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Biohydrogen production from sugarcane bagasse by integrating dark- and photo-fermentation

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HIGHLIGHTS

- Acid and enzyme pretreated SCB was utilized to produce H_2.
- Effective removal of fermentation inhibitors by adsorbent resin.
- SSFF and two-step fermentation was effectively utilized for H_2 production.

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ABSTRACT

Hydrogen production from sugarcane bagasse (SCB) by integrating dark-fermentation by *Enterobacter aerogenes* MTCC 2822 and photo-fermentation by *Rhodopseudomonas* BHU 01 was investigated. The SCB was hydrolysed by sulphuric acid and the hydrolysate detoxified by passing through adsorbent resin column (Amberlite XAD-4) to remove the inhibitory furfural, and subjected to dark-fermentation. The cellulosic residue from acid hydrolysis was hydrolysed by the new isolate *Cellulomonas fimi* to release sugars for H_2 production by *E. aerogenes*, through simultaneous saccharification, filtration and fermentation (SSFF). Cumulative H_2 production during dark-fermentation and SSFF was 1000 and 613 ml/L, respectively. The spent media of dark-fermentation and SSFF were utilized for photo-fermentation by *Rhodopseudomonas* BHU 01. The cumulative H_2 production was 755 ml/L for dark-fermentation and 351 ml/L for SSFF spent medium.

1. Introduction

Hydrogen, a promising alternative to fossil fuels and a clean energy carrier (Das and Veziroglu, 2001), is produced biologically via biophotolysis, dark-fermentation and photo-fermentation. Sugars such as glucose, fructose, galactose, arabinose, lactose and sucrose are the widely studied substrates for biohydrogen production (Kumar and Das, 2000; Lin and Jo, 2003; Rai et al., 2012). As the cost of the substrate is of prime concern for the economics of biohydrogen production, there is need to go for cheaper and abundant feedstocks for making the process cost-effective. The use of organic wastes from industries and agriculture not only supports green energy generation but also helps in bioremediation (Venkata Mohan, 2009). A large fraction of wastes from municipal, industrial and agricultural sector comprising lignocellulosic biomass, is renewable and inexpensive, and thus well suited for biofuel production. One of the major lignocellulosic material found in large quantities especially in a tropical country like India, is sugarcane bagasse (SCB) produced during cane milling (Singh et al., 2007).

The bagasse is composed of cellulose (40–45%), hemicellulose (30–35%), lignin (20–30%). In general, 1 metric ton of sugarcane generates 280 kg of bagasse, about 50% of this residue is used in sugar mills/distilleries as the energy source while the remaining is composted or used in paper industry (Peng et al., 2009). Cellulose in bagasse is a highly ordered molecule consisting of linear insoluble polymers of repeating β-1, 4 linked β-D glucopyranose units. The main problem in the use of cellulose is its crystalline structure that makes it difficult to hydrolyze. This necessitates use of novel techniques to achieve optimum hydrolysis and the extraction of fermentable sugars from the SCB cellulose. Hydrogen production from lignocellulosic feedstock involves pre-treatment by acid/alkali or by other means to open up its crystalline structure, followed by enzymatic hydrolysis of cellulose and hemicellulose(s) to yield sugars that can be utilized for fermentative H_2 production (Panagiotopoulos et al., 2013a). For enzymatic hydrolysis of the lignocellulosic material, most studies used the expensive commercial enzymes which add to the cost of fermentation and thus limit commercialization of cellulose-based hydrogen production (Stork et al., 2009). The enzymatic hydrolysis and fermentation can be

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carried out by various approaches but with certain compromises and drawbacks. However, a relatively new approach involving simultaneous saccharification filtration and fermentation (SSFF) seems advantageous and alternative to most of other methods as the enzyme inhibition and compromise between the optimum conditions for the enzymatic reactions and the fermenting organism are avoided. In addition, it also facilitates reuse of the fermenting organism (Ishola et al., 2013).

There are several studies related to biohydrogen production from SCB in single stage fermentation (Lo et al., 2008; Pattra et al., 2008; Lo et al., 2009; Fangkum and Reungsang, 2011), but none on the two-step fermentation process (dark- and photo-fermentation). Also, most studies used either SCB acid hydrolysate or SCB cellulose alone, but none together. The present study deals with H_2 production from SCB in a two-step process using both, SCB acid hydrolysate and SCB cellulose. SCB was hydrolyzed with dilute sulfuric acid, and the hydrolysate collected to recover the sugars released for dark-fermentative H_2 production by *Enterobacter aerogenes* MTCC 2822. The cellulosic residue left over following acid hydrolysis, was simultaneously hydrolysed by newly isolated cellulose degrading *Cellulomonas fimi* ATCC 484 to release sugars for dark-fermentation by *E. aerogenes* using simultaneous saccharification filtration and fermentation (SSFF) approach. In addition, the VFAs-rich spent medium generated following dark-fermentation and SSFF was further processed for photo-fermentative H_2 generation by *Rhodopseudomonas* BHU 01 strain.

2. Methods

2.1. Acid hydrolysis of sugarcane bagasse

SCB collected from the local cane crushers was sun dried (3 d) and chopped to 2–5 mm size prior to chemical/microbial pretreatment to increase the surface area. The chopped SCB pieces were soaked separately in dilute sulphuric acid of varying concentrations (0.5–4%, v/v) in the solid to liquid mass ratio of 1:15 and autoclaved (121 °C, 15 lb/inch^2, 1 h) as suggested by Fangkum and Reungsang (2011). The solid and liquid fractions were separated through filtration. The solid fraction was washed thrice with deionized water and pressed to remove the inhibitory compounds formed during acid pretreatment. The liquid fraction was maintained at pH 6.8.

2.2. Isolation and molecular characterization of cellulose degrading bacterial strain

The general methodology adopted was that of Gupta et al. (2012). Samples were collected from cow dung composting sites at different spots, serially diluted (10^{-6}) in sterile phosphate buffer saline (PBS: 10 mM KCl, 150 mM NaCl, pH 7.4) and inoculated in the broth medium containing 1% carboxy methyl cellulose (CMC), 1% peptone, 0.24% $(NH_4)_2SO_4$, 0.20% K_2HPO_4 and 0.03% $MgSO_4.7H_2O$ followed by incubation for 7 d in an incubator shaker (30 °C, 100 rpm). The resultant broth was spread over CMC agar plates containing (per litre) K_2HPO_4 0.5 g, $MgSO_4.7H_2O$ 0.25 g, CMC 2.0 g, agar 15 g (pH 6.8–7.2) to isolate the bacterial clones capable of utilizing cellulose as the sole carbon source.

The bacterial isolates were further purified by transferring individual clones to the fresh CMC plates. For cellulase activity, the isolates were streaked on CMC agar and incubated at 30 °C (48 h). Following incubation, the plates were flooded with Congo red (1%, w/v; 20 min.) and thoroughly washed with 1 M NaCl solution. A clear zone around the growing colonies against the dark-red background, ascertained the cellulase +ve clones. Colonies showing Congo red discoloration were taken as the cellulase +ve clones, and adopted for further study. For molecular characterization of cellulase +ve strain, genomic DNA was extracted using the method of Sambrook and Russell (2001), precipitated with chilled ethanol, washed with 70% ethanol, dried and finally dissolved in TE buffer (pH 8) and stored at −20 °C until use. Amplification of 16S rDNA was performed using the primers reported by Yin et al. (2010). Amplified products were resolved on 1.5% agarose gel, excised, and processed for documentation. The amplified products were purified with the SV-Gel PCR purification system (Promega). Sequencing of amplified 16S rDNA was performed by double pass sequencing with the same primers as above by ABI Prism Big dye terminator V 3.1 cycle sequencing kit (Applied Biosystems, USA) under the following temperature profile: initial denaturation at 96 °C (1 min.) followed by 25 cycles consisting of denaturation at 96 °C (30 s), annealing at 55 °C (5 s) and elongation at 60 °C (4 min.). For sequencing, the amplified sample was purified and run in 3130 XL Genetic Analyzer Automated DNA sequence (Applied Biosystems, USA). A comparison of the sequence with the homologous strains in Gene Bank was performed using the Basic Local Alignment Search Tool (BLAST).

The microscopic and Gram-staining studies of the newly isolated bacterial strain indicated it to be Gram −ve and rod shaped. The bacterium was able to grow well at 30 °C under microaerobic condition, thus is a mesophilic and facultative anaerobe. The BLAST of 16S rDNA sequence on NCBI, indicated 100% similarity with *C. fimi* ATCC 484 (Accession No. NR074509). Hence, the isolated bacterium was designated as *C. fimi* ATCC 484.

2.3. Saccharification by C. fimi

For saccharification by *C. fimi*, the bacterium was grown in the medium used for isolation except the CMC being replaced by H_2SO_4 (2%, v/v) hydrolysed SCB. The experiments were carried out in 250 ml Erlenmeyer flasks containing 100 ml medium (pH 7.0) and 10% (w/v) SCB as the carbon source. The medium was autoclaved (121 °C,15 min.), and inoculated with 10 ml (200 μg protein/ml) of overnight grown *C. fimi* cells, incubated at 30 °C in an orbital shaking incubator (100 rpm) for 72 h. Bacterial growth was measured as dry cell weight, and the extent of saccharification as the amount of reducing sugars released during bacterial growth. The bacterial biomass (dry cell wt.) and reducing sugars released, were plotted against time to evaluate the time-dependence of enzymatic hydrolysis of SCB.

2.4. Hydrogen producing bacterial strains and growth conditions

For dark-fermentation, *E. aerogenes* MTCC 2822 (from IMTECH, Chandigarh, India) was used as the volatile fatty acids (VFAs) and H_2 producing bacterium. The bacterium was maintained in the growth medium as prescribed by Microbial Type Culture Collection and Gene Bank, IMTECH, Chandigarh (India) containing (per litre): beef extract, 0.5 g; yeast extract, 1 g; peptone, 5.0 g and NaCl, 0.5 g. The growth medium pH was initially adjusted to 6.8 and the organism grown in dark at 30 °C in a gyratory incubator shaker (200 rpm).The strain was maintained on nutrient agar slants by weekly aseptic transfers.

Rhodopseudomonas BHU 01 was adopted for photo-fermentation of *E. aerogenes* spent medium. The organism was grown photo-heterotrophically in the medium as described by Rai et al. (2012): yeast extract, 0.5 g; DL-malate, 1.87 g; KH_2PO_4, 1.0 g; $MgSO_4.7H_2O$, 0.4 g; NaCl, 0.4 g; sodium succinate, 2.0 g and $CaCl_2$ $2H_2O$, 0.5 g (per litre). NH_4Cl (0.1 g/L) was initially added to anaerobic cultures for initiation of bacterial growth. The organism was grown anoxically at 34 °C in light from a tungsten lamp (8.5 W/m^2) kept at a distance of 30 cm. The initial pH of the growth medium was maintained at 6.8.