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An eco-friendly process integration for second generation bioethanol production from laccase delignified Kans grass

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ABSTRACT

The field of second generation production process has immense potential to utilize and convert lignocellulosic biomass to biofuels and biochemicals. Among these, enzyme pretreated biomass specifically treated by oxido-reductive group of enzymes is gaining attention towards bioethanol production. Until now research has been focused towards biofuels and biochemicals production based on the physical, chemical and physic-chemical methods. Biological processes on the other hand are eco-friendly and sustainable in nature and are gaining more attention towards biofuels/biochemicals generation. Moreover, biomass feasibility in terms of availability and sugar content is still one of the major challenges in bioenergy sector. The present article emphasizes process integration for bioethanol production by utilizing laccase pretreated lignocellulosic feedstock. In the present study we have attempted to combine the different processes together for bioethanol production and compared it with the single process. The fermentation process has been optimized through response surface methodology that resulted in 63.2 gL^{-1} of ethanol for partial simultaneous saccahrification and fermentation (P-SSF) and 57.91 gL^{-1} of ethanol for simultaneous saccharification and fermentation (SSF) within 25–28 h. The surface area, pore size, and pore volume of the fermented biomass is found to be decreased after SSF and P-SSF that indicates extensive action of enzymes. Microscopic study showed surface distortion of the biomass after fermentation that revealed the effective action of saccahrifying enzymes on biomass during hydrolysis and fermentation. The obtained X-ray diffraction pattern showed the utilization of amorphous and crystalline cellulose which initially increased to 8.03% and thereafter decreased to 23.49%. Thus, the findings obtained in the present study supports the feasibility of the enzymatically pretreated biomass for bioethanol production.

1. Introduction

Based on the potential for partial replacement of petroleum fuels and being a green technology that minimizes greenhouse gas emission, biomass based ethanol production is gaining increasing attention [1,2]. However, when bioethanol production from energy crops such as corn, sugarcane, maize, wheat and sugar beet led to food versus fuel issue in developing countries, lignocellulosic biomass of agricultural origin were considered as a potential source for the production of second generation or 2G ethanol [3]. Non-edible lignocellulosics such as grass, wood, crop residues, bark, saw dust, oil palm fronds etc. [4,5] do not compete with food or fodder resources, and are considered as the best starting material for bioethanol production in developing countries because of seasonal availability and low cost. In India, the total land area available for cultivation of energy crops is about 51.09 Metric hectares which can produce 510 Metric tonnes of Kans grass biomass per year at 10 tonnes per hectare per year (t/ha/year) from the total land available for energy crops cultivation [6]. India, with its huge population, cannot afford to utilize its food resources such as grains or sugars for fuel production and hence, must depend on the renewable lignocellulosic biomass.

Lignocellulosic bioethanol generation comprises three steps: first step involves, biomass pretreatment that helps to remove or degrade lignin and accompanied with increased biomass digestibility, low hemicelluloses loss and slight relative increase in biomass crystallinity [7].

The second step in bioethanol generation comprises the conversion of cellulose polymer and hemicelluose into fermentable sugars such as hexoses and pentoses through hydrolytic enzymes. Fermentation of the hexose and pentose sugars produced after the conversion of cellulose and hemicelluloses constitutes the third and final step of the bioethanol production process. Separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) being the two different modes of fermentation of hexoses and pentoses into ethanol

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were followed after biomass pretreatment [8]. The current production processes of ethanol are associated with some of the limitations that either have direct or indirect impact on the efficiency or yield of the process. The major limitations are lack of efficient biomass pretreatment technology for maximum lignin degradation, loss of hemicelluloses, efficiency and cost of cellulolytic enzymes, cellulase to xylanase cocktail optimization for enhanced hydrolysis [9], and development of yeast strain for co-fermentation of hexose and pentose sugars etc [8].

Simultaneous saccharification and fermentation initially contains celluloytic enzymes, substrate, and yeast cells at determined concentrations [8]. The major limitations existed in the SSF process is the mis-match of the optimum working temperature of the celluloytic enzymes (optimum temperature, 50 °C) and yeast (optimum temperature, 37 °C). Since both the celluloytic enzymes and yeast cells have to survive on a common environment during SSF, it is not possible to maintain the two optimum temperatures at a time. Such type of limitations can be overcome either by developing thermotolerant yeast sustaining at high temperatures or introduction of pre-hydrolysis or saccahrification step before fermentation termed as partial simultaneous saccahrification and fermentation (P-SSF). It is already reported that high temperatures adversely affect the growth and viability of fermenting yeast that indicated more ground research on genetic engineering towards the development of robust thermotolerant microorganisms [10]. The major advantage of partial-saccharification step before fermentation is the release of reducing sugar from delignified biomass before cooling down to optimum fermentation temperature of yeast [1,11]. These types of process modifications led to high degree of saccahrification and subsequently improved ethanol titre with less incubation time making the whole process more efficient.

In our previous work, we have established the conditions for enzymatic delignification that resulted in high reducing sugar during saccharification at low enzyme dose [7]. We extend here the application of enzymatic delignification in terms of utilization of laccase delignified Kans grass for bioethanol production through SSF and P-SSF and also investigated whether partial-saccharification/hydrolysis followed by SSF of delignified biomass can improve the ethanol production as advocated by others. Further, the effects of partial-saccahrification/hydrolysis on sugar release and ethanol production were validated through spectroscopic techniques. There are few reports on bioethanol generation through P-SSF mode. To the best of our knowledge there are no research reports on optimization of the P-SSF for 2G ethanol generation by exploiting laccase delignified substrate. The present work also highlights the eco-friendly manner of biomass conversion to biofuel.

2. Materials and methods

2.1. Substrate

Kans grass (*Saccharum spontaneum*) was collected locally from the Indian Institute of Technology, Kharagpur, India followed by sun drying and pulverization (Pulverizer, Murhopye Scientific Company, Mysore, India) to obtain powder biomass of 0.2 mm size. Enzymatic delignification of Kans grass was carried out in a 50 mL Erlenmeyer conical flask, containing required amount of substrate and enzyme (laccase) under a defined reaction conditions. Samples were taken out after a fixed incubation period followed by solid liquid separation. The oven dried biomass was subsequently used for estimation of residual lignin, cellulose and hemicelluloses respectively. The powder material obtained after delignification contains residual lignin 3.20% (w/w), cellulose 40.02% (w/w) and hemicellulose 24.06% (w/w) [7]. The delignified dried biomass was utilized as starting material for bioethanol production through P-SSF and SSF.

2.2. Biochemical characterization of delignified biomass

Cellulose, hemicellulose and residual lignin content of the delignified biomass were reported according to our previous work [7]. The ethanol and reducing sugar content were estimated by potassium dichromate and dinitrosalicylic acid (DNS) method respectively [12,13].

2.3. Enzyme and microorganism

The delignified biomass was subjected for partial saccharification step catalysed by a concoction of cellulase-xylanase produced from *Trichoderma reesei* Rut-C30 followed by fermentation through *Saccharomyces cerevisiae*. The activity of cellulase and xylanase was estimated following the standard assay protocol [14,15]. The enzyme titre of crude cellulase and xylanase is 30 IU/mL. One unit of enzyme activity (IU/mL) has been defined as the amount of enzyme required to produce 1 μmol of reducing sugar per minute under the assay conditions. *S. cerevisiae* was maintained in the medium containing glucose (2%, w/v) and yeast extract (5%, w/v) at pH 5.2 and 37 °C.

2.4. Simultaneous saccahrification and fermentation of delignified biomass

The SSF of the delignified Kans grass was performed in a 50 mL Erlenmeyer conical flask with required amount of substrate (5–40% (w/v)), cellulolytic enzyme dose (IU/g) and yeast (1–12% (v/v)) under defined reaction conditions. The conical flasks were then covered with parafilm to maintain semi-anaerobic environment for yeast followed by incubation at 37 °C at an interval of 12–96 h. After completion of the fermentation time the broth containing bioethanol was separated from the biomass followed by centrifugation at 2000 rpm for 5 min. The obtained supernatant was used for the estimation of ethanol and reducing sugar.

2.5. Partial hydrolysis or saccharification of delignified biomass

Hydrolysis time and enzyme dose are the two main factors of partial hydrolysis/saccharification step that minimise the temperature mismatch during P-SSF. The delignified biomass hydrolysis/saccahrification time was varied from 1 to 6 h followed by reducing sugar estimation. The reaction was carried out on a 50 mL Erlenmeyer conical flask with required amount of biomass and cellulolytic enzyme at 50 °C. After each 1 h interval, samples were withdrawn and brought down to room temperature to stop the reaction. Reducing sugar was estimated for every 1 h interval. After the hydrolysis time was fixed, cellulase dose was varied from 25 to 150 IU/g under the defined reaction conditions. Samples were withdrawn after the fixed time interval for reducing sugar estimation to identify the minimum dose of cellulase that results into in maximum release of reducing sugar.

2.6. Methodology and optimization of P-SSF of delignified biomass

The P-SSF experiments were performed in a 50 mL Erlenmeyer conical flask that included an enzymatic partial hydrolysis/saccahrification step at 50 °C followed by SSF under defined time interval. The temperature was lowered to 37 °C before commencement of the fermentation and yeast was added. In order to maintain the semi-anaerobic conditions for yeast the flasks were covered with parafilm and incubated at 37 °C for specific incubation time (12–96 h). Thereafter, solid-liquid separation was carried out to extract the broth from the biomass followed by centrifugation (2000 rpm, 5 min). The obtained liquid was used for the analysis of ethanol.

2.7. Optimization and evaluation of SSF and P-SSF

Optimization and evaluation of SSF of the delignified Kans grass was carried out by considering five process parameter namely, solid loading