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Pretreatment optimization of *Sorghum pioneer* biomass for bioethanol production and its scale-up

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HIGHLIGHTS

- Wide range of acid pre-treatments were studied for sorghum hydrolysis.
- Influence of combination of temperatures acid concentrations optimized.
- Statistical model was successfully developed for enhanced biomass hydrolysis.
- Selected yeast use both hexose and pentose for ethanol production.
- Even scale up at 25 L resulted in 96% of ethanol conversion efficiency.

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ABSTRACT

Based on one parameter at a time, saccharification of delignified sorghum biomass by 4% and 70% v/v sulfuric acid resulted in maximum 30.8 and 33.8 g% sugar production from biomass respectively. The Box Behnken Design was applied for further optimization of acid hydrolysis. As a result of the designed experiment 36.3 g% sugar production was achieved when 3% v/v H_2SO_4 treatment given for 60 min at 180 °C. The process was scaled-up to treat 2 kg of biomass. During the screening of yeast cultures, isolate C, MK-I and N were found to be potent ethanol producers from sorghum hydrolyzate. Culture MK-I was the best so used for scale up of ethanol production up to 25 L capacity, which gave a yield of 0.49 g ethanol/g sugar from hydrolyzate obtained from 2 kg of sorghum biomass.

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1. Introduction

Human population is increasing on earth and their basic necessity of life, such as food, fuel and other commodities is also increasing gradually. On the other hand, reserved crude oil, the main source of fuel is depleting and its price is rising. So, there is a need to find out an alternate option for fuel. This has lead to the current focus on bioethanol research as it is a better choice (Matsakas and Christakopoulos, 2013a). Bioethanol from lignocellulosic waste is one of the most studied research topics in present scenario. Advantages of the lignocellulosic biomass are its availability, cost effectiveness, renewable and moreover, that does not have a negative effect on food production (Naseeruddin et al., 2013). Major components of the lignocellulosic biomass are cellulose, hemicellulose and lignin, which are tightly held by β-1, 4 glycosidic bonds of cellulose, strong hydrogen bonds and van der Waals forces between hemicellulose and lignin, which make lignocellulose a highly crystalline polymer (Qiu and Aita, 2013).

Bioethanol production from lignocellulosic biomass is comprised of several processes, which includes pre-treatment, chemical and enzymatic hydrolysis, fermentation, and ethanol recovery by distillation. Pre-treatment serves to break the complex structure of lignocellulose by removing lignin and hemicellulose content of biomass. Pre-treatment of biomass render it with high porosity and low cellulose crystallinity, which triggers the availability of cellulose for enzymatic hydrolysis. Sequential physical, chemical and biological treatments are necessary for the generation of sugars from lignocellulose; but the cost of treatment, setup requirement and methodical developments are the crucial aspects for biomass pre-treatment (McIntosh and Vancov, 2010). World agricultural production is assessed to be about 3.8 billion Mg (Mega gram and 1 Mg = 1000 kg) per year total crop residues, which consisted of 74% cereals, 10% sugar crops, 8% legumes, 5% tubers and 3% of oil crops. India is the second largest producer of sorghum, globally. In the country, the cultivation of sorghum in

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~11 million hectare land leads to 10–11 million tons/annum production with 15 tons stover production per hectare (Sathesh-Prabu and Murugesan, 2011). Sorghum can be easily cultivated, as the crop has low susceptibility to temperature, salinity, alkalinity, drought and low requirements of fertilizer as well as irrigation. Moreover, due to short cultivation time of 3–5 months, it can be easily cultivated between two main crops (Matsakas and Christakopoulos, 2013b). According to Dogaris et al. (2012), sorghum is a potential C4 energy crop as its juice and bagasse, both, can be used as a source of sugars. Initially, acid hydrolysis was considered as a very complex process for plant biomass pre-treatment due to solid substrate and a liquid catalyst (Herrera et al., 2004), but current scenario confirms that acid hydrolysis with a steam explosion to the biomass makes the process feasible for commercialization due to additional hemicelluloses derived sugar (Chen et al., 2013). Currently, *Saccharomyces* sp. is most widely used for production of bioethanol due to its low nutrition requirement, pH and higher sugar tolerance, and higher sugar to ethanol conversion. Kang et al. (2014) has focused on developing new strains with higher temperature tolerance and minimum inhibitor production during fermentation. So, the present study focuses on the generation of high fermentable sugars from the sorghum biomass using combinations of different temperature-acid concentration treatments, and utilization of the obtained sugars from the delignified biomass for ethanol fermentation using various yeasts isolates. Both, pretreatment and ethanol production were also scaled up.

2. Methods

2.1. Collection of biomass and its characterization

Sorghum pioneer biomass were procured regularly from the Animal Nutrition Department of Anand Agricultural University, Anand, Gujarat, India. Harvesting of sorghum was initiated when flowering in 25% of the field was found. Collected sorghum stover was chopped in a hammer mill to reach a size of 3–4 cm, which was further crushed with mixer grinder and passed through a 1 mm sieve. Hot water treatment was given for 150 min at 75 °C to extract available free sugars, remaining biomass was used for delignification. Biomass characterization was carried out according to Sluiter et al. (2010).

2.2. Delignification and saccharification of the sorghum biomass by acid hydrolysis

Free sugar extracted biomass were dignified by alkali treatment with 5% (w/v) NaOH at 100 °C for 60 min. Biomass were then washed with tap water for neutralization and the neutral pH was confirmed by pH strip. Biomass were dried in oven at 60 °C till constant weights were achieved. In the first method, 5 g of delignified biomass was mixed with 100 mL 0.2–10.0% (v/v) sulfuric acid and treated in a muffle furnace (NOVA make, India) at 225 °C for 30 min. The biomass hydrolyzate was neutralized to pH 7.0 ± 0.2 using 10% (w/v) NaOH and was analyzed for reducing sugars using DNSA method (Miller, 1959). For biomass saccharification, in another method, delignified biomass (5 g) was treated with 10–70% (v/v) 100 mL sulfuric acid at 75 °C in hot air oven (NOVA make, India) for 30 min; biomass hydrolyzate was analyzed as mentioned above.

2.3. Optimization of saccharification with 1–5% (v/v) sulfuric acid treatment using Box Behnken Design (BBD)

The influences of H_2SO_4 concentration, temperature and contact time as well as their interactions were studied using Box Behnken Design (Design Expert® version 8.0.6.1) for their responses and obtained responses were statistically analyzed. Summary of the design is shown in Table 1. The software calculated 17 permutations of these factors; sets were prepared according to the Box Behnken Design by addition of required H_2SO_4 and 10 g biomass and incubated at different temperatures for stipulated time duration. After completion of the reaction, the pH was adjusted to 7.0 ± 0.2 with calcium hydroxide. Samples were filtered and the filtrate was analyzed by DNSA method (Miller, 1959) for estimation of released reducing sugar.

2.4. Maintenance and characterization of the cultures

Four potent cultures were used for the study of ethanol fermentation. Cultural characteristics were studied after 48 h of incubation on modified ethanol fermentation medium consisting (g/L): dextrose, 200; KH_2PO_4, 5.0; $MgSO_4$, 2.5; peptone, 10.0; agar powder 30.0 and pH 6.5 adjusted with HCl (Gaur, 2006). Standard strain of *S. cerevisiae* was obtained from National Chemical Laboratory (NCL), Pune, India, for the comparison. All the isolates and *S. cerevisiae* were maintained on GYA slants at 4–8 °C. Potent culture (MK-I) was identified by 18S rRNA gene sequencing.

2.5. Screening of the isolates for ethanol production ability

All the isolates were studied for their ethanol fermentation capability by separately inoculating in the modified ethanol fermentation medium. To evaluate the ethanol production capability, 10% v/v having $\sim 9 \times 10^7$ cells/mL of 18 h old inoculum of all the isolates, was inoculated in an anaerobic bottle (125 mL capacity) having 90 mL of sterile modified ethanol fermentation medium. The system was incubated at 30 °C in incubator (NOVA make, India) for 96 h, after incubation cell mass was removed from the fermented broth. The broth was neutralized using 2.5 N NaOH and used for distillation. Ethanol was estimated from the distillate by the dichromate method (Caputi et al., 1968).

2.6. Fermentation technique and optimization of various parameters

Inocula were prepared from freshly grown 18 h old active culture of *S. cerevisiae*, culture C, MK-I and N. For submerged fermentation, 100 mL medium was prepared in double mouth 125 mL anaerobic bottles; pH adjustment was done using 1 N HCl or NaOH. Bottles were sealed with butyl rubber cork and aluminium seal, sterilization was done at 121 °C, 15 psi for 15 min. Then, 10% v/v inoculum having $\sim 9 \times 10^7$ cells/mL was inoculated into the fermentation medium by sterile syringe and incubated at 30 °C for 96 h. Sterile needle with silica tubing was inserted in the butyl rubber cork for removal of CO_2 from the medium. The other end of the silica tube was dipped in a conical flask containing 10 N NaOH. After the incubation period, fermented broth was centrifuged at 6000g for 10 min, cell mass was collected and dried in oven (NOVA make, India) at 60 °C until constant weight was obtained. Cell free medium was adjusted to pH 7.0 with 2.5 N NaOH and distilled. The distillate was used for ethanol estimation. Various parameters of ethanol fermentation, i.e., incubation time 24–96 h, initial sugar concentration 12–20% (w/v), inocula size 2.5–10% (v/v) and pH of the medium 4–6 were studied for optimization.

2.7. Lab scale fermentation technique for alcohol production from sorghum biomass

Pool of all extracts of sorghum biomass (Sections 2.1 and 2.2) were mixed and used for the ethanol fermentation in 125 mL capacity anaerobic bottles as described in Section 2.6. Individual

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