Simultaneous Cellulase Production, Saccharification and Detoxification Using Dilute Acid Hydrolysate of *S. spontaneum* with *Trichoderma reesei* NCIM 992 and *Aspergillus niger*

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Abstract Bioethanol production from lignocellulosic materials has several limitations. One aspect is the high production cost of cellulases used for saccharification of substrate and inhibition of fermenting yeast due to inhibitors released in acid hydrolysis. In the present work we have made an attempt to achieve simultaneous cellulases production, saccharification and detoxification using dilute acid hydrolysate of *Saccharum spontaneum* with and without addition of nutrients, supplemented with acid hydrolyzed biomass prior to inoculation in one set and after 3 days of inoculation in another set. Organisms used were *T. reesei* NCIM 992, and *Aspergillus niger* isolated in our laboratory. Cellulase yield obtained was 0.8 IU/ml on fourth day with *T. reesei*. Sugars were found to increase from fourth to fifth day, when hydrolysate was supplemented with nutrients and acid hydrolyzed biomass followed by inoculation with *T. reesei*. Phenolics were also found to decrease by 67%.

Keywords Acid hydrolysate · Cellulase · Detoxification · *Saccharum spontaneum* · Saccharification

Introduction

With the increasing shortage of petroleum reserves, cellulosic materials have been recognized as one of the most

promising alternatives to supply our chemical and energy needs [1]. The benefits of developing biomass-to-ethanol technology have been previously stated to increase national energy security, reduce greenhouse gas emission, use of renewable resources give foundation to a carbohydrate-based chemical process industry and provide macroeconomic benefits for rural communities and society at large [2].

Cellulosic biomass, sometimes called lignocellulosic biomass, is a heterogeneous complex of carbohydrate polymers and lignin, a complex polymer of phenyl propanoid units [3]. Lignocellulosic biomass typically contains 55–75% carbohydrates by dry weight. The presence of lignin in some cell walls imparts further strength, and provides resistance against pests and diseases. Cellulose and hemicellulose are potential sources of fermentable sugars [4]. The presence of lignin in the cell wall, however, impedes hydrolysis of the carbohydrates. Biomass having less competition with food crops would be realistically more appropriate for bioethanol production. As such lignocelluloses from agricultural, industrial and forest or weeds have been identified as potential alternative, sustainable sources of biofuel and other value added products. Enzymatic hydrolysis of cellulose is a very promising and is known to have many advantages over acid hydrolysis for saccharification of lignocelluloses to sugars, but the rate of enzymatic hydrolysis is considerably slower than that of acid hydrolysis [5].

Saccharum spontaneum (wild sugarcane), a wasteland weed, is a tall perennial grass with deep roots and rhizomes, up to 4 m height. It is believed to be a predecessor of the important species *S. officinarum* L. (cultivated sugarcane) [6]. It has the world wide distribution extending across three geographic zones i.e. East Zone, Central Zone, West Zone and other countries infesting millions of acres,

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often causing abandonment of fields [7]. This particular plant has been selected because of following attributes:

- it has no economic value,
- it grows as an extensive weed in India,
- it is drought and flood tolerant,
- it can grow even on sandy soils,
- it is competitive over other weeds as it has an extensive root system that allows its firm establishment in the soil and possesses lightest of seeds that is of high avail in the seed dispersal to distant places and its easy establishment at the new place and low lignin content.

Therefore, we have used *S. spontaneum* as the cheap raw material for cellulase production and saccharification. The present study was carried out to investigate simultaneous cellulase production, saccharification and detoxification of acid hydrolysate obtained from *S. spontaneum* using *Trichoderma reesei* NCIM 992 and *Aspergillus niger*.

Materials and Methods

Microorganisms and Maintenance

Trichoderma reesei NCIM 992 was procured from National Collection of Industrial Microorganisms (NCIM), Pune, India. *A. niger* was an isolate of our laboratory and both were maintained on Potato Dextrose agar at 4°C.

Raw Material

Saccharum spontaneum was collected from the outskirts of Hyderabad, India. Dry stem pieces including leaf sheath were processed using pulverizer to attain a particular size between 4 and 6 mm, then it was washed with running tap water until the washings were clear and dust free and then gently dried at $50 \pm 0.5^\circ\text{C}$ for overnight.

Analysis of Chemical Composition of *S. spontaneum*

The cellulose, lignin and hemi cellulose fractions of pulverized *S. spontaneum* were determined according to Technical Association of the Pulp and Paper Institute TAPPI, test methods (1992).

Delignification

Hundred grams of dry *S. spontaneum* was soaked in 0.2 M NaOH (1:10 ratio w/v), and kept at room temperature i.e. $30 \pm 2^\circ\text{C}$ for 16 h. The contents were filtered with two layers of muslin cloth and the solid residue was repeatedly washed with water until the pH of the filtrate became

neutral. The residue was dried at $50 \pm 0.5^\circ\text{C}$ for overnight and subsequently used for acid hydrolysis experiments.

Preparation of the Acid Hydrolysate

Acid hydrolysis of delignified *S. spontaneum* was carried out in single phase, 50 g of *S. spontaneum* was taken in 1 l conical flask and 500 ml of 3% H_2SO_4 was added. Hydrolysis was performed at 110°C for 30 min. The hydrolyzed substrate was filtered and washed to neutral pH and the hydrolysate was used for the submerged fermentation for simultaneous Cellulase production, saccharification and detoxification.

Preparation of Fungal Inoculum

For cellulase production, conidia of *T. reesei* NCIM 992 and *A. niger* were harvested from 14 to 20 day old stock cultures by adding 5 ml sterile distilled water to the agar slants and re-suspending them. In order to prepare the inoculum, 1 ml of spore suspension (4×10^7 spores ml^{-1}) was inoculated into 150 ml inoculum medium [8] and incubated on a rotary shaker with an agitation rate of 150 rpm at 30°C for 4 days. A 10% (v/v) inoculum was transferred to the production medium.

Submerged Fermentation Using Acid Hydrolysate for Simultaneous Cellulase Production and Detoxification

Two different fungal fermentations were carried out: Fifty ml of acid hydrolysate was taken in eight 100 ml conical flasks. Eight conical flasks were divided into two sets of four conical flasks each. One set of conical flasks contained Mandels mineral salts solution [9] and other set was without media. Then among the each set of four conical flasks, two flasks were inoculated with *A. niger* and the other two conical flasks with *T. reesei* (NCIM 992). From the above four flasks, two flasks with and two flasks without nutrients were taken and 2.5 g of neutralized acid treated biomass was added prior to inoculation. Then to the other two flasks (with nutrients and without nutrients) acid treated biomass was added after 3 days of inoculation. The pH was maintained at 6.0 in all the eight flasks and autoclaved at 110°C for 20 min prior to inoculation.

Analytical Methods

Filter paper activity (FPU) was determined according to [10]. Filter paper cellulase activity (FPase) was assayed by incubating the suitably diluted crude enzyme extract (0.5 ml) with 1.5 ml citrate buffer (50 mM, pH 4.8) containing ash less Whatman no. 40 filter paper strips (50 mg,

1 × 6 cm) at 50°C for 60 min. One unit (U) of enzyme activity is defined as the amount of enzyme, which produces 1 μmol reducing sugar as glucose in the reaction mixture per minute under the above-specified conditions.

The reducing sugars liberated were quantified using the dinitrosalicylic acid (DNS) method of [11].

Total soluble Phenolic compounds were estimated by a modification of Folin ciocalteu method as described by [12].

The Filter paper activity, reducing sugar and phenolic concentration was observed from third day to fifth day of fermentation period. All experiments were carried out in triplicates and data was presented as mean values.

Results and Discussion

Chemical Composition

Saccharum spontaneum was initially characterized for its chemical composition. The pulverized material was found to contain $45.10 \pm 0.35\%$ cellulose, $22.75 \pm 0.28\%$ hemicellulose, $24.38 \pm 0.22\%$ Klason lignin and $2.82 \pm 0.15\%$ ash. The presence of cellulose and hemicellulose together make the total carbohydrate content (TCC) of the substrate 67.85%, which is also the potential sugar concentration in the pretreated substrate. It can be fairly compared with the extensively explored lignocelluloses (sugarcane bagasse, 67.15%; corn stover, 58.29%; wheat straw, 54% and sorghum straw, 61%) for ethanol production [13]. It is evident from the chemical composition that among all the lignocellulosic biomass, forests and agricultural residues, which are inexpensive source of hexose and pentose sugars, *S. spontaneum* is an ideal substrate for ethanol production. Further, the substrate gets easily hydrolyzed with high amount of sugars and less amount of inhibitors in the hydrolysate.

Cellulase Production, Saccharification and Detoxification

During acid hydrolysis of *Saccharum*, the initial amount of sugars in the hydrolysate was 15.4 mg/ml. Sugars were reduced gradually during third day to fifth day of fermentation in all the treatments coincidentally growth was also observed in all the flasks on the third day indicating that the reduction in sugars is due to utilization for the growth of the organism irrespective of addition of nutrients. However, from fourth day to fifth day the concentration of sugars was increased from 6.5 to 8.0 mg/ml in the flask where the substrate was added to acid hydrolysate with minimal medium inoculated with *T. reesei* (Fig. 1). The

enzyme produced caused the hydrolysis of cellulose present in the fermented acid hydrolysate.

According to Palmqvist et al. (1997) in the fungal fermentation with added substrate, 72% of sugars from the hydrolysate are consumed within 6 days, where as in our study 50% sugars were utilized in the flask where the substrate was added to acid hydrolysate with minimal medium and inoculated with *T. reesei*.

The phenolic content in the hydrolysates was studied from third day to fifth day of fermentation. Initially the phenolic content in all the treatments was 3.53 mg/ml (Fig. 2). By the third day, the phenolic content was decreased to 2.37 mg/ml i.e. by 67% in the flask containing hydrolysate supplemented with nutrients and acid treated substrate inoculated with *T. reesei*. The fungus was not negatively affected by the inhibitors. In fact, the inhibitors might even be useful for growth and enzyme production. The decrease of inhibitors in the hydrolysate indicates that a change in the content or structure of the phenolics has been caused by the fungus and eventually decreased the inhibitory action of the lignin-derived compounds.

Palmqvist et al. (1997) reported 30% reduction in phenolic content in the simultaneous detoxification and enzyme production of hemicellulosic hydrolysate using *T. reesei*. However we could obtain maximum reduction i.e. 67% in the hydrolysate inoculated with *T. reesei*.

Cellulolytic enzymes were produced in all the treatments of acid hydrolysate fermented with *T. reesei* and *A. niger*. The FP activity was evaluated on third, fourth and fifth day of fermentation. On third day the activity was in the range of 0.2–0.32 IU/ml. But FPU values got increased to 0.8 IU/ml in the hydrolysate where the substrate and minimal media were added and inoculated with *T. reesei* when compared to *A. niger* (0.3 IU/ml) (Fig. 3). Further on fifth day of fermentation the FPU activity decreased in the range of 0.2–0.4 IU/ml. The reason might be explained as the substrate acted as an inducer for enzyme production. Palmqvist et al. (1997) reported 0.6 IU/ml of FPU activity in the simultaneous detoxification and enzyme production of hemicellulosic hydrolysate using *T. reesei*. However in the present study we could obtain maximum 0.8 FPU/ml in the hydrolysate inoculated with *T. reesei*.

The production of cellulase enzyme is a major process and affects the overall economics of ethanol production from lignocelluloses. In general, cellulase enzyme production accounts for 40% of cost in bioethanol synthesis [14]. During acid hydrolysis, complex mixtures of microbial toxins that retard the bioconversion are generated. Two compounds, Furans (furfurals from the dehydration of pentose sugars and hydroxyl methyl furfural from the dehydration of hexose sugars) and soluble phenolic compounds are typically produced at significant concentrations (grams per liter depending on hydrolysis conditions). For

Fig. 1 Effect of different treatments of acid hydrolysate and fermentation periods on sugar concentration (mg/ml).

1 AH + Tri, 2 AH + Sub + Tri, 3 AH + M + Tri, 4 AH + M + Sub + Tri, 5 AH + Asp, 6 AH + Sub + Asp, 7 AH + M + Asp, 8 AH + M + Sub + Asp. AH, acid hydrolysate; Tri, *Trichoderma*; Sub, substrate; M—, minimal media; Asp, *Aspergillus*

Fig. 2 Effect of different treatments of acid hydrolysate and fermentation periods on phenolic concentration (mg/ml).

1 AH + Tri, 2 AH + Sub + Tri, 3 AH + M + Tri, 4 AH + M + Sub + Tri, 5 AH + Asp, 6 AH + Sub + Asp, 7 AH + M + Asp, 8 AH + M + Sub + Asp. AH, acid hydrolysate; Tri, *Trichoderma*; Sub, substrate; M—, minimal media; Asp, *Aspergillus*

Fig. 3 Effect of different treatments of acid hydrolysate and fermentation periods on FPU/ml.

1 AH + Tri, 2 AH + Sub + Tri, 3 AH + M + Tri, 4 AH + M + Sub + Tri, 5 AH + Asp, 6 AH + Sub + Asp, 7 AH + M + Asp, 8 AH + M + Sub + Asp. AH, acid hydrolysate; Tri, *Trichoderma*; Sub, substrate; M—, minimal media; Asp, *Aspergillus*

the production of cellulytic enzymes to be used in the hydrolysis, the lignocellulose-degrading fungus *T. reesei* can be used. This fungus [15] is able to metabolize pentose and hexose sugars and also oligomers, and it is insensitive

to inhibitors generated from the lignocellulosic material, its natural environment. In the present study, it was investigated whether the cellulytic fungus *T. reesei* could degrade inhibitory compounds present in a hydrolysate

obtained after acid hydrolysis of *S. spontaneum*, and thereby decrease its inhibitory effect on the ethanolic fermentation by *S. cerevisiae*. It was also investigated whether the inhibitor-containing fraction could be used as a carbon source for the production of high-quality cellulolytic enzymes to be used in the hydrolysis.

In the present study, when acid hydrolysate of *S. spontaneum* was inoculated with *T. reesei* supplemented with acid treated biomass and minimal medium, 0.8 FPU/ml was obtained, reduction in phenolic content by 67% and increase in sugar content from fourth day to fifth day was obtained.

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