Indian J. Applied & Pure Bio. Vol. 39(3), 1760-1766 (2024). **ISSN: 0970-2091**
A web of Science Journal

Lignocellulosic biomass for bio-ethanol recovery: A way towards environmental sustainability

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Abstract

The increasing population and industrialization has increased the day to day demand of energy. Global warming and other climatic disasters caused due to burning of conventional fossil fuels have laid the need for an alternative and renewable fuel. Concurrently, the worldwide demand and production of bio-ethanol, a bio-based sustainable fuel is increasing continuously. Fungal saccharification of lignocellulosic biomass takes place simultaneously with the secretion of various metabolites, which function as catalytic system to liberate soluble sugars from insoluble composite biomass. Cellulase holds a major role for converting lignocellulosic feedstock into fermentable sugar. Fungi, especially filamentous fungi preferably the *Aspergillus* species are known to produce cellulase.

Amongst the lignocellulosic biomass, rice straw is considered as one of the most attractive material for producing bioethanol because of its high cellulosic and hemicellulosic content, which can be hydrolyzed into fermentable sugars. However, the recalcitrant nature of the substrate imposes considerable challenge and limitations to bioethanol production. To combat these challenges, various pretreatment techniques such as chemical, physical and physico-chemical processes are known to be effective enough to enhance efficiency of enzymatic saccharification in order to make the complete process economically feasible. The present work deals with the isolation and selection of hypercellulase producing fungal species. These species were used along with the pretreatment techniques either alone or in combination for efficient hydrolysis of rice straw to obtain the maximum amount of the released sugar.

Key words : Lignocellulosic biomass, cellulose, pretreatment, bio-ethanol.

The past few decades have witnessed the doubling of human population[26] which is estimated to reach upto a value of 9 billion by 2050[21,24]. This has increased a worldwide concern about climate change keeping carbon-emissions as a casual factor[10]. India with its vast population density showing a growth rate every minute demands for a simultaneous increase in the rate of agricultural production. However, the rise in agricultural productivity would lead to a consequent rise in agricultural residues. Lignocellulosic biomass serves as potential option for bio-ethanol production in terms of energy ratio, their abundant availability, low cost as well as their ethanol yields[5]. Rice straw is one such main agricultural substrate residues in India[3]. In addition, day-to-day rise in gas and fuel prices, have triggered humankind to find alternative and sustainable energy resources, especially bio-ethanol fuel for automobiles[22].

The yearly availability of rice straw was approximately 731 million tons in Africa, 667.6 million tons in Asia, 3.9 million tons in Europe and 37.2 million tons in America[2]. This huge quantity of rice straw is estimated to produce about 206 billion liters volume of bio-ethanol annually[13]. The major steps involved in lignocellulosic ethanol production are effective pretreatment of the substrate, enzymatic hydrolysis by potential microorganisms and fermentation of reduced sugar[18]. The basic purpose of this research study is to find potential fungal isolate which could degrade the lignocellulosic components of rice straw to form reducing sugar and thereby conversion of these sugars to bio-ethanol.

Collection and preparation of substrates :

Rice straw harvested from agricultural fields near Patna, Bihar, underwent a rigorous cleaning process. Initially washed with distilled water to remove impurities, it was subsequently dried, chipped, and ground to 50µm and 100µm sieve sizes. The resulting material was then stored in sterile polythene bags for future use.

Isolation and purification of microorganisms:

Various agricultural sites and garbage areas were chosen as collection sites for soil samples to isolate potential hypercellulolytic fungi. The soil surface was dug 2-4 cm and soil samples were aseptically collected and stored in sterile bags for subsequent analysis.

The collected soil samples were then cultured using the spread plate technique[1] on Potato Dextrose Agar (PDA) media. These plates were then incubated at a temperature of 28 ± 2°C for duration of 5 days.

To obtain purified cultures, these mixed colonies were transferred onto individual plates containing the same PDA media[12]. This step allowed for the isolation of individual fungal strains for subsequent characterization and experimentation.

Screening of purified plates :

The isolated fungal strains were screened for their cellulose producing ability. Each of the fungal isolate was streaked individually on minimal agar media where Carboxymethyl cellulose was used as the only carbon source[7]. The plates were incubated at 28 ± 2°C for proper growth after which 1%

congo red dye was flooded on the plates. Following the initial incubation period of 15 minutes, the plates were subjected to a washing step using 1M NaCl solution. Upon completion of the washing step, transparent hydrolytic zones were carefully observed and noted. These zones indicated areas where the hypercellulolytic fungi had enzymatically degraded the cellulose present in the medium, resulting in the formation of transparent regions. This observation served as a qualitative indicator of cellulolytic activity and aided in the further characterization of the isolated fungal strains.

Enzyme assays :

Fungal isolates displaying the most extensive hydrolytic zones were selected for enzyme assays to further characterize their cellulolytic activity. Various enzyme assays, including Filter Paper Activity (FPase) and endoglucanase assays were conducted[9,16,20]. In these assays, the fungal isolates were tested for their ability to produce cellulolytic enzymes, which are capable of breaking down cellulose into simpler sugars. The amount of reducing sugar released during the enzymatic reactions was quantitatively measured. This provided valuable insights into the efficiency and potency of the cellulolytic enzymes produced by the fungal isolates, facilitating a comprehensive understanding of their cellulolytic capabilities.

For F Pase assay, filter paper was used as a substrate. Modified Mandels medium[19] was prepared to which appropriate size of Whatmann filter paper was added. The mixture was allowed to incubate at 28°C in a shaker incubator for 5days at 180rpm. After the incubation period, the reaction mixture was procured and filtered. 1ml of the filtered mixture was added to equal amount of citrate buffer (pH 4.8) to which 3ml of DNSA reagent[8] was added and incubated at 50°C for 30 mins. Amount of reducing sugar was observed.

For endoglucanase assay, CMCase was used as a substrate. 0.5g of carboxymethyl cellulase was added to Modified Mandels medium and incubated at 28°C for 5 days. The reaction mixture was filtered after the completion of the incubation period and the supernatant was restored. 1ml of this supernatant was mixed with equal amount of citrate buffer and boiled in waterbath for 15mins. 3ml of DNSA reagent was added to the mixture and amount of reducing sugar was noted.

Pretreatment of substrate :

Rice straw was pretreated using different techniques such as physical and chemical treatments were adapted to break the cellulose-hemicellulose and lignin matrix.

Milling and steam explosion treatments were used as physical treatments[17]. For this, 10g of the rice straw were finely chopped and sieved to desired mesh size. Further, it was transferred to Erlenmeyer flask to which 250ml of distilled water was added and passed through steam for 30 mins.

For alkaline pretreatment 10% of sodium hydroxide was dissolved in 250ml of distilled water to which the chopped rice straw was added. The mixture was continuously stirred with a magnetic stirrer for about 2 hours[15]. It

was further filtered and washed with distilled water until it was completely alkali free, dried and stored.

Isolation & purification of microorganisms:

Initially, 96 fungi were isolated and purified on Potato Dextrose Agar plates using the streak plate technique. Among these, a hypercellulolytic fungal isolate identified as *Aspergillus terreus*, named BA13.5, demonstrated the highest cellulase activity. Other research studies have highlighted fungi as the most efficient cellulase degraders among various microorganisms for many natural polymers. Extensive research has focused on a diverse range of strains from *Penicillium* and *Trichoderma* species in this context[25]. In recent years, *Aspergillus* species have also gained attention for their significant cellulase production, rendering them promising for industrial applications[4,12,14].

Following isolation, the fungal strains underwent screening to assess their hypercellulolytic capabilities. This screening involved a series of assays and observations aimed at identifying strains proficient in cellulose degradation. Those strains showing significant hypercellulolytic activity were then chosen for in-depth analysis and characterization, thereby advancing the exploration of cellulolytic fungi for potential biotechnological uses.

Screening of the purified isolates :

Using Congo red dye screening, 57 fungal isolates were identified as hypercellulase producers. Among them, isolate BA13.5 displayed the largest hydrolytic zone, measuring approximately 27mm in diameter. This exceptional cellulolytic activity underscores the promise of isolate BA13.5 for future investigation and utilization in cellulose degradation processes.

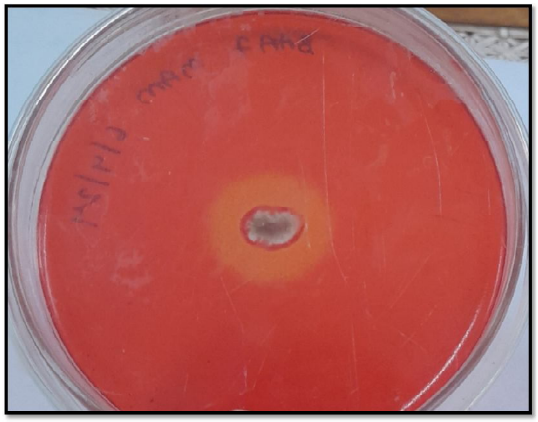

Fig. 1.Transparent hydrolytic zone by BA13.5

Enzyme assays :

During the filter paper assay of BA13.5, no considerable activity was detected within the first 24 hours of incubation. However, after 72 hours of incubation, a significant rise in enzyme activity was observed, peaking at 0.210 IU/ml at 120 hours of incubation. Interestingly, reducing the incubation period resulted in a decline in enzyme activity in the reaction mixture. These findings mirror those reported by Dashtan *et al*., where the filter paper activity after a 96-hour incubation period was measured at 0.028 IU/ml[6].

BA13.5 exhibited a notable increase in endoglucanase activity starting from 72 hours of incubation, with this concentration continuing to rise significantly as the incubation time prolonged. It reached its peak at 144 hours of incubation, recording a value of 0.419 IU/ml.

These findings highlight the cellulolytic potential of isolate BA13.5, particularly in terms of its endoglucanase activity, emphasizing its significance for further exploration and potential biotechnological applications.

These results align with the research conducted by Yadav *et al.*[27], where the endoglucanase amount after 160 hours of incubation was observed to be 0.788 IU/ml. However, they contrast with the findings presented by Gunjikar *et al.*[11], where the endoglucanase activity after 72 hours of incubation was noted to be 0.523 IU/ml.

One IU of activity toward the substrate mentioned above was defined as µmole of glucose (endoglucanase, exoglucanse activity or p-nitrophenol (β-glucosidase) released per minute under the above assay conditions by using glucose, xylose or p-nitrophenol, respectively as standard curve. Reducing sugar estimation was carried out by dinitro salicylic acid (DNSA) method.

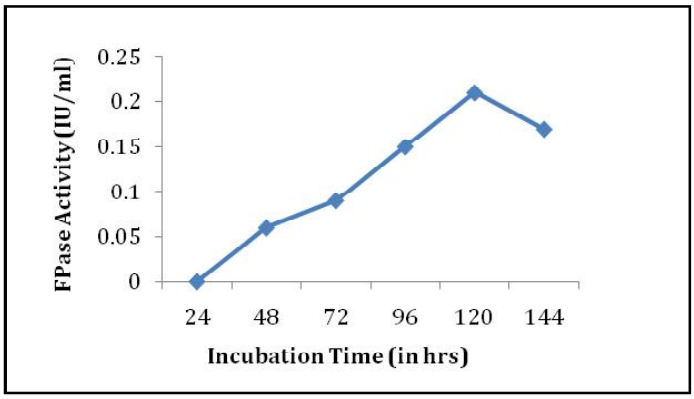

Fig. 2. FPase activity of BA13.5

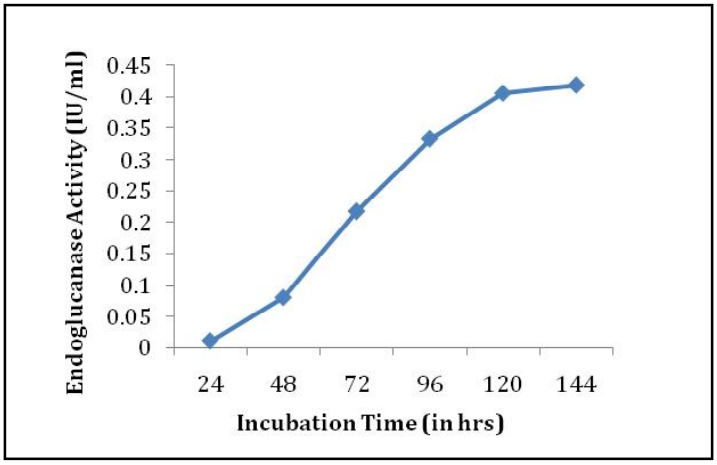

Fig. 3. Endoglucanase activity of BA13.5

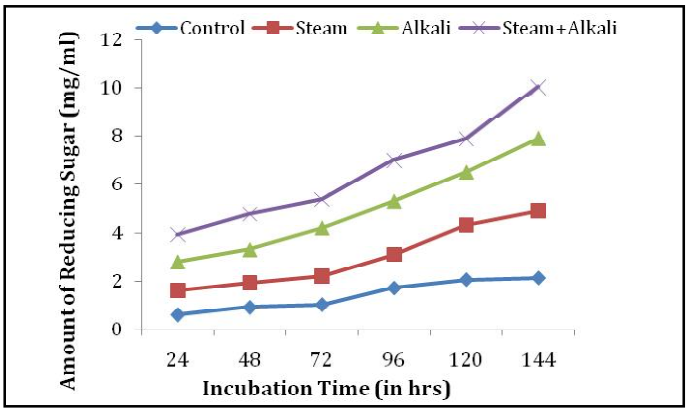

Fig. 4. Amount of reducing sugar released after different pretreatment techniques

Pretreatment of substrate :

Several pretreatment techniques, including milling, steam explosion, alkali treatment, and their combinations, were utilized to evaluate their effects on cellulose degradation, quantified by the release of reducing sugars.

Among these techniques, the steam alkali combination treatment demonstrated the highest yield of reducing sugar, measuring at 10.03 mg/ml. This outcome suggests that the combined application of steam and alkali led to the most efficient breakdown of cellulose, resulting in a substantial release of reducing sugars. This observation highlights the potential of steam alkali pretreatment as a promising method for enhancing enzymatic cellulose hydrolysis, providing valuable insights for optimizing bioconversion processes in various biotechnological applications.

Based on morphological and phylogenetic analyses, isolate BA13.5 was identified as *Aspergillus terreus*. It exhibited promising enzymatic activities, with FPase activity peaking at 0.210 IU/ml after 120 hours of incubation, and endoglucanase activity reaching 0.419 IU/ml within the same incubation period.

Moreover, the combination of steam and alkali pretreatment emerged as the most effective method, yielding a significant amount of reducing sugars (10.03 mg/ml). This finding suggests its potential for enhancing cellulose degradation and subsequent bioethanol production.

Given these results, further investigations utilizing isolate BA13.5 will focus on developing cost-effective techniques for industrial bioethanol production. This research aims to harness the cellulolytic capabilities of *Aspergillus terreus* BA13.5 to optimize bioconversion processes, ultimately contributing to the advancement of sustainable biofuel production methods.

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