

Bioethanol Production from Rice Straw and Cellulose Degradation using *Aspergillus terreus* and *Trichoderma harzanium*

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On account of its minimal expense and a high potential for change into energy-producing products, lignocellulosic biomass is the future of bioenergy and energy. Notwithstanding, their true capacity is restricted by the utilization of inefficient and unstable enzymes. Therefore, the present work aim to investigate the cellulose degradation potential of two isolated fungal species *Aspergillus terreus* and *Trichoderma harzanium* followed by bioethanol production from acid-thermal pre-treated rice straw (RS). The experiments were conducted in two phases. In phase-I of experiments, the isolated cellulose-degrading fungal species (*Aspergillus terreus* and *Trichoderma harzanium*) were employed for enzymatic hydrolysis of pretreated RS which was separated into two fractions namely: (Hydrolysate liquid) HL and (Residual pulp) RP. In phase-II of experiments, the enzymatically hydrolyzed substrate was subjected to yeast fermentation for bioethanol production. Using 18S rRNA sequencing, the microbial diversity study of the isolated species is covered in detail. The results revealed that the isolated fungal species *A. terreus* and *T. harzanium* resulted in 80 % of cellulose degradation. The highest bioethanol yield of 0.38 and 0.42 g/g of glucose was obtained from HL using *Aspergillus terreus* and *Trichoderma harzanium* treatment followed by yeast fermentation. The bioethanol yield of 0.17 g/g of cellulose was obtained from HL using both the fungal species.

Keywords: *Aspergillus terreus*; Bioethanol; Cellulose; Rice straw; *Trichoderma harzanium*.

Fossil fuels represent >80% of the total energy supplies in the world. The transportation area consumes around 29% of this, provoking worries about fossil product supplies, as well as its effect on the greenhouse effect, global warming, and environmental change. The current trend is towards the development of new techniques using renewable energy sources such as solar, wind, geothermal, and biomass¹. Urbanization and

modern development, then again, increased garbage production, which has social, environmental, and economic consequences. It can affect human health and populace security assuming it is misused, as well as expanding the deficiency of unrefined components, water, and fossil fuels². It can likewise be monetarily disadvantageous on the off chance that the capital consumptions of new infrastructures are exceptionally high, and how much trash to be

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collected and treated surpasses the executive's capacity. Accordingly, bio-based arrangements are being looked to further develop energy security, lessen petroleum product reliance, diminish CO₂ emissions, and decrease waste generation. Because of its low-cost and high potential for transformation into energy-producing products, where lignocellulosic biomass is the future of bioenergy and fuels³. However, their potential is limited by the use of inefficient and unstable enzymes.

Cellulase is found in a variety of microorganisms, however, most of them are sluggish or unsuitable for industrial conversion. The development of an efficient process requires the selection of suitable microorganisms. The significant expense of cellulase enzymes is one of the main obstacles in the conversion of lignocelluloses to bioethanol. Cellulases have a great deal of potential in the modern world. Glucose produced from cellulosic substrate could be used as a substrate for additional fermentation or another interaction that produces significant-end products. Cellulases, like ethanol, methane, butanol, Single-cell protein (SCP), and others, have been utilized in waste-water treatment, food processing, detergent formulation, feed preparation, textile manufacture, and other industries for a very long time. An important stage in the real-time application is the screening of optimal enzymes with high catalytic activity and stability. Plant cellulose can be metabolized by a variety of bacteria and fungi⁴⁻⁵. The consolidated response of bacterial and fungal groups to cellulose in assorted soils, then again, stays unknown. Under both aerobic and anaerobic soil conditions, soil microorganisms can degrade cellulose. Therefore, bioethanol production could be a reasonable choice for actually using agricultural waste. As far as biomass accessibility, rice straw, corn straw, wheat straw, and sugarcane bagasse are the most widely recognized agricultural wastes. Besides, cellulases produced from different microbial sources contrast altogether with regards to strength, catalytic efficacy, and subsequently cellulose hydrolysis rate. A few techniques have been utilized to resolve this issue, including the quest for microbes with higher cellulase production capacities, the improvement of microbial cellulase synthesis

through growth parameter optimization, and the advancement of effective cellulolytic systems through hereditary and protein engineering, all of which can fundamentally diminish the expense of the saccharification process⁶⁻⁷.

Stalks, stems, leaves, seedpods, husks, bagasse, and roots are a portion of the agricultural waste feedstocks with a ton of guarantee for biofuel generation⁸⁻⁹. Bioethanol is one of the most common biofuels produced using lignocellulosic biomass. Due to its minimal expense and a high potential for change into energy-producing products, lignocellulosic biomass is the future of bioenergy and fuels. In any case, their true capacity is restricted by the utilization of wasteful and unsteady fuels. Cellulase is found in a variety of microorganisms, however, most of them are sluggish or unsuitable for industrial conversion. As a result, researchers are looking into the many habitats of cellulase-producing bacteria to find effective and innovative cellulase or glycoside hydrolases. Bacteria and fungi are the two microbial species that are capable to degrade products of cellulose such as cellobiose, especially glucose. *Actinobacteria* and *Streptomyces* are the two dominant cellulolytic community groups found in soil. Because most species of the *Aspergillus* genus (*Aspergillus niger*) can manufacture cellulases under a variety of conditions, this genus can remove and overwhelm the enzyme industry and is notable for producing high-quality cellulase¹⁰⁻¹².

After acid-thermal pretreatment, rice straw was separated into hydrolysate liquor (liquid fermentation) and residual pulp (solid-state fermentation) for bioethanol production. Therefore, the bioethanol production potential of the isolated fungal species for bioethanol production was evaluated using residual pulp and hydrolyzed liquid of rice straw as substrate. Using 18S rRNA gene sequencing analysis, the microbial diversity study of the isolated species is covered in detail. Therefore, the present work aims to look into the pretreated rice straw's ability to produce bioethanol. The two isolated cellulose-degrading fungi such as *Aspergillus terreus* and *Trichoderma harzanium* are used to examine the effects of an acid-thermal pre-treatment followed by enzymatic hydrolysis.

MATERIALS AND METHODS

Substrate collection

Dry rice straw (RS) of 1.5 kg was collected from the agricultural fields in the Warangal District of Telangana State, where the predominant cultivated crop is the paddy. Rice straw was cut into small pieces about 1 to 3 cm size using scissors before pretreatment. The raw RS was analyzed for total solids (TS), hemicellulose, volatile solids (VS), cellulose, lignin, nitrogen, carbon, hydrogen, and sulfur as per the standard methods.

Acid thermal pretreatment

Soaking the rice straw in the acidic solution followed by thermal pretreatment releases the monomeric units such as glucose from cellulose. Acid thermal pretreatment initially results in the breakdown of lignin bonds leading to the accessibility of cellulose for microorganisms to produce glucose which is one of the main feed components for the yeast to produce bioethanol. Therefore, acid thermal pretreatment was adopted in this study. Small pieces of 1 kg RS was soaked overnight in 7L of 1% H₂SO₄. The soaked RS in acid solution was then thermally pretreated in an autoclave at 1bar and 120°C for 20 minutes i.e., 1 cycle. The acid-thermal pretreated RS was pulpy and contained brownish liquid. The pretreated slurry was then filtered out to separate the hydrolysate liquid (HL) and the residual pulp (RP). Both the HL and RP were analyzed for physicochemical characteristics as per the standard analytical procedures mentioned in APHA¹³.

Isolation and identification of cellulose-degrading fungi

Isolation of cellulolytic fungi

The Comprehensive survey of different decomposing soils such as plant decomposing soil, vegetables decomposing soil, maize decomposing soil, paddy decomposing soil, wood decomposing soil, grass decomposing soil, flower decomposing soil, litter decomposing soil, sugarcane decomposing soil, dump yard decomposing soil and composting soil collected from different places of Warangal District, Telangana, India were analyzed for the presence of cellulose-degrading fungi. Each soil sample of 1.0 g was used for the serial dilution by using NaCl as diluent prepared with double distilled water up to 10⁻¹-10⁻⁸ dilutions.

Among these dilutions, 10⁻³ and 10⁻⁴ were taken for the isolation of different fungi by using the pour plate method containing potato dextrose agar. To accomplish uniform circulation of the sample, the media was tenderly poured into the petriplates and incubated at 27°C for 5 days. Antibiotics and rose bengal were used to reduce bacterial growth and fungal colonies. The fungi developed between the 3rd and 7th day were then isolated and identified by staining the isolated and cultured colonies with cotton blue and lactophenol by covering them with a glass coverslip. They were viewed under a light microscope with low power (10X) and high power (40X) objective lenses. A laboratory handbook for beginning mycology was used to identify morphological traits of fungus species¹⁴⁻¹⁵. The morphologically identified species were further confirmed by molecular identification using 18S rRNA gene sequencing analysis. The incidence, frequency, and abundance of individual fungi were also calculated in percentage using the following equations Eq. 1, Eq. 2, and Eq. 3.

$$\text{Incidence (\%)} = \left[\frac{\text{No. of colonies of a species in all the plates}}{\text{Total no. of colonies of all the species in all the plates}} \right] \times 100 \quad \dots(1)$$

$$\text{Frequency (\%)} = \left[\frac{\text{No. of observations in which a species appeared}}{\text{Total no. of observations}} \right] \times 100 \quad \dots(2)$$

$$\text{Abundance (\%)} = \left[\frac{\text{Total no. of colonies of a species in all the observations}}{\text{Total no. of colonies in all observations}} \right] \times 100 \quad \dots(3)$$

Identification and screening of cellulolytic fungi by Congo red assay

The isolated fungi were screened for cellulolytic properties by cultivating on Czapek's agar medium (Cellulose: 30 g; NaNO₃: 3 g; KH₂PO₄·3H₂O: 1.3 g; MgSO₄·7 H₂O: 0.5 g; KCl: 0.5 g; FeSO₄·7 H₂O: 0.001 g; ZnSO₄·7 H₂O: 0.001 g; CuSO₄·7 H₂O: 0.0005 g; Yeast extract: 5 g; 20 g agar-agar and 1000 mL deionized water and pH around 6.3±0.2) by Petri plate method. At 30±2°C, all of the plates were incubated for 4 days. All Petri

dishes were immersed with anaqueous solution of Congo red (1% percent w/v) for 15 minutes at the end of the incubation period, then Congo red solution was drained off. The plates were then subsequently treated by flooding them with 1 M NaCl for 15 minutes. The creation of a clear zone around the colonies is a cellulose degradation signal.

CMCase activity assay

Each fungal isolate was developed in a 150 mL Erlenmeyer flask containing 50 mL of basal salt medium modified with CMC (10 g/L) as the main carbon source to approve its cellulose-degrading limit. The pH of the medium was adjusted to 6.5. Every flask was inoculated with old PDA plate culture and refined for 5 days at $30\pm 2^\circ\text{C}$. The crude enzyme was separated prior to being centrifuged for 10 minutes at 11,000 rpm. The enzyme activity was resolved to utilize Miller's technique¹⁶, which comprised 0.2 mL of crude enzyme solution in addition to 1.8 mL of 1% carboxymethyl cellulose (CMC) in a 100 mM sodium phosphate cushion (pH 5.5). The reaction was then incubated in a water bath at 50°C for 25 minutes. The reaction was terminated by adding 3.0 mL of Dinitrosalysilic corrosive (DNS) reagent and setting the reagent tubes in a water bath at 100°C for 15 minutes to permit color advancement. Utilizing a standard curve, the OD was estimated at 575 nm, and the concentration of reducing sugars was assessed and computed as glucose.

Selection of strains for experimental studies

Among all the 50 selected fungal strains collected, 4403 colonies were isolated using different dilutions. Among 4403 colonies, 9 fungal species belonging to different genera were screened for cellulolytic properties. Based on the diameter of the cellulolytic zone that appeared on Czapek medium plates, two fungal species such as *Aspergillus terreus* strain and *Trichoderma harzianum* strain were selected for further studies (enzymatic hydrolysis and bioethanol production) and characterized phenotypically with the help of cultural and morphological characteristics. The morphological features were further confirmed by molecular identification using 18S rRNA sequencing analysis and the obtained sequences were deposited in NCBI.

Experimental design and operational procedure

The experiments in the present study were

conducted in two phases. In phase I of experiments, the isolated cellulose-degrading fungal species (*A. terreus* and *T. harzianum*) were employed for enzymatic hydrolysis of pretreated RS which was separated into two fractions namely: HL and RP. In phase II of experiments, the enzymatically hydrolyzed substrate was subjected to yeast fermentation for bioethanol production. The design of the experiments is shown in Fig. 1.0

Enzymatic pretreatment using isolated cellulose-degrading fungal strains: *Aspergillus terreus* and *Trichoderma harzianum*

The hydrolysate liquor (HL) and residual pulp (RP) were subjected to enzymatic hydrolysis using the isolated *A. terreus* and *T. harzianum* fungal strains. The experiments were performed in batch mode in 250 mL Erlenmeyer flask with an effective volume of 200 mL from here on referred to as 'Reactors', labeled as R1 for *A. terreus* and R2 for *T. harzianum* to investigate the impact of the isolated strains on the degradation of cellulose. Two control reactors R1-Control using *A. terreus* and R2-Control using *T. harzianum* were also operated in parallel using cellulose as a carbon source. Therefore, a total of 6 reactors namely R1-Control; R1-HL; R1-RP; R2-Control; R2-HL, and R2-RP were operated in parallel for 5 days at mesophilic temperature ($32\pm 2^\circ\text{C}$). The reactors were fed with 150 mL of HL in R1 reactors and 150 mL of RP in R2 reactors. The R1 reactors were inoculated with 50 mL of *A. terreus* fungal strain in the form of a broth (fungal strains enriched in cellulose-degrading media) and an R2 reactor with 50 mL of *T. harzianum* strain respectively. In the control reactor, about 10 g of cellulose was used as feed which was considered control in the phase I experimental setup. The initial and final samples on day 1 and day 5 respectively were analyzed for cellulose, glucose, volatile fatty acids and soluble Chemical Oxygen Demand (COD) content to understand the performance of isolated fungal strains in degrading cellulose.

Yeast fermentation of enzymatically treated HL and RP for bioethanol production

In the second phase of experiments, the enzymatically hydrolyzed substrate was subjected to yeast fermentation for the production of bioethanol¹⁷. Similar to the phase I experiments, a set of 6 reactors were operated in parallel to understand the bioethanol production potential

of the enzymatically treated substrate through yeast fermentation. Commercial grade yeast was purchased and enriched in a liquid medium containing yeast fermentation media. The supernatant liquid from both the reactors R1 and R2 was separated and fed to another set of reactors labeled R3 and R4. The supernatant from R1 (*A. terreus* treated) and R2 (*T. harzianum* treated) reactors were fed to R3 and R4 reactors respectively. The reactors (R1 and R2) containing HL and RP respectively were subjected to liquid and solid-state fermentation. The reactors R3 (control; HL and RP) and R4 (control; HL and RP) were inoculated with 50 mL of enriched yeast. The reactors were operated for 4 days to observe the bioethanol production from RS after enzymatic pretreatment. The initial and final samples on day 1 and day 5 respectively were analyzed for pH and bioethanol production to understand the efficiency of enzymatic pretreatment followed by yeast fermentation in bioethanol production.

RESULTS AND DISCUSSION

Characteristics of the raw substrate and impact of acid thermal pretreatment

The characteristics of rice straw before and after acid thermal pretreatment is tabulated and the average values are presented in Table 1.0. As can be seen from Table 1.0. the cellulose, hemicellulose, and lignin content in the acid-thermally pre-treated RS decreased from 34–24%; 23–13%, and 17–7% respectively. The carbon content in raw RS was found to be 41% suggesting that the RS is suitable for biofuel production. However, the presence of lignin hinders the easy accessibility of the cellulose and hemicellulose to the microorganisms for their conversion to its monomers for bioethanol production. The Chemical Oxygen Demand was 45 g/L in HL and 40 g/L in RP indicating that the pre-treated substrate is organic-rich. The VFA in HL and RP was 12 g/L and 7 g/L respectively. Overall, the acid-thermal pre-treatment has positively impacted the substrate in increasing the accessible organic matter in the form of soluble Chemical oxygen Demand, Volatile Fatty acids, and Volatile Solids which are essential food to microorganisms for bioethanol production.

Activity test of isolated cellulolytic fungal strains

Among all the 50 selected fungal strains

collected, 4403 colonies were isolated using different dilutions. The isolated microorganisms belonged to fungal species which represented 9 genera (*Alternaria*, *Aspergillus*, *Cladosporium*, *Curvularia*, *Fusarium*, *Mucor*, *Rhizopus*, *Penicillium*, and *Trichoderma*) among the isolates. Among 4403 colonies, 9 fungal species belonging to different genera were screened to be positive for cellulolytic properties. The growth region of the cellulolytic activity of the isolated fungal strains, stained on Congo red in the Czapek medium plates is shown in Fig. 1.2. Based on the diameter of the cellulolytic zone that appeared on czapek medium plates, two fungal species such as *A. terreus* and *T. harzianum* were selected for further studies and characterized phenotypically with the help of cultural and morphological characteristics. The diagnostic features were further confirmed by molecular identification using 18S rRNA sequencing analysis and the obtained sequences were deposited in NCBI with accession numbers MZ617278 and MZ620641.

Phylogenetic identification and genetic characterization of the isolated fungal strains using 18S rRNA sequencing

The 18S rRNA sequencing of the isolated fungal strains from soil samples was carried out and labeled as HJSMKU-01 and HJSMKU-02 respectively. The genetic characterization and the phylogenetic tree of the two isolated fungal species are shown in Fig. 1.3. Fig. 1.3(A) Fig. 1.3(B). The phylogenetic tree of both the isolated strains confirms the fungal strains isolated from the soil as *A. terreus* and *T. harzianum* respectively. These two fungal strains were known to degrade cellulosic material which is evident from the activity test and was capable of producing bioethanol. The gene bank submission number of both the species is SUB10066977 and SUB10067688 while the NCBI numbers are allocated for the isolated species from soil which were identified as *A. terreus* and *T. harzianum* are MZ617278 and MZ620641 respectively.

Performance evaluation of enzymatic pretreatment of the substrate using isolated strains

The performance evaluation of enzymatic pretreatment of RS (HL and RP) and synthetic cellulose (carbon source) using *A. terreus* and *T. harzianum* in terms of cellulose, glucose, Volatile

Fatty Acids, and soluble Chemical Oxygen Demand profiles is shown in Fig. 1.4. As can be seen from Fig. 1.4(a) that there is a considerable decrease in the cellulose content (%) in the substrate after enzymatic pretreatment. For instance, in control reactors R1 and R2 with synthetic cellulose as substrate, about 80 % of cellulose reduction was observed i.e., from 10 g to 8 g. The 80% reduction

in cellulose has resulted in 6 g/L of glucose conversion using both *A. terreus* and *T. harzanium*. Interestingly, the performance of *A. terreus* on the reduction of cellulose with HL and RP as substrate was 92% and 80% respectively. Improved cellulose reduction using HL in comparison to RP could be attributed to the fact that HL was in liquid form while RP was solid which might have decreased

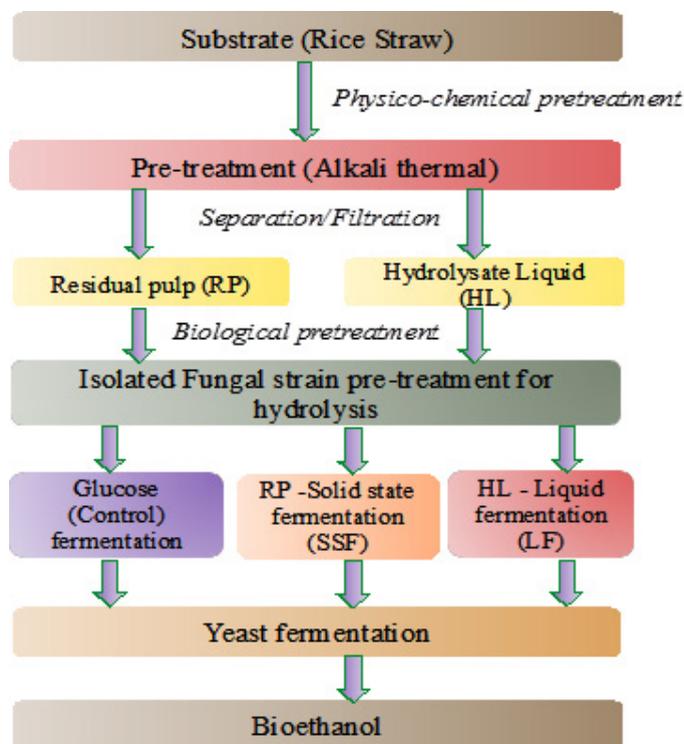


Fig. 1.0. Design of experiments for bioethanol production

Table 1.0. Raw and pre-treated slurry characteristics of the substrates

Parameter	Unit	Raw Rice Straw (RS)	Acid-thermal treated RS	Hydrolysate liquor (HL)	Residual pulp (RP)
Total Solids (TS)	%	94	15	9	75
Volatile Solids (VS)	%	85	13	8	70
Cellulose	%	34	24	18	32
Hemicellulose	%	23	13	6	23
Lignin	%	17	7	2.5	15
COD	g/L	ND*	ND*	110	140
VFA	g/L	ND*	ND*	12	7
sCOD	g/L	ND*	85	45	40
Carbon (C)	%	41	36	ND*	ND*
Hydrogen (H)	%	6.7	4.5	ND*	ND*
Nitrogen (N)	%	0.2	0.5	ND*	ND*
Sulfur (S)	%	0.2	0.1	ND*	ND*

*not determined

the accessibility of cellulose to the fungi. Similarly, the performance of *T. harzanium* on the reduction of cellulose with HL and RP as substrate was 93% and 82% respectively. A marginal difference in the cellulose reduction efficiency was observed in both species. However, there exists a difference in the formation of glucose from cellulose during enzyme hydrolysis i.e., 12.15 g/L and 16 g/L using *A. terreus* and 10.8 g/L and 21.6 g/L using *T. harzanium*. The main mechanism behind the conversion of cellulose to glucose using cellulose-degrading fungi is that the cellulose is a polymer consisting of monomeric glucose units linked by α -1, 4- glycosidic bonds in a linear mode which can only be accessible to microorganisms upon pretreatment. The breakdown of cellulose to glucose is the result of the coordinated activity of endocellulases, exocellulases (cellobiohydrolases, glucohydrolases), and beta-glucosidases. Endocellulases randomly hydrolyze the internal glycosidic bonds resulting in a rapid decrease in polymer length and a gradual increase in the reducing sugar concentration¹⁷⁻¹⁸. Exocellulases hydrolyze cellulose polymers by releasing mostly cellobiose from either the reducing or non-reducing ends, resulting in rapid reducing sugar release with little change in polymer length. On cellulose, endocellulases and exocellulases work together to form cellooligosaccharides and cellobiose, which are then broken into glucose by beta-glucosidase⁵⁻⁶. Therefore, cellulose which is the most abundantly available organic matter in agriculture wastes is a valuable raw material that can be used as feed or can be converted to fuel and chemicals.

The enzymatic pretreatment using *A. terreus* and *T. harzanium* on the soluble Chemical oxygen Demand and Volatile Fatty acids profile was illustrated in Fig. 1.4(b). VFAs are the short-chain carboxylic acids mainly comprising acetic acid, propionic acid, and butyric acid which can also be converted into bioethanol¹⁹. The sCOD which is the completed biodegradable fraction of COD has improved significantly after enzymatic hydrolysis in reactors operated with RP because the cellulose content in the RP was relatively higher than that of HL in the reactors. Therefore, the activity of *A. terreus* and *T. harzanium* was superior in the reactors with cellulose-rich substrate i.e., RP on the other hand, HL also contained cellulose which resulted in the sCOD but was relatively lower than the HL reactors. The percentage increase in sCOD in reactors fed with HL was 77% using *A. terreus* and 69% using *T. harzanium* whereas in reactors with RP, it was 55% and 68% using *A. terreus* and *T. harzanium* respectively. This illustrates that the activity of *T. harzanium* on RP was more while it was HL for *A. terreus*. In contrast to the sCOD profile, the VFAs were more in reactors fed with HL compared to RP.

Bioethanol generation potential of treated RS using solid-state and liquid state yeast fermentation

The performance evaluation of enzymatic pretreatment of RS (HL and RP) and synthetic cellulose (carbon source) using *A. terreus* and *T. harzanium* in terms of cellulose, glucose, VFA, and sCOD profiles is shown in Fig. 1.5. As can be seen from Fig. 1.5(a) the glucose formation

Table 1.1. Screening of cellulolytic activity

Isolated fungal species	Activity for cellulose degradation	Diameter of the cellulolytic zone (cm)
<i>Alternaria</i>	++	2.1
<i>Aspergillus</i>	+++	4.5
<i>Cladosporium</i>	+	1.5
<i>Curvularia</i>	++	2.5
<i>Fusarium</i>	+	0.8
<i>Mucor</i>	++	1.9
<i>Rhizopus</i>	++	2.8
<i>Penicillium</i>	+	1.1
<i>Trichoderma</i>	+++	4.2

Moderate growth: +; Good growth: ++; Maximum growth: +++

and reduction are inversely related, i.e., in control reactors the formation of glucose is relatively lower compared to HL and RP reactors but the conversion of glucose to bioethanol using yeast through fermentation process is more in control reactors followed by HL and RP reactors. This could be due to two main reasons; one is the substrate inhibition and the other is the presence/formation of other intermediates that might have hindered the process. The pH in the reactors after

A. terreus enzyme treatment was 3.8, 4.8, and 4.5 in control, HL, and RP reactors respectively whereas the pH is 5.0, 4.9, and 4.8 in *T. harzanium* reactors. However, after yeast fermentation, a reduction in pH was observed in all the reactors which are indicative of the production of bioethanol. As bioethanol can be produced at a pH range of 3.5 to 6.0 depending on the type of substrate and inoculum used. Comparable bioethanol production was observed in control, HL, and RP reactors.

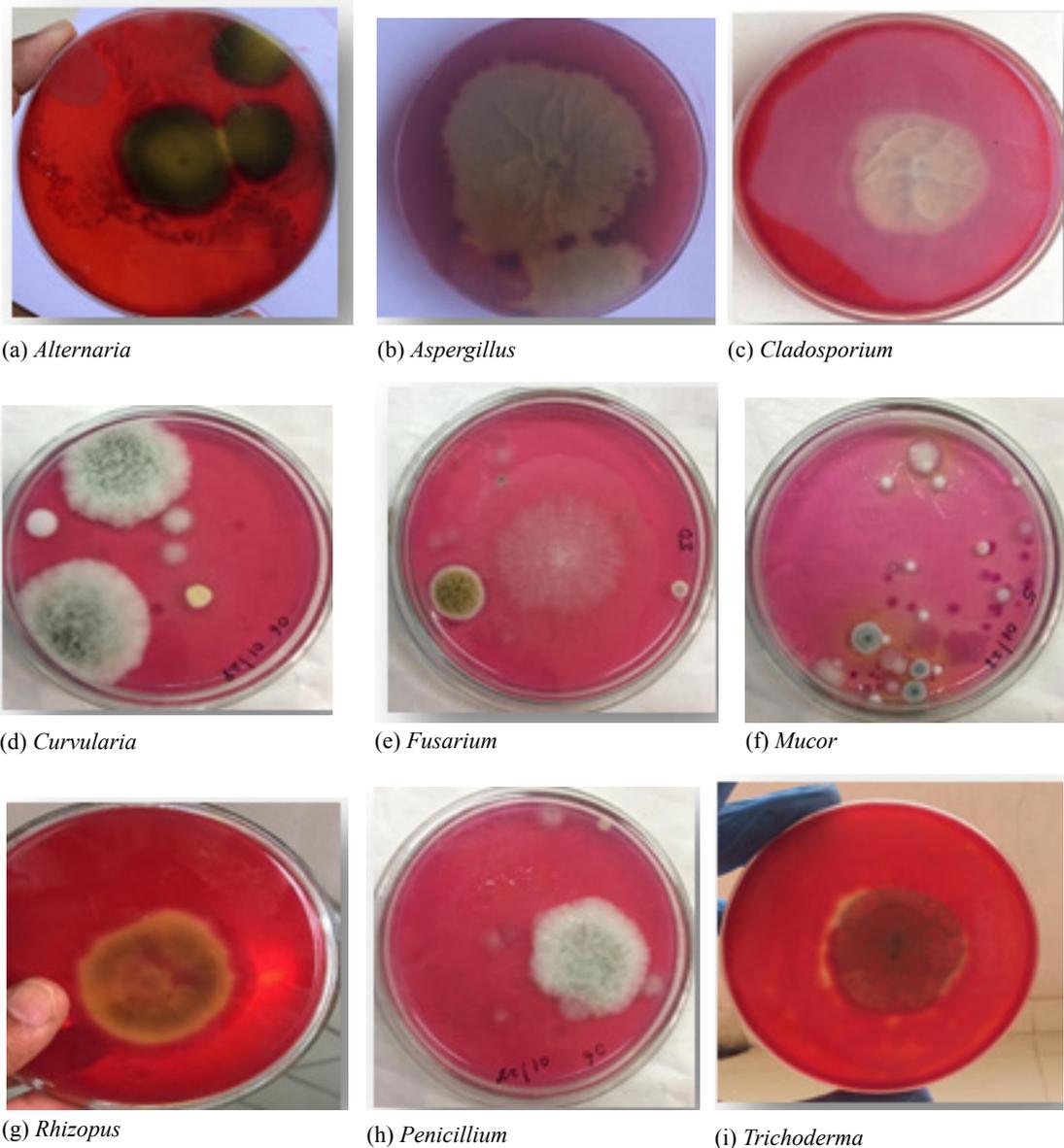
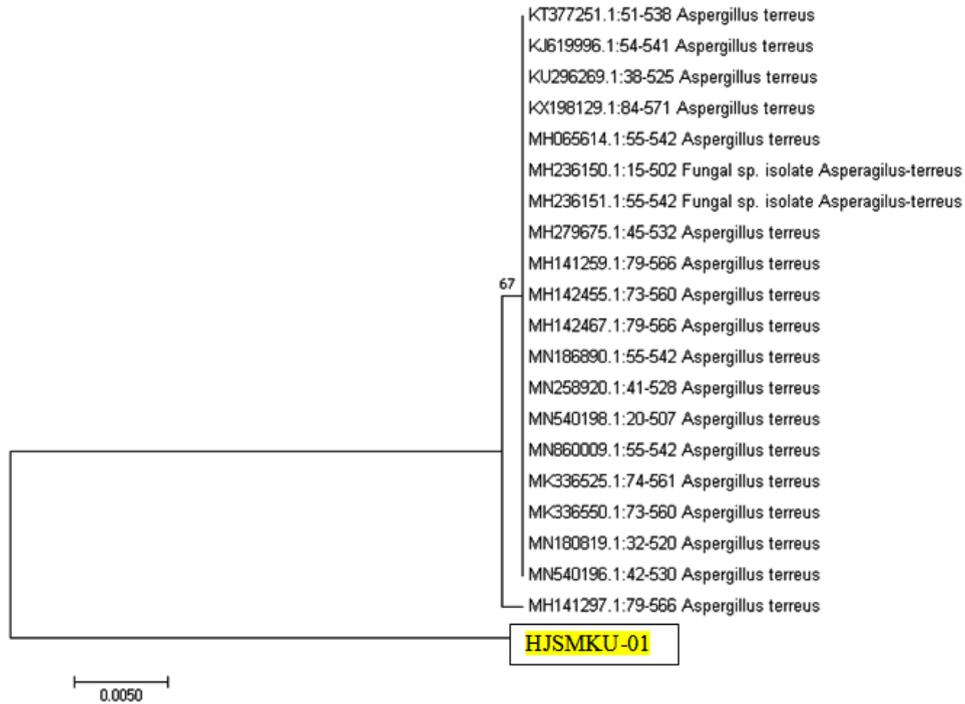
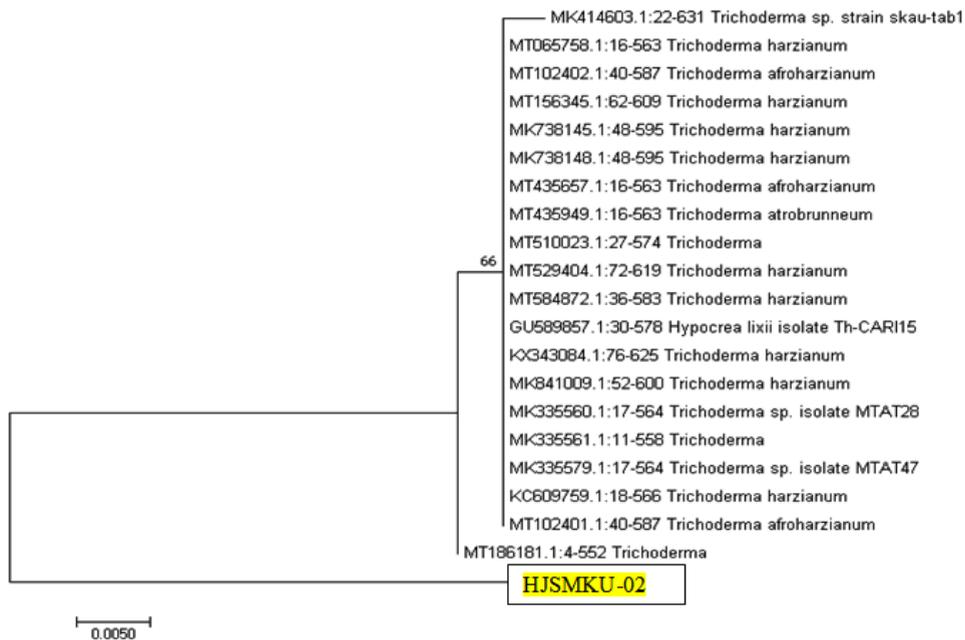


Fig. 1.2. Plates showing the growth of cellulolytic activity of the isolated cellulose-degrading fungi on Czapek medium



(A) Phylogenetic tree of isolated fungal strain *Aspergillus Terreus*

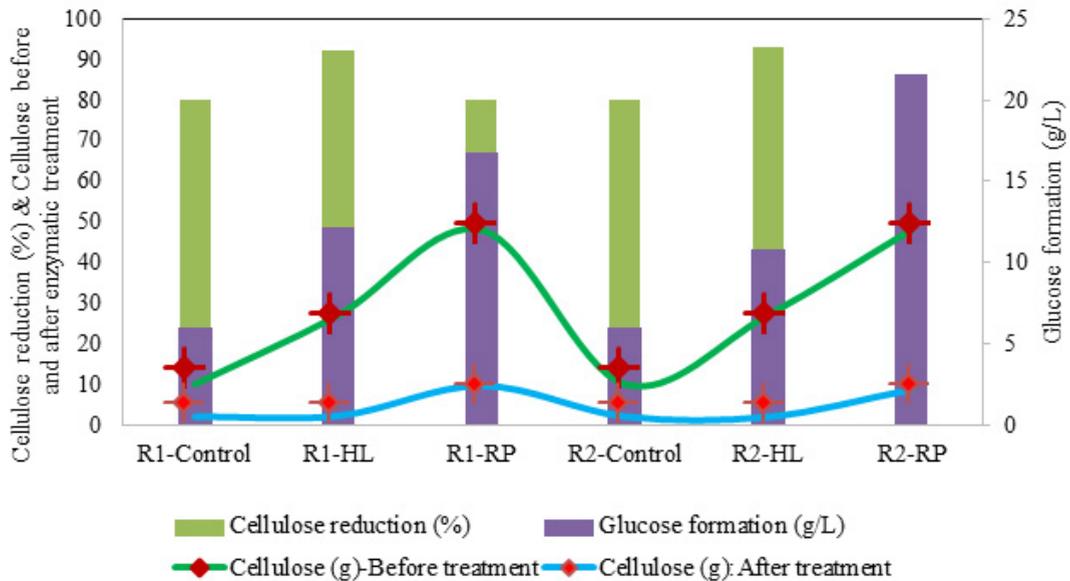


(B) Phylogenetic tree of isolated fungal strain *Trichoderma harzianum*

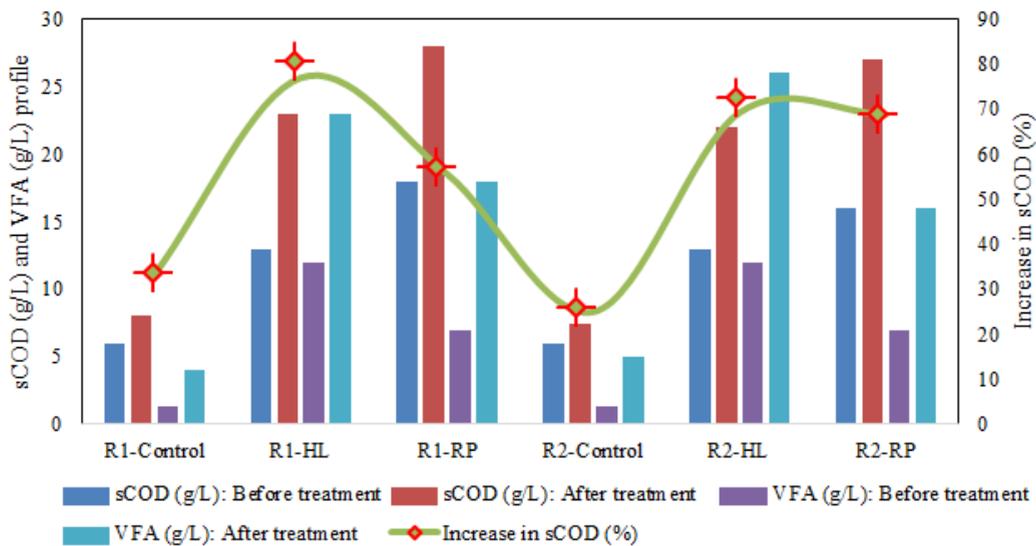
Fig. 1.3. Phylogenetic tree of the isolated fungal strains

However, the *T. harzanium* treated RP of rice straw has resulted in more bioethanol of 5.4 g/L whereas it is 4.7 g/L in the case of *A. terreus*. Interestingly, the performance of *A. terreus* and *T. harzanium* in the reactors with HL as the substrate is the same with 4.5 g/L of bioethanol production. The

control reactors with synthetic carbon sources have resulted in higher bioethanol production of 5.0 g/L and 5.5 g/L in both the cases of *A. terreus* and *T. harzanium* treatment which is obvious because the substrate was pure, unlike the other reactors which were fed to HL and RP of pretreated RS.



(a) Impact of pretreatment on cellulose degradation and conversion to glucose

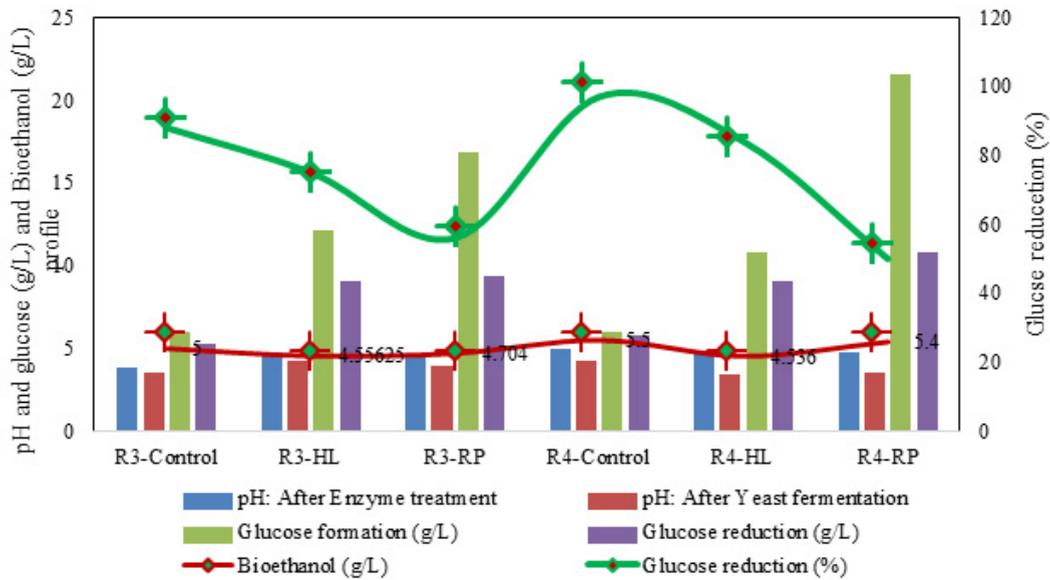


(b) Impact of pretreatment on cellulose degradation and conversion to glucose

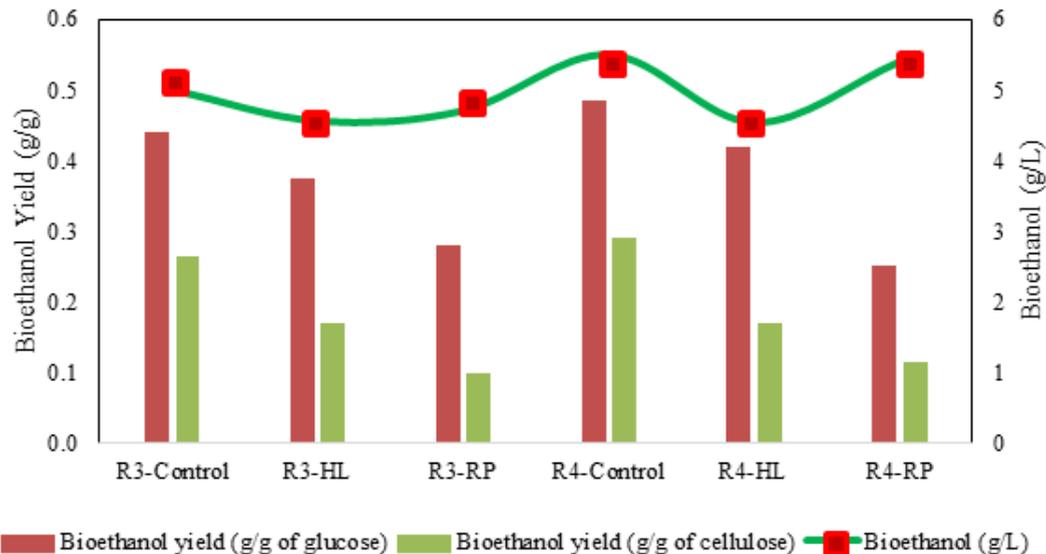
Fig. 1.4. Impact of acid thermal pretreatment followed by enzymatic hydrolysis on the degradation and conversion of cellulose to glucose, sCOD, and VFA

The bioethanol yield obtained from the reactors using yeast fermentation of the acid thermal followed by enzymatically treated fractions of RS (HL and RP) is reflected in Fig.1.5(b). As can be seen from Fig. 1.5(b) that the bioethanol yield of 0.44, 0.38, and 0.28 g/g of glucose was

obtained from reactors R3-control, R3-HL, and R3-RP whereas it was 0.49, 0.42, and 0.25 g/g of glucose in reactors R4-control, R4-HL, and R4-RP. The bioethanol yield of 0.26, 0.17, and 0.10 g/g of cellulose were obtained from reactors R3-control, R3-HL, and R3-RP whereas was 0.29, 0.17, and 0.10 g/g of cellulose were obtained from reactors R4-control, R4-HL, and R4-RP.



(a) pH and glucose profile during yeast fermentation



(b) Bioethanol yield profile during yeast fermentation

Fig. 1.5. Performance evaluation of yeast fermentation using enzymatically acid thermal followed by enzymatically treated HL and RP

0.11 g/g of cellulose in reactors R4-control, R4-HL, and R4-RP. In summary, it can be stated that the bioethanol production using synthetic cellulose as substrate and the application of acid-thermal followed by enzymatic pretreatment is superior compared to that of real substrates such as the fraction of RS i.e., HL and RP.

CONCLUSIONS

Valorization of agriculture wastes for bioethanol production by overcoming the substrate limitations for efficient degradation of cellulose and hemicellulose in the presence of recalcitrant lignin through the development of cellulose-degrading microorganisms is one of the promising approaches as the conversion of lignocelluloses to bioethanol using high-cost commercial cellulase enzyme makes the entire process expensive. Therefore, this study demonstrated the isolation, identification, and of two potential cellulose-degrading fungal species isolated from soil potential and tested for cellulose degradation of RS followed by bioethanol production via yeast fermentation. The 18S rRNA gene sequencing study showed that a total of 19 species capable of degrading cellulose were isolated and among them, about 9 species were found to be good for cellulose degradation. Among the 9 species, two species namely *Aspergillus terreus* and *Trichoderma harzanium* have shown maximum growth for cellulose degradation which were used as potential organisms in the study. Bioethanol production from the HL and RP of the acid-thermal followed by enzymatic hydrolysis pre-treatment of RS was used as feed. The isolated fungal species *A. terreus* and *T. harzanium* resulted in the degradation of cellulose by 80%. Yeast fermentation of hydrolyzed HL and RP resulted in the highest bioethanol yield of 0.38 and 0.42 g/g of glucose from HL using *A. terreus* and *T. harzanium* treatment. The bioethanol yield of 0.17 g/g of cellulose was obtained from HL using both the fungal species upon yeast fermentation.

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