Harnessing cellulolytic potential of a new *Aspergillus terreus* AP02 strain for developing integrated pre-treatment and saccharification process for efficient lignocellulosic ethanol production

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Abstract

The global need for sustainable energy solutions emphasizes the importance of efficient lignocellulosic biomass conversion into biofuels. In this study, a potent cellulase-producing fungal isolate, Aspergillus terreus AP02, was isolated from rotting wood compost and identified through ITS sequencing and phylogenetic analysis. The crude enzyme extract (AT-CS) obtained from solid-state fermentation displayed a broad enzymatic profile including filter paper activity (1.09 ± 0.067 FPU/ml), CMCase (0.55 ± 0.043 IU/ml), avicellase (0.14 ± 0.009 IU/ml), β-glucosidase (0.45 ± 0.021 IU/ml) and endo-xylanase (8.96 ± 0.77 IU/ml), indicating strong cellulose and hemicellulose degrading potential. An integrated pre-treatment and saccharification (IPS) process was developed using AT-CS and optimized via "One Factor at a Time" (OFAT) approach. Optimal conditions were determined as: 1% (v/v) acid loading, 10% (w/v) biomass loading, 1.0% (w/v) PEG-6000, 45 min pre-treatment, 30 FPU/g enzyme dose and 48 hours saccharification time wherein a maximum 0.57 ± 0.016 g/g reducing sugar was released from rice straw.

The resulting RS hydrolysate was fermented using Saccharomyces cerevisiae NCIM 3570 and produced 15.82 ± 0.84 g/L ethanol, with 93.93% fermentation efficiency (yield: 0.48 g/g sugar) and 0.659 g/L/h productivity. These findings demonstrated the potential of isolate A. terreus AP02 for cellulolytic enzymes production and feasibility of an optimized IPS strategy for developing agricultural waste based lignocellulosic biorefinery for second-generation bioethanol production.

Keywords: Lignocellulosic biomass, Cellulase, Integrated Pre-treatment and Saccharification, Fermentation, Bioethanol.

Introduction

Global demand of transport fuel is increasing due to the increasing population and industrialization, which is leading to more intensified fossil fuel uses. However, the intensified uses of these non-renewable reserves is significantly contributing to increased greenhouse gas emissions leading to global warming[9]. Therefore, in order to combat these environmental challenges, there is critical necessity to transition towards alternative low-carbon renewable and sustainable biofuels. Among the various alternatives, lignocellulosic biomass (LCB) derived second-generation (2G) bioethanol is emerging as promising sustainable alternative[11]. Unlike first-generation bioethanol, 2G bioethanol research utilizes non-food LCB resources which not only resolve the "food Vs. fuels issue" but also provides a carbon-neutral sustainable fuel generation[16].

Lignocellulosic biomass mainly consists of cellulose, hemicellulose and lignin and it can be produced in several tons in the forms of various agricultural residues including wheat straw, sugarcane bagasse, rice husk and corncobs as well as forestry residues and municipal organic waste[24]. India is an agriculture based economy with the annual crop-residue generation estimated to be more than 500 million metric tons[19]. These agro-industrial residues are usually disposed of or burnt at great cost to the environment, hence can be effectively valorised for biofuels leading to "waste to value" generation, constituting a sustainable pathway for the circular bioeconomy[28].

Although promising, conversion of LCB to fermentable sugars and then to ethanol is technically difficult because of its very recalcitrant structure[27]. Cellulose is a linear polysaccharide of D-glucose that is bonded by β-(1,4)-glycosidic bonds and is found in crystalline microfibrils which are closely adhered to hemicellulose and cemented in a complicated lignin matrix[14]. Hemicellulose, a heterogeneous C5 and C6 sugar polymer, is more amorphous but is essential for structural stability through cross-linking between cellulose and lignin[15].

Lignin, an aromatic, irregular polymer, is a significant enzymatic hydrolysis barrier through the creation of a rigid, hydrophobic sheath and non-specific adsorption of hydrolytic enzymes, thus significantly lowering saccharification efficiency[36].

To overcome such structural impediments, effective pretreatment is needed. Pretreatment breaks the architecture of the plant cell wall, solubilizes or transforms lignin and hemicellulose and increases the accessibility of the enzyme to cellulose[1]. Traditional pretreatment processes such as dilute acid, alkali, steam explosion and oxidative treatments, can be efficient but tend to be energy-intensive, multi-step

processing-intensive and result in fermentation-inhibitory substances like furfural and hydroxymethylfurfural (HMF)[32].

In response to these constraints, integrated pretreatment and saccharification (IPS) approaches have been emerging[31]. IPS combines biomass disruption and enzymatic hydrolysis in one step, minimizing process steps, energy consumption and inhibitor generation. Successful IPS deployment demands optimization of the most significant parameters such as biomass loading, acid concentration, enzyme dosage and saccharification time for maximal sugar production and process yield[18].

The second key to the success of LCB valorization is the choice of effective and economical enzymatic systems. Fungi, especially those obtained from natural cellulose-rich or rotting environments, are high producers of lignocellulolytic enzymes like cellulases, xylanases and β-glucosidases[20]. Solid-state fermentation provides a low-cost and effective platform for the production of crude enzyme extracts from such fungi on agro-residues as substrates[29]. These raw enzyme blends, when directly applied to pretreated biomass, provide a low-cost alternative to commercial enzyme cocktails[2].

This study aimed to isolate and to characterize an efficient cellulase producing fungus from wood decaying compost sample. The selected isolate was characterized for the different cellulolytic activities and molecularly characterized by ITS sequencing followed by NCBI-BLAST and phylogenetic tree analysis. The crude enzyme secretome of the isolate *A. terreus* AP09, was further harnessed for developing an optimized integrated pretreatment and saccharification (IPS) process using rice straw. Furthermore, the optimized IPS derived lignocellulosic hydrolysates were subsequently fermented to ethanol by using *S. cerevisiae* NCIM 3570 strain.

Material and Methods

Isolation and Screening, of Cellulase-Producing Fungal Strains: Rotting biomass compost (RBC) was aseptically harvested from a decomposed composting field close to the Gaushala locality in Gorakhpur, India, to isolate and screen cellulase producing fungal strains. Compost in one gram was dispersed in sterile distilled water and serially diluted between 10^{-1} to 10^{-7}. 100 µL aliquot from all dilutions was plated on potato dextrose agar (PDA) plates and kept at 28 ± 2 °C for 120 hours for fungal growth.

Morphologically different fungal colonies were subcultured on selective cellulase screening media containing (g/L): $NaNO_3$ – 2.5, KH_2PO_4 – 2.0, $MgSO_4$ – 0.2, NaCl – 0.2, $CaCl_2 \cdot 6H_2O$ – 0.1, agar – 20.0 and supplemented with 1% (w/v) carboxymethyl cellulose (CMC), pH adjusted to 6.0. The plates were incubated at 28 ± 2 °C for 5 days. After incubation, the plates were flooded with 0.1% (w/v) congo red solution for 15 min and then destained with 1 M NaCl to reveal zones of hydrolysis. Fungal isolates that developed clear, visible halos around the colonies, which are signs of cellulolytic activity, were chosen for further characterization.

Molecular Identification of selected fungus: The most efficient cellulase-producing fungal isolate was subjected to molecular identification through Internal Transcribed Spacer (ITS) region sequencing adopting previously described method[29]. Briefly, genomic DNA was extracted and the ITS region was amplified using universal fungal primers ITS-1 and ITS-4. PCR products were purified using the Thermo Scientific PCR product purification kit and subsequently sequenced using the Sanger sequencing.

The obtained ITS sequence was analyzed and compared with known sequences in the NCBI GenBank database for taxonomic identification and phylogenetic analysis was performed using Mega 12.0 software.

Production of Cellulase and Xylanase under Solid-State Fermentation (SSF)

Inoculum Preparation for SSF: The cellulolytic fungal isolates showing prominent hydrolysis zones on CMC agar were maintained on potato dextrose agar (PDA) slants and incubated at 28 ± 2 °C for 72 h to induce sporulation. For inoculum preparation, subsequently, 20 mL of sterile distilled water was added to the slant and the surface was scraped gently with a sterile inoculation loop to release the spores. The suspension was shaken well to achieve a uniform spore suspension. The spore concentration was finally adjusted to 1.8×10^8 spores/mL for inoculation.

SSF for enzyme production: Solid-state fermentation (SSF) was carried out using wheat bran as the substrate. Five grams of wheat bran were placed in 250 mL Erlenmeyer flasks and moistened with modified Czapek-Dox inorganic medium (g/L): $NaNO_3$ – 2.0, KCl – 0.5, $FeSO_4 \cdot 7H_2O$ – 0.01, $MgSO_4 \cdot 7H_2O$ – 0.5, KH_2PO_4 – 4.5, maintaining a substrate-to-moisture ratio of 1:2 (w/v). The contents were autoclaved at 121 °C for 15 minutes and allowed to be cool. Each flask was inoculated with 1.0 mL of the above prepared fungal spore suspension and incubated at 30 ± 2 °C for 5 days without shaking. Uninoculated wheat bran with same moisture ratio containing flasks served as control in this experiment.

Enzyme Extraction and Recovery: After fermentation, crude enzyme extraction was carried out by the addition of 50 mL of 50 mM sodium citrate buffer solution of pH 4.8 per flask. The flasks were shaken at 120 rpm for 3 hours at 30 ± 2 °C to provide efficient extraction. The obtained slurry was filtered using double-layered muslin cloth to eliminate the solid wastes. The filtrate was centrifuged at 10,000 rpm for 15 minutes at 4 °C. The supernatant thus collected was used as the crude enzyme extract of cellulase and xylanase. The experiments were carried out in triplicate and data were reported as mean values to ensure the statistical validity.

Secretome Profiling of *A. terreus* Crude Secretome (AT-CS): Total protein content of *A. terreus* crude secretome (AT-CS) was quantified with Bradford assay[7]. Enzymatic activities toward lignocellulosic biomass degradation such as FPase, CMCase (carboxymethyl cellulase), Avicellase, Xylanase, pNPGase (4-nitrophenyl β-D-glucopyranosidase) and pNPXase (4-nitrophenyl β-D-xylopyranosidase) were measured by adopting the established method[34]. These enzyme assays gave an integrated profile of hydrolytic capability of AT-CS.

Enzymatic Saccharification of Rice straw: Dilute acid-pretreated rice straw (DAP-RS), treated at 1% v/v H_2SO_4 at 121 °C for 45 minutes, was employed as lignocellulosic substrate for saccharification. Compositional analysis conducted according to National Renewable Energy Laboratory (NREL) procedures[33] showed DAP-RS containing 46.2% w/w cellulose, 3.1% w/w xylan and 25.51% w/w lignin. Saccharification was conducted by suspending biomass in 50 mM sodium citrate buffer of pH 4.8 with a biomass loading of 5.0% w/v. AT-CS was supplemented at an enzyme loading of 20 FPU/g biomass. The reaction mixtures were incubated at 50 °C for 48 hours with shaking at 180 rpm. The slurries were then centrifuged at 10000 rpm for 10 minutes at 4 °C after incubation. The supernatants were harvested and assayed for released sugars with the 3,5-dinitrosalicylic acid (DNS) method[22]. Percentage saccharification was calculated as reported previously[21] using following formula:

$$\text{Saccharification \%} = \frac{\text{Reducing sugars (mg/ mL)} * 0.9 * 100}{\text{Initial substrate concentration (mg/mL)}}$$

The polysaccharides had been converted into monosaccharides using a factor of 0.9, which accounted for the absorption of water during hydrolysis.

Optimization of process parameters for integrated pre-treatment and saccharification (IPS): The integrated pre-treatment and saccharification (IPS) process was optimized by using **"**one factor at a time (OFAT)" approach using crude AT-CS secretome. The process parameters such as acid loading (0.5, 1.0, 1.5, 2.0 and 2.5% v/v), biomass loading (5, 10, 15, 20, 25 % w/v), surfactant (0.5, 1.0, 1.5, 2.0 and 2.5% w/w), pre-treatment time (15, 30, 45, 60, 75 min), enzyme loading (10, 20, 30, 40, 50 FPU/g) and saccharification time (12, 24, 48, 72, 96, 120 h) were studied and optimized by using "one factor at a time (OFAT)" approach. For each IPS optimization experiments, after pretreatment, the entire pre-treated slurry (pH adjusted to 7.0) was directly mixed with AT-CS enzyme and incubated for saccharification at 50°C. After every parameter's optimization experiment, 1.0 ml of slurry was centrifuged at 10,000 rpm for 10 min and obtained supernatant was analysed for reducing sugar using DNS method[22].

RS Hydrolysates Fermentation: Seed inoculums were prepared by growing yeast strain, *S. cerevisiae* NCIM 3288 in yeast extract peptone dextrose broth (composition in g/L; yeast extract, 10.0; peptone, 20.0; dextrose 20.0; pH 5.0) for 18 h at 30 °C and at 150 rpm. 200 ml of hydrolyzate (pH 5.6) was supplemented with yeast extract (5 g/L) and inoculated with 5% (v/v) of 18 h old seed inoculums in 250 ml rubber cork capped Erlenmeyer flasks and incubated for 72 h at 30 °C and 150 rpm. After every 6 h, 2 ml aliquots of the fermented media were sampled and centrifuged at 10,000 x g for 10 min, the supernatant obtained was analyzed for ethanol by dichromate method[8], for sugar consumption by 3,5-dinitrosalicylic acid (DNS) method[22] and for biomass by dry cell weight method[12].

Results

Isolation and Primary Screening of Cellulolytic Fungi: Four morphologically different fungal isolates were recovered from rotten, cellulose-based compost material. The isolates were screened at the primary level for cellulolytic activity with CMC agar amended with congo red dye. Isolate AP02 showed the highest hydrolytic zone (data not shown), indicating the highest extracellular cellulase activity. To measure cellulase production quantitatively, solid state fermentation (SSF) was conducted and overall cellulase activity was determined in the filter paper assay. Isolate AP02 showed a much higher activity of 1.57 ± 0.09 FPU/ml which surpassed other isolates and validated its status as an efficient cellulase producer (Table 1).

Molecular Identification and Phylogenetic Analysis: For identification of isolate at taxonomic level, the ITS region of AP02 was sequenced and amplified. NCBI BLAST showed 94.20% identity with *Aspergillus terreus*, indicating it to be a close relative or a new strain within the species complex. The top hits from BLAST are listed in figure 1 which shows the ten most similar alignments retrieved from the database. To confirm and represent the evolutionary relatedness, a phylogenetic tree was prepared by the Neighbor-Joining method with 1000 bootstrap replications in MEGA 12.0 software. The tree thus obtained (Figure 2) positioned AP02 near established *A. terreus* strains, confirming its taxonomic placement within this species.

Enzymatic profiling of *Aspergillus terreus* AP02 crude Secretome (AT-CS): The crude secretome of AP02, known as AT-CS, was analyzed by enzymatic profiling to determine its ability to break down lignocellulosic biomass. As shown in table 1, the secretome had a diverse array of cellulolytic enzymes. The concentration of total proteins was 0.48 ± 0.032 mg/ml and FPA activity was determined at 1.09 ± 0.067 FPU/ml. Besides cellulase components, the strain also showed high endo-xylanase activity (8.96 ± 0.77 IU/ml), showing its dual potential to hydrolyze both cellulose and hemicellulose. The presence of CMCase (0.55 ± 0.043 IU/ml), avicellase (0.14 ± 0.009 IU/ml) and β-glucosidase (0.45 ± 0.021 IU/ml) (determined with pNPG) suggests a whole cellulase system required for synergistic lignocellulose hydrolysis.

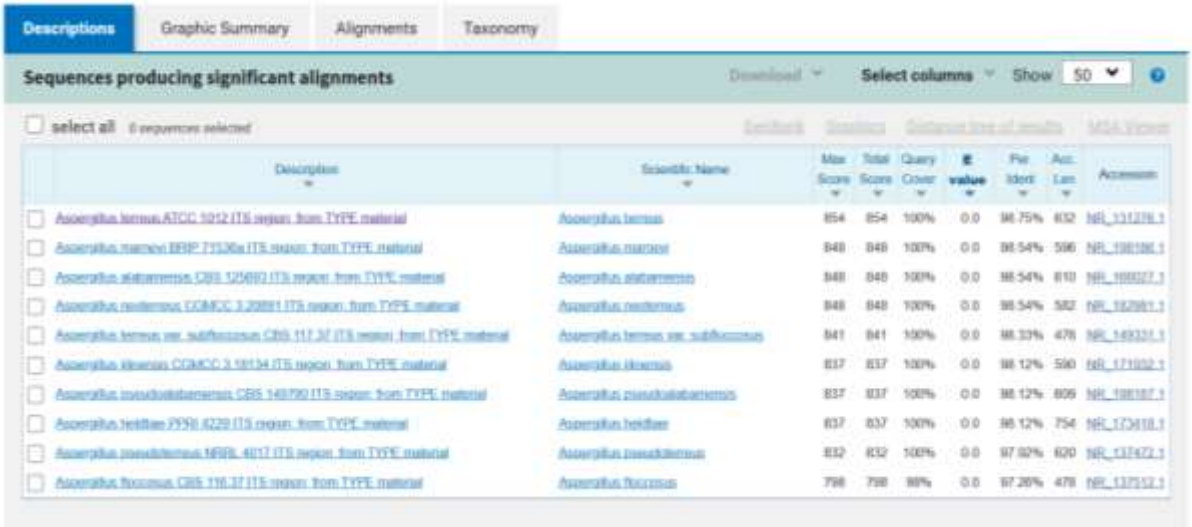

Fig. 1: Top 10 matches for the ITS sequence derived from AP02 fungal isolate. ITS sequence was submitted to NCBI Nucleotide BLAST (blast) and blast analysis was performed. Based on maximum identity isolate AP02 was identified as *Aspergillus terreus* strain.

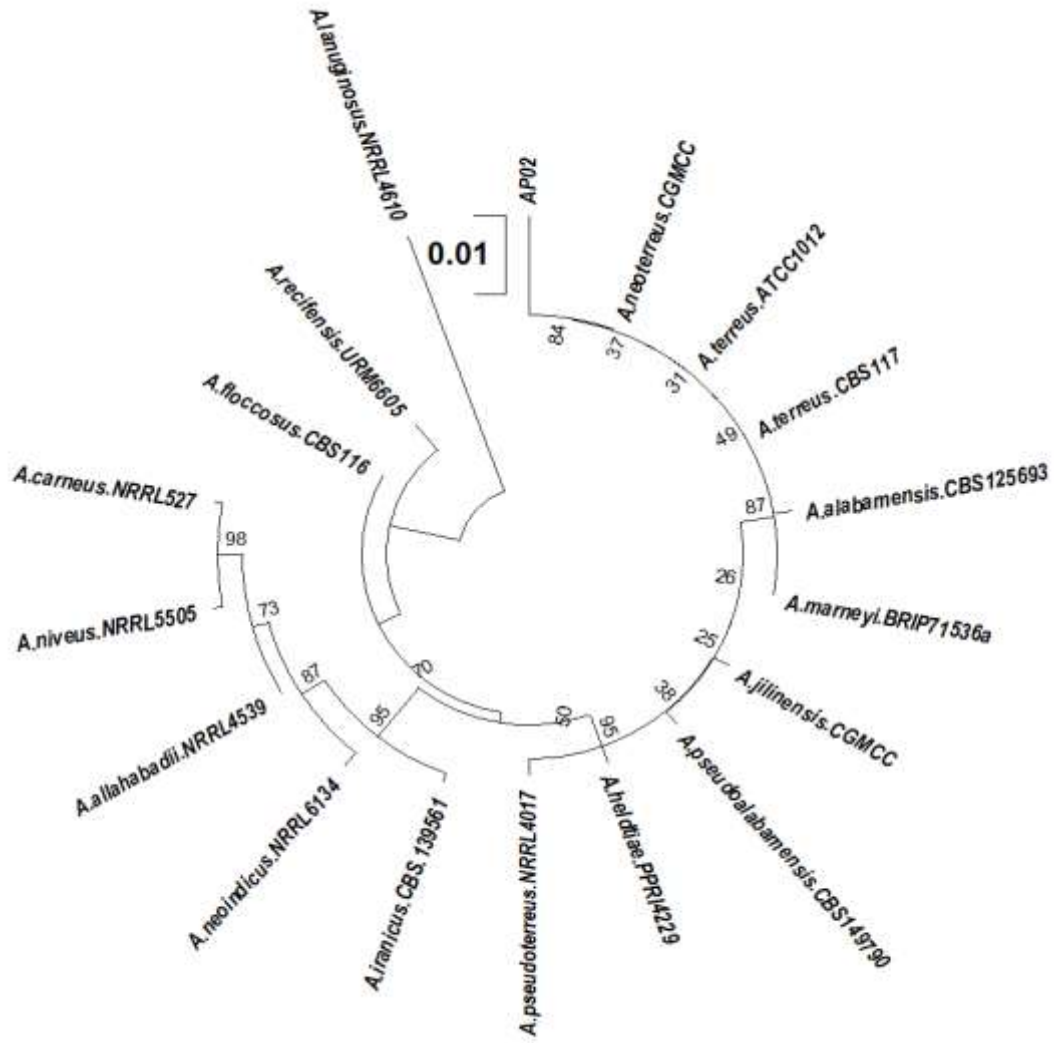

Fig. 2: Phylogenetic analysis of ITS sequence derived from AP02 fungal isolate. ITS sequences (of AP02 and top matching sequences of blastn) were aligned by ClustalW in Mega 12.0. The aligned sequences were submitted to MEGA 12.0 and phylogenetic tree was generated using Neighbor-Joining method by selecting test of phylogeny: Bootstrap method (using 1000 bootstrap replicates) and substitution model: Tamura-Nei method.

Table 1
Enzymatic profile of secretome produced by *A. terreus* AP02. (FPA: Filter paper activity; FPU: Filter paper unit; CMCase: Carboxymethyl cellulase activity; pNPG: 4-Nitrophenyl β-D-glucopyranosidase)

S.N.	Components	Enzyme concentration/activities
1	Protein Concentration (mg/ml)	0.48 ± 0.032*
2	FPA activity (FPU/ml)	1.09 ± 0.067
3	CMCase activity (IU/ml)	0.55 ± 0.043
4	Avicellase activity (IU/ml)	0.14 ± 0.009
5	pNPG activity (IU/ml)	0.45 ± 0.021
6	Endo-xylanase (IU/ml)	8.96 ± 0.77

* Mean + SD (for n =3)

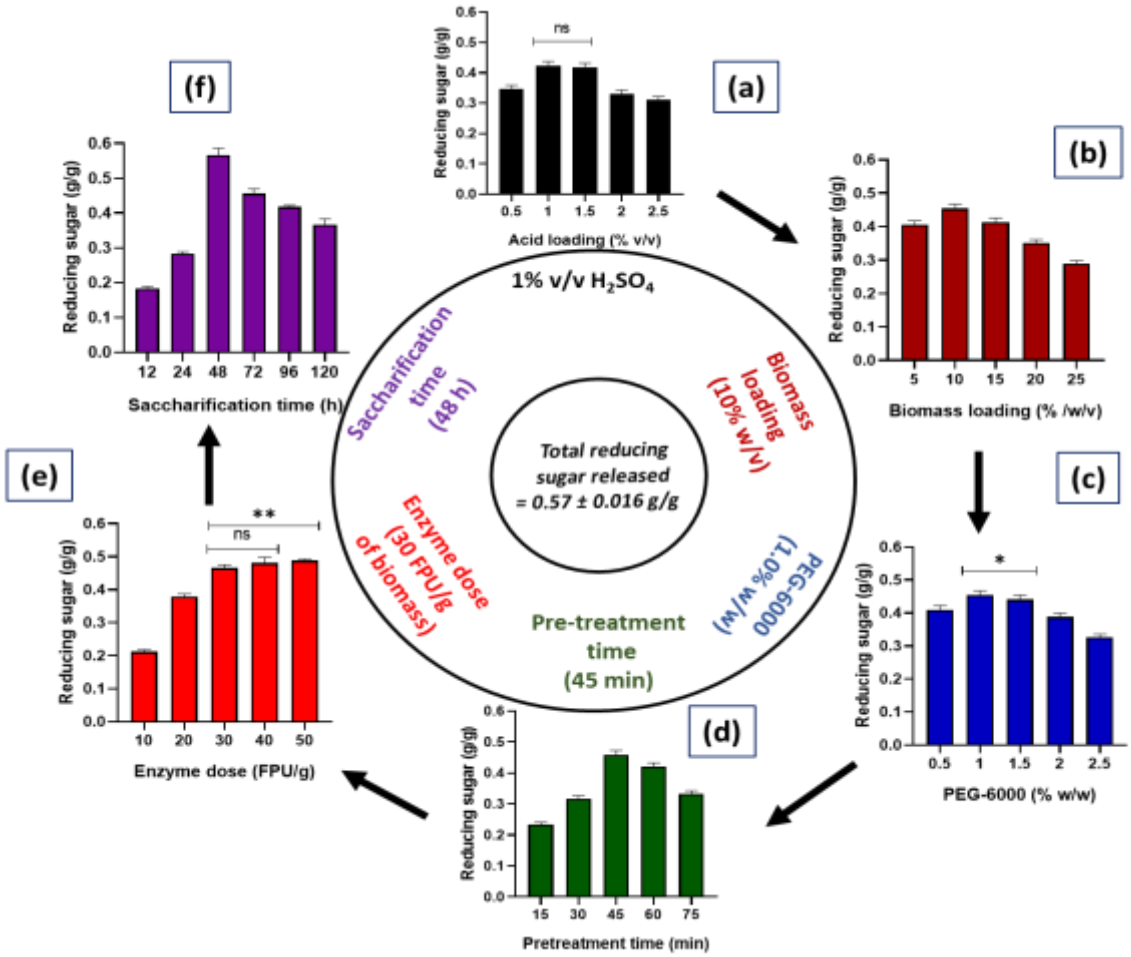

Fig. 3: Optimization profile of integrated pre-treatment and saccharification process using "One factor at a time" approach. a) Effect of Acid loading; b) Effect of biomass loading; c) Effect of PEG-6000 loading; d) Effect of pre-treatment time; e) Effect of enzyme doses and f) Effect of saccharification time.

Table 2
Fermentation profile of IPS-RS hydrolysate using *S. cerevisiae* NCIM 3570 in 24 h.

S. N.	Parameters	Results obtained
1.	Initial Sugar (g/L)	57.12 ± 1.84
2.	Residual Sugar (g/L)	24.17 ± 1.06
3.	Sugar consumption (g/L)	32.95 (57.7%)
4.	Ethanol Concentration (g/L)	15.82 ± 0.84
5.	Ethanol Productivity (g/L/h)	0.659
6.	Ethanol Yield (g/g sugar)	0.48
7.	Ethanol Efficiency (%)	93.93%

Optimization of process parameters for integrated pre-treatment and saccharification (IPS): To enhance sugar recovery, a set of optimization experiments were conducted following "One Factor at a Time" (OFAT) approach towards six major parameters: acid loading, biomass loading, PEG-6000 content, pre-treatment duration, enzyme concentration and saccharification time (Figure 3a–f). The outcome of this optimization is depicted in each parameter having a unique effect on the release of sugar with the best conditions witnessing a significant rise in fermentable sugar yield. The key function here was served by enzyme dosages, showing 30 FPU/g of RS as optimum dose, above which there was no significant sugar released observed (Fig. 3f). Likewise, optimum pretreatment time (45 min) and saccharification time (48 h) also showed significant impact on reducing sugar yield (Fig. 3d and 3f). PEG-6000 played a crucial role by enhancing enzymatic hydrolysis through reduced non-productive binding of enzymes to lignin. Integrated process that carried out the pre-treatment along with saccharification as one step, dramatically lessened process complexity and duration.

Fermentation Performance of RS hydrolysate: The high-sugar RS hydrolysate produced under optimized IPS conditions was employed as a substrate for ethanol fermentation by *Saccharomyces cerevisiae* NCIM 3570. As indicated in table 2, the initial sugar content was 57.12 ± 1.84 g/L, which reduced to 24.17 ± 1.06 g/L after 24 hours, indicating a sugar uptake of 32.95 g/L (57.7%). The obtained ethanol concentration was 15.82 ± 0.84 g/L, which is equal to an ethanol yield of 0.48 g/g sugar and an ethanol efficiency of 93.93%, showing that the hydrolysate was successfully fermented into ethanol. These results confirm the efficacy of the upstream enzymatic hydrolysis process and the fermentability of the produced sugars.

Discussion

This study proves the remarkable cellulolytic activity of *Aspergillus terreus* AP02, a newly isolated fungus from compost with high cellulose content. The isolate showed a high filter paper activity (1.57 ± 0.09 FPU/ml) at primary screening and was far better than other isolates in our study. The cellulase activity here is considerably greater than previously reported values for *A. terreus* NRRL 1960 (0.12 FPU/ml)[17] and are comparable to well-studied *Trichoderma reesei* Rut C30 (1.6 FPU/ml)[6]. High enzyme activity indicates that AP02 may be an efficient in-house cellulase producers in industrial set ups. The better enzyme production is perhaps as a result of the special metabolic features of our isolate.

Molecular identification by ITS sequencing indicated a 94.20% match to *A. terreus*, situating AP02 in this species group but hinting at novelty. This is an interesting observation when viewed against observations by Alshehri et al[5], who found cellulolytic *Aspergillus* strains of 90% ITS sequence similarity to described species. The phylogenetic analysis had AP02 clustering together with *A. terreus* isolates, but still holding a unique branch, as per certain observations made before for an *Aspergillus* isolate[4]. This genomic distinction can be used to explain the increased enzymatic activity seen in our isolate, which calls for a more in-depth genomic study to determine the genetic underpinnings of its better cellulolytic activity.

The enzymatic activity of AP02 crude secretome depicted a complete lignocellulolytic system, with notably stellar endo-xylanase activity (8.96 ± 0.77 IU/ml). Maximum of 0.46 and 0.26 IU/ml of CMCase and FPase respectively was achieved with the use of *Rhizopus stolonifer* on cassava waste in SSF condition after incubation of 10 days[30]. Sugarcane bagasse, in application as substrate for enzyme production by *Trichoderma viride*, yielded optimum levels of enzyme activity of 3.229 U/mL for CMCase and 1.009 U/mL for FPase at high moisture content[25]. Balanced production of cellulase components (CMCase 0.55 IU/ml, β-glucosidase 0.45 IU/ml) indicates effective synergy in biomass degradation, solving a general limitation in fungal enzyme systems in which β-glucosidase activity tends to be rate-limiting[10]. This well-balanced enzyme profile would dramatically enhance saccharification effectiveness in industrial processes.

Optimization of the integrated pre-treatment and saccharification (IPS) process proved the pivotal role of enzyme doses, pretreatment time, saccharification time and PEG-6000 in improving enzymatic hydrolysis (Fig. 3). The 35% increase in sugar yield was with the addition of PEG (from 42.3 to 57.1 g/L). Likewise, Aggarwal et al[3] also reported the 35.96% saccharification by the crude secretome of isolate *Aspergillus niger* BK01[3].

In yet another study, Yadav et al[35] had a highest saccharification yield of 74.19% from SCB, resulting in 34.6 g/L of reducing sugar at 72 hours with 6% SCB loading, 20 FPU/g enzyme and 0.075% surfactant. They observed no noteworthy differences among free and immobilized enzymes, since free enzyme hydrolysis gave 70.71% saccharification and 33 g/L of reducing sugar[35].

The fermentation efficiency of the hydrolysate was outstanding, with *S. cerevisiae* NCIM 3570 reaching 93.93% theoretical ethanol yield. This is significantly higher than the 80% and 89% fermentation efficiencies obtained for softwood hydrolysates fermentation by *S. cerevisiae* XR122N strain[13] and sugarcane bagasse hydrolysate by *S. cerevisiae* TP-1 strain[23]. The 0.659 g/L/h ethanol production rate is noteworthy, particularly 25% higher than reported in other lignocellulosic hydrolysates (0.35-0.57 g/L/h)[23]. It indicates that our IPS process successfully optimized inhibitor formation and fermentable sugar yield, solving two key problems with the production of lignocellulosic ethanol.

Taken togather, isolate *Aspergillus terreus* AP02 exhibits higher lignocellulolytic enzyme production than most reported fungal strains, with its well-balanced cellulolytic

enzyme profile. The optimized IPS process engineered in this research addresses major shortcomings in biomass conversion, especially through the optimized pretreatment and saccharification time, enzyme doses and PEG-6000 application. Future research should be directed towards process scaling up and studying strain improvement strategies to further improve enzyme yields and thermal stability.

Conclusion

This study highlights *Aspergillus terreus* AP02 isolate as a potential cellulase-producer's fungus able to effectively break down rice straw using its crude enzyme extract (AT-CS). The optimized IPS process allowed maximum reducing sugar production, which in turn was converted to 15.82 ± 0.84 g/L ethanol with a 93.93% efficiency by *S. cerevisiae* NCIM 3570. The results show an economical and environmentally friendly method for second-generation bioethanol production from crop residues, providing a promising strategy for lignocellulosic biomass valorization.

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