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Simultaneous pretreatment and saccharification of bamboo for biobutanol production

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ABSTRACT

With the depletion of the fossil fuels and rising global living standards biofuels research is gaining impetus since it is environmental friendly, sustainable and leads to rural employment generation. In this context, biobutanol presents a feasible option since its energy content is more than that of ethanol and moreover it can be distributed in the existing gasoline distribution pipelines. In the present study, efforts have been made to develop a process for the production of fermentable sugars through simultaneous pretreatment and saccharification. This process is advantageous since it leads to reduction in the overall processing time required for reducing sugars production. Bamboo was chosen as the lignocellulosic which was treated with concoction of laccase extracted from *Pleurotus djamor* and cellulase-xylanase extracted from *Trichoderma reesei*. The potential of these sugars for biobutanol production has been studied using *Clostridium beijerinckii* ATCC 55025-E604. Process parameters governing the system viz., temperature, incubation time, pH, cellulase: laccase ratio in the enzyme cocktail and solid loading were optimized using response surface methodology and the optimum values determined were 54.54 °C, 8 h, 4.92, 8.5 (v/v) and 14.74% (w/v) respectively leading to a reducing sugar content of 503.16 mg/g with 72.44% saccharification efficiency. Sugar fractionation quantified through HPLC revealed that the reducing sugar contained 36.89% glucose, 38.81% cellobiose and 24.29% pentoses (xylose and arabinose). Broth containing reducing sugar was further fermented using *C. beijerinckii* resulting in butanol content of 6.45 g/L.

1. Introduction

Biofuels production from lignocellulosic biomass is a green and effective technology targeting the alleviation of the dependence on fossil fuels for meeting energy demands (IEO, 2010). Among liquid biofuels such as ethanol, butanol and biodiesel, butanol is considered to be a superior biofuel in terms of higher energy content, capability to be utilized solely as a transportation fuel without blending with gasoline and also due to its property to be transported through the existing gasoline pipelines (Lee et al., 2008). Current know-how on biofuels production from lignocellulosic biomass includes sequential processing of the biomass for delignification and saccharification in order to obtain reducing sugar rich broth which is ultimately utilized for fermentation (Jeya et al., 2012). Among these steps, pretreatment is the major hurdle since the recalcitrant lignin layer present within the biomass poses hindrance towards the carbohydratases for efficient hydrolysis of the polysaccharides and thus an efficient delignification would expose more carbohydrates to carbohydratases ultimately leading to efficient biofuels production (Kuila and Banerjee, 2014). Moreover, the cellulases have non-productive binding towards lignin which impedes saccharification efficiency of lignocellulosic biomass (Gao et al., 2014) and hence the requirement for delignification is further substantiated for an effective saccharification process. Conventionally delignification is practiced through physical, chemical, physico-chemical and biological means. Among these options, biological route is the most process friendly considering the fact that there is no production of furfurals and hydroxymethyl furfurals which otherwise inhibits the fermentation step (Yi et al., 2012; Wang et al., 2013).

Sequential processing of the lignocellulosic biomass for reducing sugars production is both energy intensive and time consuming. These issues can be solved by utilizing laccase mediated delignification at mild operating conditions simultaneously with cellulases in order to obtain reducing sugar yield comparable to the sequential processing thereby reducing the overall processing time. Temperature and pH optima's of laccase and cellulases lies within the range

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40–60 °C and 3–7 (Stoilova et al., 2010; Althuri and Banerjee, 2016; Madhavi and Lele, 2009), thus they can be used simultaneously for reducing sugars production. In this study bamboo was chosen as the lignocellulosic biomass for simultaneous pretreatment and saccharification (SPS). Bamboo is a fast-growing perennial evergreen grass which has high holocellulose content (up to 70% of dry weight) and thus can be explored as a promising feedstock for production of biobutanol (Sun, et al., 2015). India is the second largest producer of bamboo in the world with 80.4 MMT (Million Metric Tons) of total growing stock of which 18.0 MMT is harvested annually. Among this reserve, 5.4 million tons of residues are generated of which 3.3 million tons are available as surplus (Sindhu et al., 2014). Considering the huge amount of surplus residue, bamboo is considered to be a potential substrate for studying the simultaneous pretreatment and saccharification process followed by biobutanol production. Butanol is produced in anaerobic fermentation by employing the bacterial strains of clostridium family. Clostridium strains are saccharolytic bacteria, gram positive, spore-forming, obligate anaerobes and have the ability to utilize starch, hexoses, pentoses di- and polysaccharides sugars (Bharathiraja et al., 2017). Clostridium family has several wild strains among which 4 species viz. *Clostridium acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* are major contributor for industrial butanol production (Kumar and Gayen, 2011).

Therefore in this study, an attempt has been made to optimize a process where simultaneous application of enzymes viz., laccase, and cellulases can be performed to yield sugars with an intention to reduce the overall process time. The sugars obtained from this venture have been further used for fermentation using *Clostridium beijerinckii* for butanol production. Bamboo was used to evaluate the scope of the approach since no publications are available on butanol production from this lignocellulosic through the SPS route using *C. beijerinckii*. The effect of solid loading, enzyme ratio, pH, temperature, and incubation time required for sugar yields were studied using in-house laccase and cellulases produced from *Pleurotus djamor* and *Trichoderma reesei* RUT C30 respectively and the feasibility of SPS hydrolysate of bamboo for butanol production was also evaluated using *C. beijerinckii* ATCC 55025-E604.

2. Materials and methods

2.1. Raw material

Bamboo (*Bambusa bambos*) was collected from the campus of IIT Kharagpur (22°31′ N, 87°30′ E), West Bengal, India The biomass was dried at 60 °C till constant weight and then pulverized using a hammer mill fitted with a sieve of 0.2 mm pore size.

2.2. Enzymes

Laccase used in the study was extracted from a solid state fermentation (SSF) of *Pleurotus djamor* (Bhattacharya et al., 2011). Cellulases were extracted from the SSF of *Trichoderma reesei* RUT C30 (Das et al., 2008).

2.3. Laccase and cellulase assay

In order to estimate the activity of laccase, 1 mM solution of 2,2′-azino-bis (3 ethylbenzothiazoline-6-sulphonic acid) (ABTS) prepared in 0.1 M sodium acetate buffer (pH 4.5) was used as the substrate and its oxidation within a reaction mixture was estimated spectrophotometrically at 436 nm over a period of 1 min (Bhattacharya et al., 2011). One IU of laccase activity can be defined as the capability of the enzyme to oxidize 1 μmole of ABTS per minute (ε_{436} = 29300 M^{-1} cm^{-1}).

Cellulase activity was analyzed using 1% (w/v) carboxymethyl cellulose (CMC) prepared in 10 mM sodium acetate buffer, pH 5.0 as the substrate and the reaction was performed at 50 °C for 30 min (Cohen et al., 2005). The progress of the reaction was stopped by the addition of 3 mL 3,5-dinitro salicylic acid (DNS) regent followed by boiling the mixture in a boiling water bath for 15 min. After boiling the reaction mixture, the contents were cooled to room temperature and then its optical density (OD) was measured at 540 nm. One IU of cellulase activity can be defined as the capability of the enzyme to release 1 μmole of glucose per min. The activities of β-glucosidase, FPU and xylanase were assayed using protocols of Zhang et al. (2007), Ghose (1987) and Jeffries et al. (1998).

2.4. Biochemical composition and proximate analysis of the lignocellulosic biomass

In order to probe the composition of bamboo, proximate, biochemical and ultimate analysis was performed. The biomass (as received) was used for moisture and total solids content determination (Sluiter et al., 2008). Dry biomass obtained after moisture analysis was further used for determination of ash content (Singh et al., 2013). Ultimate analysis of the biomass viz., elemental C, H, N, S and O content was conducted using CHNS analyzer (Singh et al., 2013). During CHNSO analysis, dry biomass as obtained after moisture analysis was used. Oxygen content of the biomass was determined by deducting the total of C, H, N and S content from 100 (Althuri and Banerjee, 2016). Amount of extractives within the biomass was estimated by acetone extraction (Yang et al., 2006). Biochemical components of bamboo viz., cellulose and hemicellulose which determines the carbohydrate pool of the lignocellulosic was determined according to “semimicro determination method” and anthrone method respectively (Updegraff, 1969; Marlett and Lee, 2006) while lignin which represents the recalcitrant component of the biomass was estimated by titration method (Rajak and Banerjee, 2015). Energy content of raw bamboo was estimated in a bomb calorimeter (Eastern Instruments, Kolkata, India) by calculating their calorific values (Singh et al., 2013; Rajak and Banerjee, 2015).

2.5. Simultaneous pretreatment and saccharification (SPS)

For conducting SPS experiment, one gram of bamboo having particle size ≤ 0.2 mm was taken in a 50 mL Erlenmeyer flask into which required volume of cellulase: laccase (within the range of 6–10 (v/v)) was added and the reaction was performed at an appropriate temperature (in the range 50–60 °C). During experimentation, solid loading was maintained in the range 10–20% (w/v), pH of the enzymes was maintained in the range 4–6 and incubation time was kept in the range 6–10 h. One variable at a time approach was adopted initially for screening the range of individual parameters conducive for maximum reducing sugar production. These factors were chosen since they have a marked influence on the activity of all the enzymes used in this study. After the requisite incubation time of SPS experimental set, solid liquid separation was done by squeezing the reaction mixture through a muslin cheese cloth followed by centrifugation of the broth at 4000 rpm for 5 min. The supernatant collected was used for estimating reducing sugar content.

2.6. Reducing sugar estimation in SPS broth

The supernatant collected from the broth of SPS experimental set was estimated for reducing sugar content by dinitrosalicylic acid (DNS) method (Miller, 1959). High performance liquid chromatography (HPLC, Agilent 1100 series) equipped with Hi-Plex H column (300 × 7.7 mm and 8 μm) was used for analysing the SPS broth. Sug-