rice straw, rice straw hydrolysate and Kitchen food waste showed 78.4, 197.4 and 263.3 NmL/gVS H_2 yield respectively as showed in Table 2. In (Fig. 8), a comparison of the experimental cumulative H_2 volume and theoretical cumulative volume from glucose in 14 days is shown. The experimental H_2 volume is relatively similar to theoretically estimated H_2 volume of the glucose. Similar H_2 volume was observed for other substrates too (data not showed). The overall results of *Clostridium Butyricum* CBT-1 strain are notable, because only few studies reported biohydrogen potential from a pure culture. However, the capability of H_2 potential from lignocellulosic biomass (rice straw and rice straw hydrolysate in this study) is more interesting. The H_2 yield from rice straw by *Clostridium Butyricum* CBT-1 is higher than $Ca(OH)_2$ and acid treated biomass straw as reported previously (Nasirian et al. 2011, Reilly et al. 2014). The chemicals treatment are used to enhanced the hydrogen yield from waste biomass (Reginatto and Antônio 2015). As a 68.1 ml H_2/g TVS cumulative H_2 yield was obtained from HCl treated agriculture straw (Fan et al. 2006). Similarly, from untreated cornstalk waste a 61.4 mL/g of cumulative H_2 yield was observed using *Clostridium thermocellum* as a single culture. Even, *Clostridium thermocellum* and *Clostridium thermosaccharolyticum* co-culture produced 75 mL of H_2/g H_2 yield (Li and Liu 2012, Li et al. 2012). This is noteworthy, that most of the studies either used mix microorganism of anaerobic sludge as inoculum or expensive chemothermal pretreatments. As, H_2 production potential of *Clostridium Saccharolyticus* from wheat straw hydrolysate treated at 130°C for 30 min has been reported (Ivanova et al. 2009).

In addition, the hydrogen yield of *Clostridium Butyricum* CBT-1 is higher than previously reported studies. The application and H_2 yield of *Clostridium Butyricum* CBT-1 proved that pure active and biomass degrading culture can avoid the necessities of expensive chemicals pretreatment of waste straw carbon sources. *Clostridium Butyricum* CBT-1 showed overall 357.15, 293.5, 299.8, 184.4 and 236.2% more hydrogen yield compared to untreated rice straw sample. In our previous study, pure ligninolytic culture *Brevibacillus agri* AN-3 showed 293.7% increase of hydrogen yield from wheat straw compared to untreated wheat straw sample (Shah et al. 2018a). The bioH_2 production is directly proportional to the concentration of VFA consumed during anaerobic digestion process. The level of VFA produced and consumed reflected the performance of substrates fermentation assay and elucidate the H_2 metabolic pathways (Luo et al. 2019). In this study, no VFAs concentrations were detected at the completion of glucose, cellulose and starch fermentation assay, signifying the total consumption of VFA to products by the tested *Clostridium Butyricum* CBT-1. However, in sample of rice straw hydrolysate and kitchen food waste, a considerable level of VFA were detected. The higher concentration was of butyrate, iso-butyrate, acetate, and propionate. The detection of butyrate and acetate VFA production in *Clostridium Butyricum* CBT-1 proved it ideal candidate for bioH_2 production. Similar results of VFA (butyrate and acetate) is reported in the H_2 producing microorganisms specifying similar H_2 catabolic pathways (Shin et al. 2004, Fang et al. 2006). The leftover VFA and slurry after anaerobic fermentation process is a rich source of

beneficial nutrients for plants, crops and vegetables growth. Which can be use as an organic fertilzers to enhance their growth and yield (Hamid et al. 2021). The overall results of this study highlight the importance of isolation of pure culture capable of different biomass hydrolysis and fermentation into value added metabolites. This strategy can be used to collect specific strains for pure bioproducts as well as a biocatalyst for hydrolysis of complex biomass at large scale.

Table 2
Cumulative yield of hydrogen (NmL/gVS) estimated through modified Gompertz Model.

	P	L	Rmax	R^2
Glucose	393.18	1.69	40.6	0.997
Cellulose	314.79	1.606	37.24	0.998
Starch	325.62	1.602	36.32	0.998
KFWS	263.3	2.23	29.7	0.997
RS Hydrolysate	197.4	1.85	25.5	0.998
Untreated RS	78.4	4.01	10.6	0.989

4. Conclusion

In this study, *Clostridium Butyricum* CBT-1 strain was isolated and purified that was capable of expressing amylase, cellulase and peroxidases enzymes. These enzymes activities were determined from a significant (82.4 and 78.5%) amount of decolorization of azure B and crystal violet dyes. In addition, the strain showed considerably high H_2 potential from glucose, starch, cellulose, KFWS and rice straw hydrolysate. Especially, the *Clostridium Butyricum* CBT-1 cumulative H_2 yield was significant from rice straw hydrolysate and kitchen food waste. It produced 180–240% more H_2 volume from rice straw hydrolysate and kitchen food waste compared to the untreated rice straw. These results indicates a promising line for enhancing H_2 yield of pure anaerobic culture from organic waste biomasses. Although, culture conditions optimization may also play an important role to uplift the yield of specific product from similar anaerobic culture.

Declarations

Authors contribution

Tawaf Ali Shah did investigation, conceptualization, experiments, writing and manuscript preparation, L Zhiyu, and Andong Zhang helped in writing and manuscript preparation Li Zhihe provide supervision and editing, Di Lu, did results and samples analysis; Wang Fan and H. Xuan did samples analysis, equipment and resources management.

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Data availability

The authors confirm that the data supporting the findings of this study are available within the article.

Ethics approval and consent to participate

This study does not involve any humans or animals during experimentation, so it is not applicable in this study.

Consent for publication

This study does not contain data from any individual person.

Competing interests

The authors declare no competing interests.

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Figures

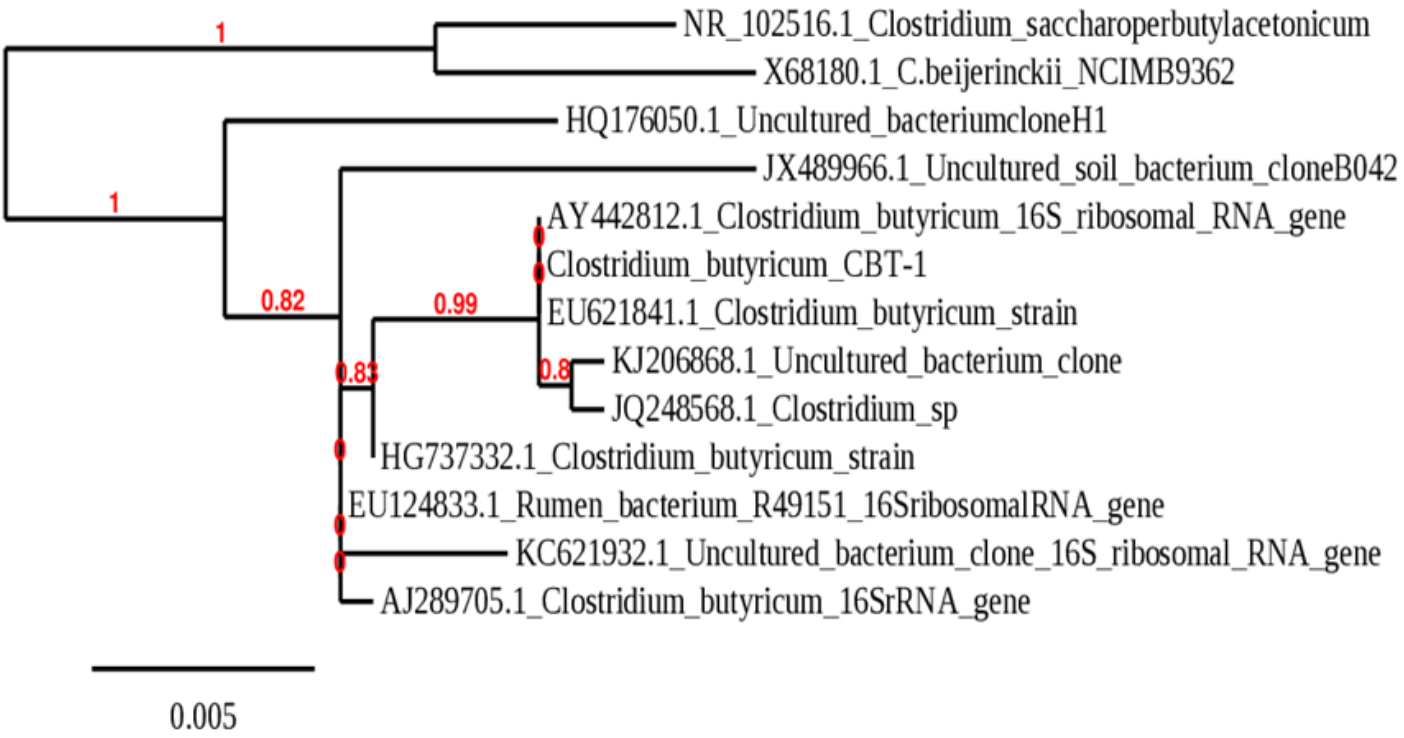

Figure 1

Similarity report of *Clostridium butyricum* CBT-1 with other similar *Clostridium butyricum* strains and bacterial culture based on 16S rRNA phylogeny

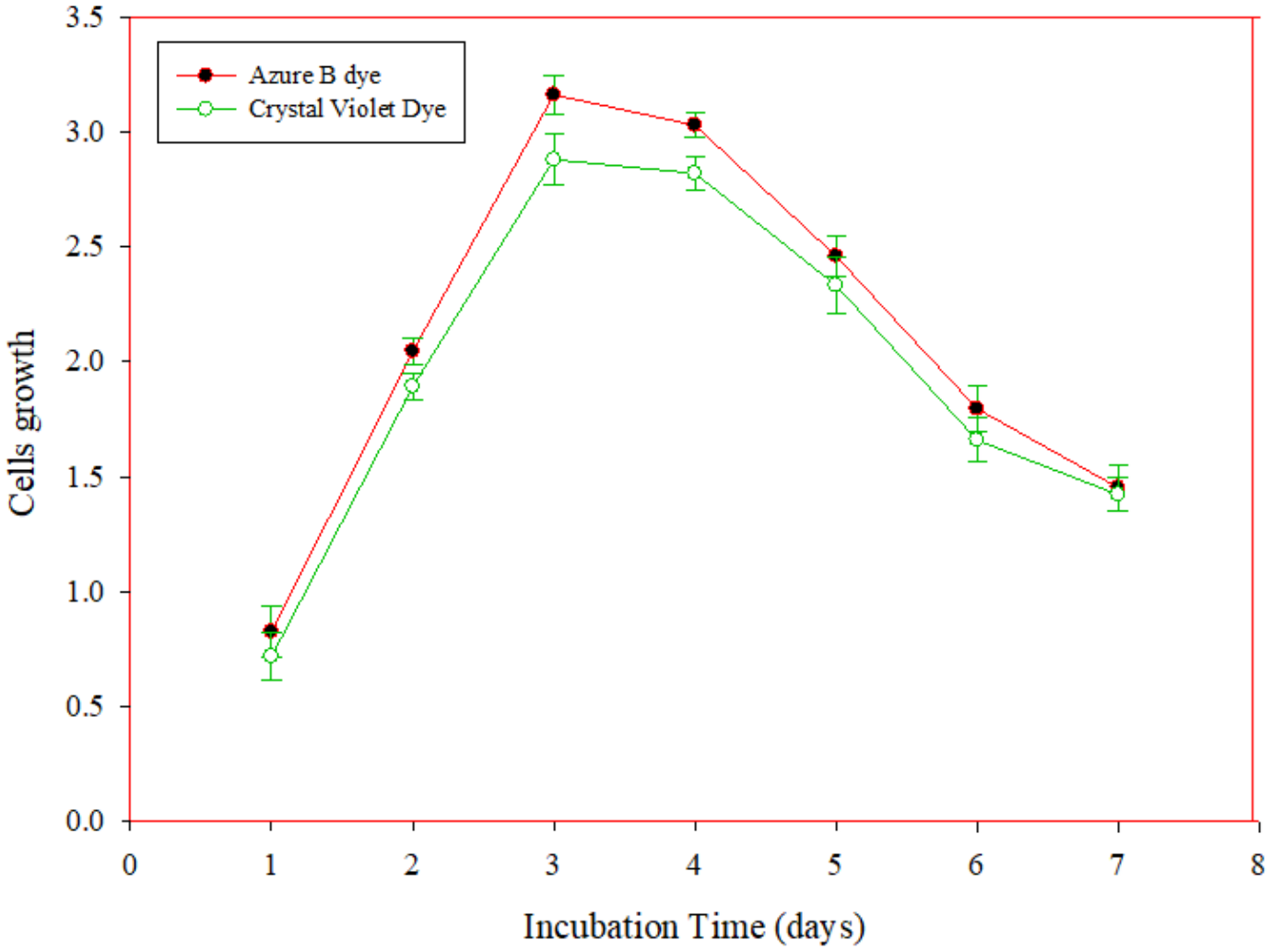

Fig. 2 CBT-1 growth potential on Azure B and Crystal violet dye .

Figure 2

See image above for figure legend

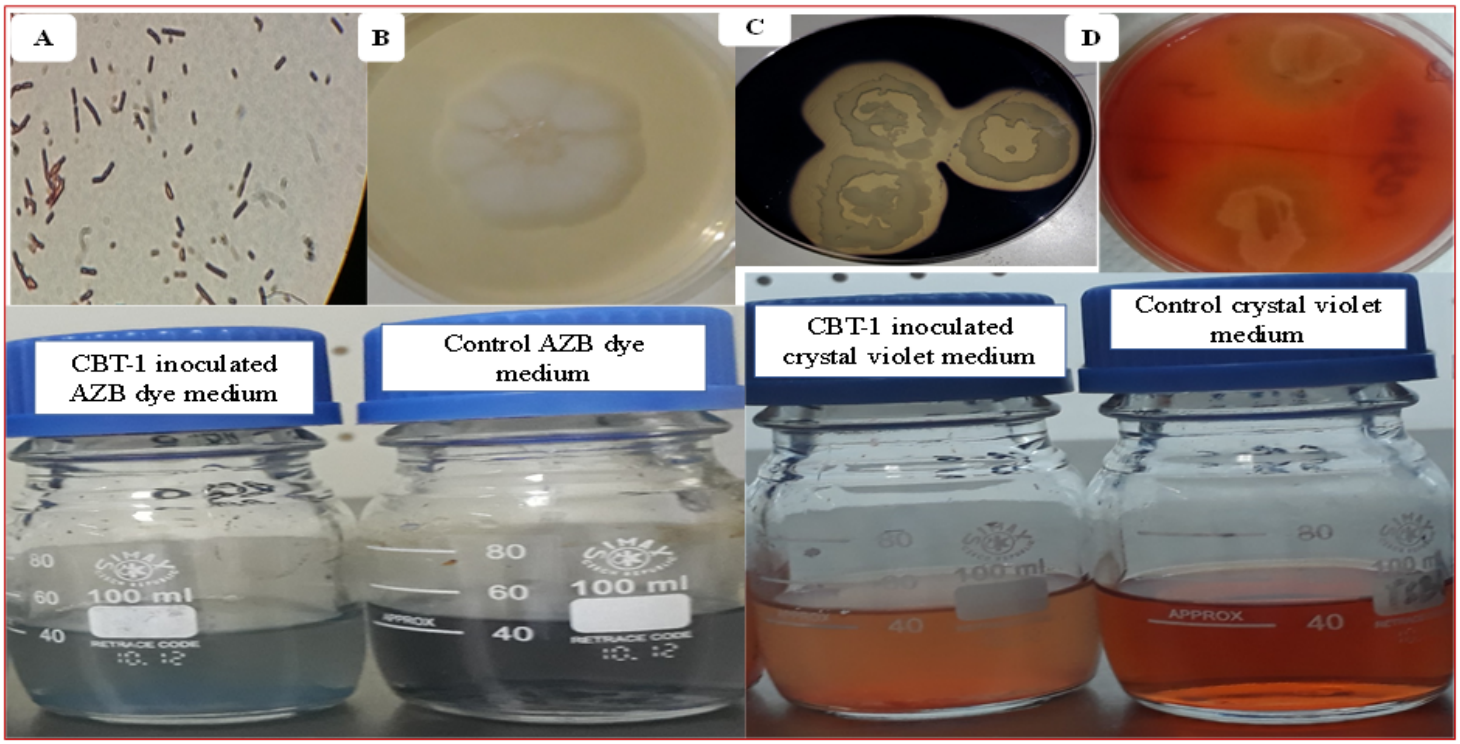

Figure 3

In the above figure, A= showed gram positive rod shape cells, B= white lobe shape cells, C= indicate positive for amylase on starch agar plate flooded with iodine solution, D = indicate cellulase positive on CMC plate, Azure B dye inoculated with CBT-1 strain culture, crystal violet dye inoculated with CBT-1 and their control medium

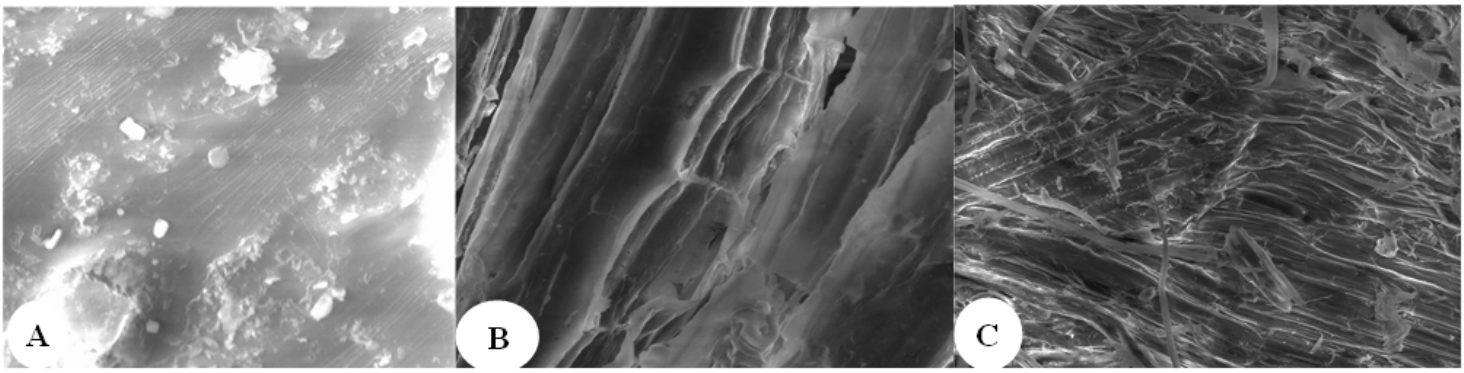

Figure 4

SEM images of rice straw, A= control untreated rice straw, B= 1% NaOH treated, C = both 1%NaOH and 1%H_2SO_4 acid treated image of rice straw

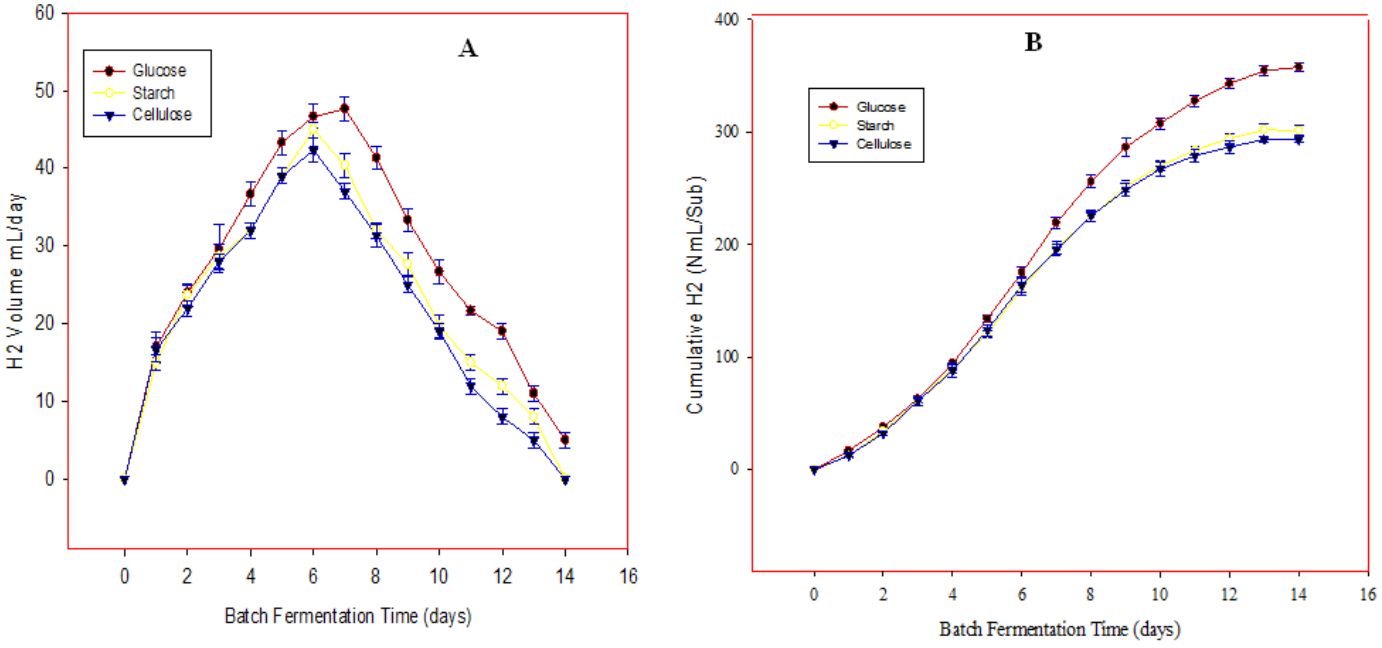

Fig. 5 Daily H2 yield from glucose, starch and cellulose and cumulative H2 yield from glucose, starch and cellulose (B) respectively

Figure 5

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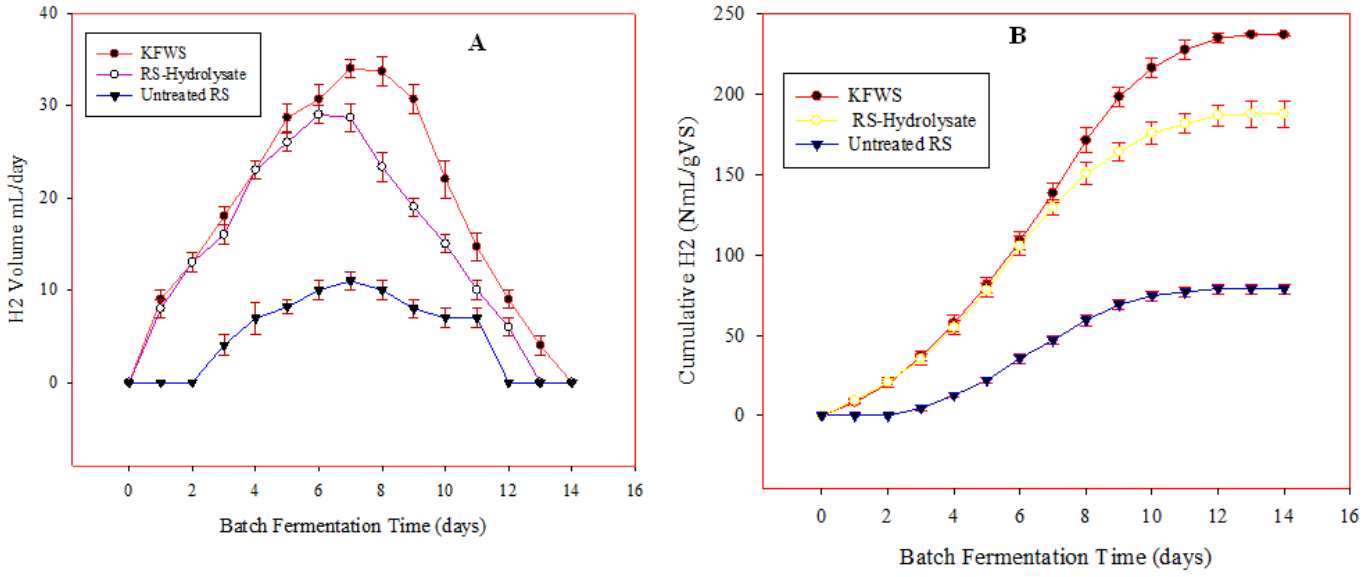

Fig. 6 Daily H2 yield from untreated RS, RS hydrolysate and KFWS (A), and cumulative H2 yield from untreated RS, RS hydrolysate and KFWS (B) respectively

Figure 6

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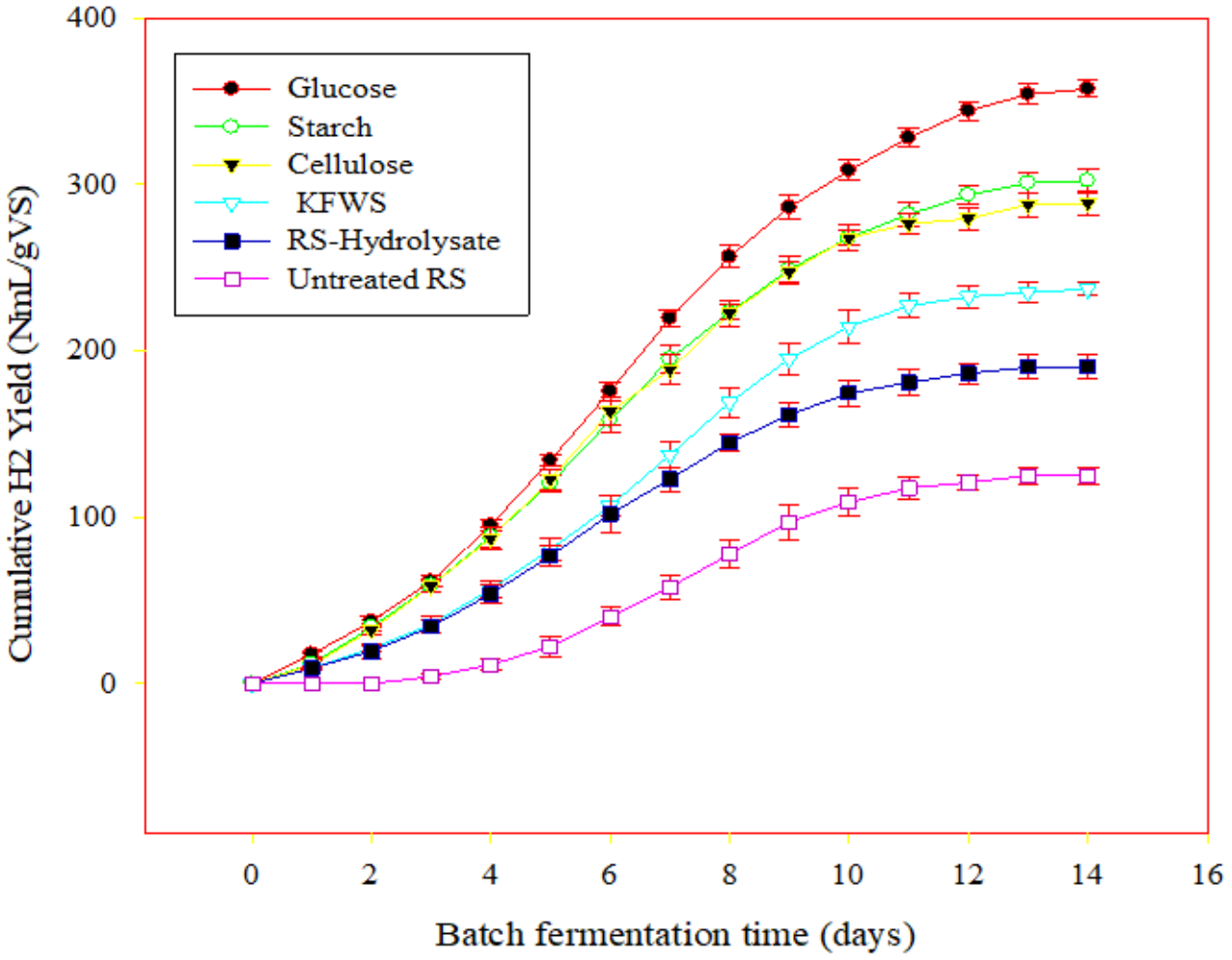

Fig. 7 Comparison of cumulative H_2 yield from glucose , starch , cellulose , KFWS , RS hydrolysate and untreated RS respectively

Figure 7

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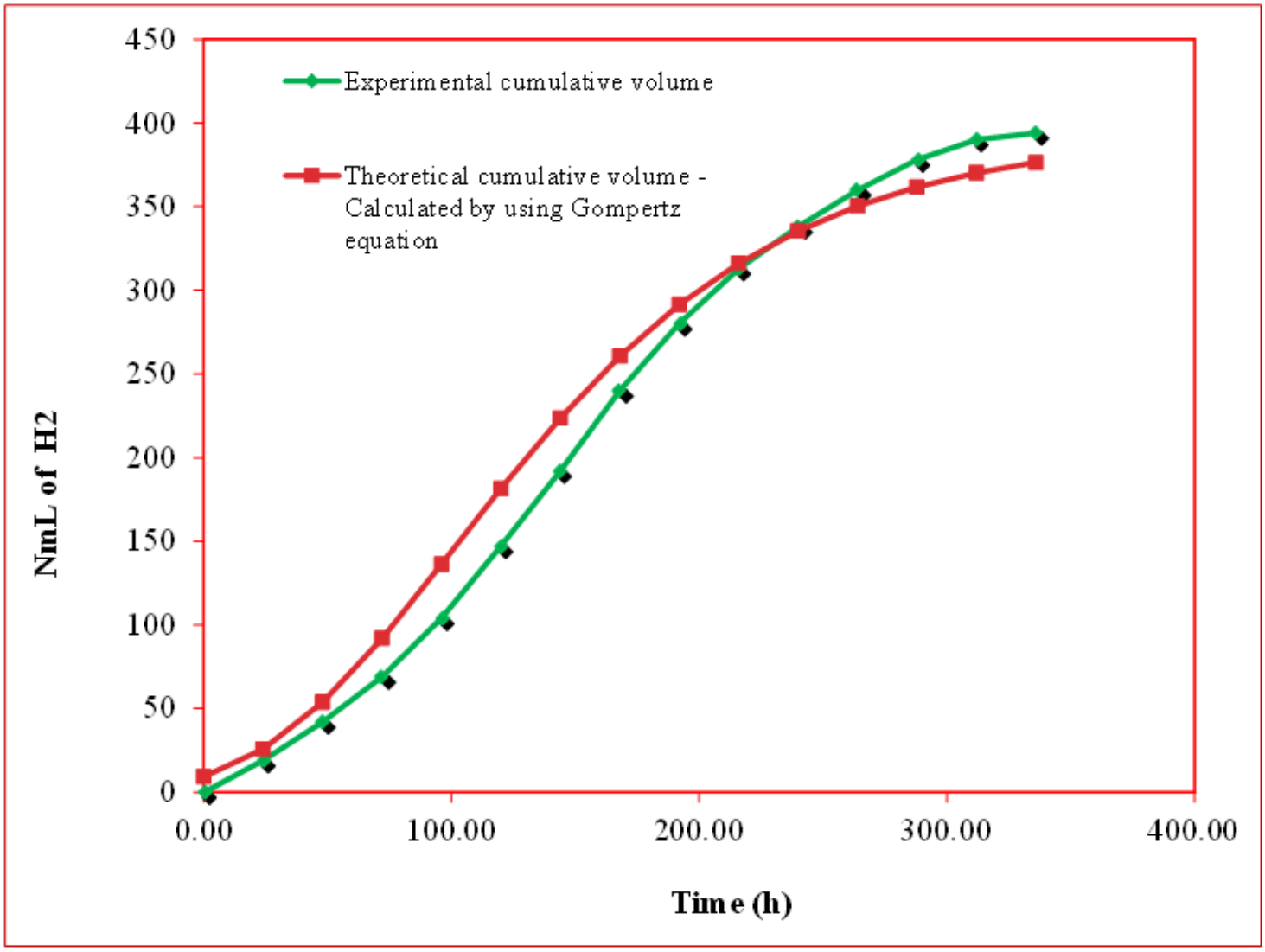

Figure 8

Comparison of experimental cumulative H_2 volume and theoretical cumulative volume from glucose in 14 days represented in hours yield

Supplementary Files

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