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Screening and evaluation of cellulytic fungal strains for saccharification and bioethanol production from rice residue

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ABSTRACT

In this study, microwave-assisted alkali and acid pretreated rice straw *were* used to improve fermentable sugar yield by enzymatic saccharification (ES) employing cellulolytic fungal strains and subsequent bioethanol production by using fermenting yeast. The cellulolytic fungal strains *Trichoderma reesei* NCIM 1052, 1186, 992, *T. reesei* ITCC 4025, 6413, *Aspergillus niger* ITCC 302, *A. acculeatus* ITCC 5078, *A. fumigates* ITCC 4768 and *Fusarium Solani* ITCC 6397 were used for enzymatic saccharification (ES) of acid/alkali pretreated rice straw to optimize the sugar recovery. *T. reesei* NCIM 1052 was found superior as compared to the other fungal strains in terms of FPase, CMCase activities, and reducing sugars yield from pretreated rice straw. The strains of *Saccharomyces cerevisiae* NCIM 3186, and *Pichia stipitis* NCIM 3499 were used for subsequent fermentation to produce bioethanol. The saccharification of alkali pretreated rice straw (2% v/w NaOH) by *T. reesei* NCIM 1052 resulted in the highest fermentable sugar yield (55.6 g/l) and ultimately, the higher ethanol concentration after 72 h of fermentation with *P. stipitis* NCIM 3499 (25.3 g/L) as compared to other yeast strains. This study also exhibits the high potential for economic generation of ethanol from rice straw.

1. Introduction

Energy is most crucial for the socio-economic development of a nation. Fluctuation in the prices of crude oil severely affects the economics of the country, especially of developing countries like India. Since energy is mainly conventional fossil fuel-driven, which are limited, non-renewable resources. Therefore, researchers have to explore renewable sources of energy to meet demand worldwide. Previously, the maximum study has pointed to concentrate on producing an economical and eco-friendly bioethanol production means [1]. Currently, in vehicles, blended ethanol is used as an alternative fuel to raise the octane number and enhance fuel efficiency [2,3], also reducing CO_2 emission [4]. Though, bioethanol generation from agricultural and food crops such as maize, potato, and sugarcane (1st generation biofuels) has resulted in an unwanted direct conflict with the food supply and distribution [5]. A switch to a more plentiful non-edible crop matter should help to lessen the burden on the food crops. Therefore, with a remarkable production estimated at 1×10^{10} MT per annum worldwide [6], lignocellulosic biomass is considered as the only foreseeable, sustainable, feasible and renewable source of energy and value-added chemicals [7].

Consequently, the major emphasis is being given on lignocellulosic biomass, a viable feedstock for ethanol generation by the microbial fermentation [8]. In the past, farmers burned rice straw as the most common economical method of management. Presently, the burning of crop residues in the open field is a significant concern as it is creating several health issues [1,9]. Residue burning also contributes to global warming and have a negative impact on soil health. Now, agro-residues based energy generation is

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attracting the focus. Therefore, rice straw can be a potential source for our future energy requirements [3,10,11].

Rice is the most important cereal crop after wheat and corn, as per Food and Agriculture Organization (FAO) statistics, global consumption of milled rice in 2016–17 was about 477.77 MT [12]. Near about 1–1.5 kg of the straw is generated from each kilogram (kg) of paddy harvested. Globally, almost 478–716 MT of rice residue generated each year and used for several purposes. Moreover, the cellulosic part plant biomass still an untapped reservoir of sugars for an ethanol generation [8,13]. It has high cellulose and hemicelluloses content that can be quickly hydrolyzed into sugars for ethanol production. The chemical composition of rice straw contains almost 30–56% cellulose, 10–27% hemicellulose, 3–30% lignin, 3.6–7.2% protein, and 9% silica [14]. In hemicellulose, pentoses are dominant sugars, in which xylose (14.8–20.2%) is the essential sugar [15].

As compared to first-generation ethanol production, bioethanol from biomass requires a more complicated upstream process [13,16]. The components of biomass, such as cellulose and hemicellulose, are tightly bound by layers of lignin, which prevents the enzymatic hydrolysis [17,18]. Bioethanol production from rice straw mainly involves four steps: 1) Pretreatment to remove lignin 2) Enzymatic hydrolysis of pretreated biomass to produce sugar monomers 3) Fermentation of sugar monomers to ethanol by fermenting yeast 4) distillation. Pretreatment of rice straw has already been reported for help to break the lignin bonds to expose the cellulose and hemicellulose for the enzymatic reaction. The pretreatment methods are categorized as physical, chemical, and biological methods. Pretreatment is one of the costliest steps involved in the conversion of biomass to sugars [8,16] while present research on bioethanol is driven by the need to reduce the overall cost to make the process economically feasible and competitive. Therefore, an effective method of pretreatment is required to release the cellulose and hemicellulose. Several methods have been developed by the researchers to remove the lignin from lignocellulosic biomass. Most of the methods are having limitations like low sugar recovery, substantial capital investment, extreme reaction conditions, high processing cost, time-consuming, etc.

In acid pretreatment, the formation of inhibitors like furfural, 5-hydroxy methyl furfural, and high capital cost due to corrosion of equipment are the main limitations [19]. Oxidative pretreatment results typically in losses of carbohydrate polymers due to the non-selective nature of oxidative reagents [20]. Pretreatment with organic solvents is very costlier to be employed for biomass though pure lignin could be obtained as a byproduct through this processing technology [21]. Biological pretreatment is cost-effective and environment-friendly but unappealing for industries due to slow conversion rate [22,23]. Extensive reviews on pre-treatment process methods and the use of these technologies for pretreatment of various lignocellulosic biomass are given by Refs. [16,23–26].

Pretreatment with dilute acid includes the use of H_2SO_4, HNO_3, or HCl to break down the hemicellulose components and open cellulose for enzymatic digestion [16,27]. Microwave irradiation has been extensively applied due to its high heating efficiency and smooth operation. This method offers several advantages such as low energy requirements, uniform, and selective processing, ability to start and stop the process instantaneously and improved saccharification efficiency. Microwave irradiation could alter the ultrastructure of lignocellulosic materials and increase the susceptibility of the substrate by increasing the activity surface for enzymes [28]. Earlier studies Previous observed that microwave radiation could alter the supermolecular structure of lignocellulosic material to improve suitability and reactivity [29,30]. However, the traditional heating methods are slow in structural changes of biomass because of the low heating rate and the heating mode [31]. Pretreatment with alkali is more proper and economically feasible as it requires lower pressure and temperature conditions, and reduces the sugar degradation to a much greater extent as compared with acid pretreatment [32,33]. Alkali pretreatment involves salvation and saponification, leading into a swollen state of biomass with increased internal surface area and decreased the degree of polymerization [34,35] therefore, in this study; alkali pretreatment has been applied for delignification of straw. Pretreatment with alkali does not produce inhibitors like acid pretreatment, and sugar losses are also comparatively low. Therefore, a combination of microwave heating with acid/alkali can be a suitable alternative for pretreatment of lignocellulosics. Zhu et al. [30] have reported that microwave-assisted alkali pretreatment of wheat straw, lower sugars losses, and higher hydrolysis rates than conventional alkali pretreatment methods.

The microwave-assisted H_2SO_4 catalytic hydrolysis of rice straw was improved by Gong et al. [10] and 26.45% sugar yield achieved. Microwave pretreatment of crop biomass was examined by several researchers [30,36]. The improvement in enzymatic activity with the microwave-assisted acid treatment of switchgrass was conducted by Hu and Wen [31], who conferred a total sugar yield equivalent to 58.5% of the maximum potential sugar released. The quantity of inhibitors (furfural and 5-hydroxymethylfurfural) generated by alkali pretreatment is also almost negligible.

The enzymatic hydrolysis is economically cheaper as compared to acid or alkaline hydrolysis due to mild operating conditions [37]. During the enzymatic hydrolysis, cellulases degrade the cellulose to reducing sugars. Several microbial strains, especially *Trichoderma, Aspergillus*, and *Fusarium solani*, can produce cellulase and convert the lignocellulosic biomass to fermentable sugars for ethanol [38]. The enormous amounts of enzymes required for converting cellulose and hemicellulose to fermentable sugars impact severely on the cost-effectiveness of this technology [39–41]. Microwave-assisted acid or alkali pretreatment is regarded as beneficial in improving the efficiency of enzymatic hydrolysis. Therefore; the study has been undertaken with objectives to screen and evaluate the efficient cellulytic fungal strains for saccharification and bioethanol production from microwave-assisted acid and alkali pretreatment rice residue.

2. Material and methods

2.1. Chemical composition of rice straw

The samples of rice straw were collected from the research field after harvesting from the Indian Agricultural Research Institute (IARI) field, New Delhi. The rice straw was chopped into small pieces and oven-dried at 65 °C. The oven-dried biomass was ground by FOSS TECATOR CYCLOTEC 1093 sample mill and screened through a 20-mesh sieve. The ground residue samples were collected in a sealed plastic bag at normal room temperature for further analysis.

The cellulose, hemicellulose, and lignin fractions of rice straw was determined by using 2022 FOSS TECATOR Fibertec Analyzer (Foss Tecator Application note, AN 380). The Analysis of total soluble sugar (TSS) in rice straw was done by using the anthrone method [42].

2.2. Microbial cultivation and screening of cellulolytic strain for enzyme activity

For carrying out enzymatic hydrolysis, nine fungal strains, i.e., *T. reesei* ITCC 4025, *Trichoderma viride* ITCC 6413, *Aspergillus niger* ITCC 302, *Aspergillus acculeatus* ITCC 5078, *Aspergillus fumigatus*

ITCC 4768, *Fusarium solani* ITCC 6397 were procured from ITCC (Indian Type Culture Collection), Division of Plant Pathology, IARI, New Delhi and *T. reesei* strains NCIM 992, NCIM 1186 and NCIM 1052 from National Chemical Laboratory, NCIM (National Collection of Industrial Microorganisms), Pune were selected based on literature survey. All the stock cultures were sub-cultured on potato dextrose agar (PDA) (Hi-Media, India) slants, at 30 °C and sub-cultured after 7 days. Completely sporulated cultures received after 7 days were sub-cultured onto fresh PDA slants.

Screening of nine fungal strains was carried out to select effective strain for enzymatic hydrolysis of rice straw. To produce enzyme extract, 1.0 ml (approximately 2×10^6 spores/ml) spore suspension of respective fungal strains was added in 250 ml Erlenmeyer flask containing 100 ml potato dextrose broth and incubated at 30 °C for 5 days. Suspension of fungal spores was prepared by adding 5 ml distilled water to the slants, and the spores were dislodged with the help of inoculation needle under aseptic conditions. The suspension was appropriately diluted and was used as the inoculum. Spore count of the fungal suspensions was set to approximately 2×10^6 spores/ml using haemocytometer. The activity of Filter paper (exo-β-glucanase) and CMCase (endo-β-1, 4-glucanase) observed at pH 4.8 in sodium citrate buffer [43].

Reducing sugars were estimated by DNSA method [44]. One International Unit of FPase/CMCase was expressed as 1 μmole of glucose produced per minute during the hydrolysis process. The fungal strains were also screened for FPase activity on acid and alkali pretreated rice straw. For that, a loopful of fungal spore suspension was added into 10 ml PDB broth in 50 ml conical flasks. After attaining the growth, the cultures were transferred into 250 ml Erlenmeyer flasks containing pretreated substrate. Out of all pretreated sample, two were selected for comparing the best-reducing sugar-producing the enzyme, one from NaOH and one from H_2SO_4 treated samples which shown best fermentable sugar percentage recovery after scarification. The hydrolysis was run for 72 h, and the hydrolysate was taken out in a fixed time interval (1, 24, 48 and 72 h), and centrifuged at 8000 rpm for 10 min. The supernatant was stored at −20 °C for analysis of reducing sugars. All the trials were performed in triplicates, and average values are listed. The best activity cultures were selected by qualitative screening of hydrolytic enzymes.

2.3. Pre-treatment of rice straw

The microwave (LG WD700 MG-5062T microwave convection oven) assisted acid/alkali pretreatment done as follows: rice straw (1 g) containing glass beakers were immersed in dilute H_2SO_4 (v/v) and dilute NaOH (w/v) at concentration of 1, 2 and 3% and a solid loading was kept in 1:10 (solid: liquid) ratio. The mixtures were poured in 250 ml glass beaker, and the beaker placed at the center of a rotating circular glass plate in the microwave oven pretreatment as mentioned above. Pretreatments were carried at residence times of 1 and 2 min, respectively, with 100 °C and 140 °C temperature. After pretreatment, the material was neutralized by using sodium carbonate, and hydrochloric acid for H_2SO_4 and NaOH treated samples, respectively. The pretreated content was filtered through a Whatman filter paper no. 1. The residues were collected and appropriately washed 3–4 times by distilled water. The filtered residue was dried and kept at 4 °C for further enzymatic hydrolysis. Collected liquid fractions were centrifuged 4 °C at 4000 rpm for 4 min, and the supernatant liquid was used to determine the sugar content [45].

2.4. Enzymatic hydrolysis of pretreated straw

The enzymatic hydrolysis of pretreated rice straw with 2.0% alkali at 140 °C for 2 min was used to produce fermentable sugars. The alkali pretreated 5% w/v rice straw was supplied with 20FPU/g of the biomass of crude mixture of the enzyme (derived from *T. reesei NCIM- 1052* and *A. niger 302*) in citrate buffer (50 mM, pH 4.8). Sodium azide of 0.3% w/v is also added to the sample to prevent the infection. Enzymatic hydrolysis of pretreated samples was carried out in 250 ml Erlenmeyer flasks in an incubator cum shaker fixed at 55 °C and rotation speed 150 rpm. The hydrolysis was run for 64 h, and the hydrolysate was taken out in a fixed time interval (16, 32, 48 and 64 h), and centrifuged at 8000 rpm for 10 min. The supernatant was stored at −20 °C for analysis of liberated reducing sugars. All the trials were performed in triplicates.

2.5. Fermentation of rice straw hydrolysate

Total soluble sugars were estimated using the colourimetric method (anthrone method) as described by Thimmaiah [42]. For carrying out fermentation of sugar-rich hydrolysate to ethanol, yeast strains *S. cerevisiae* 3186 and *P. stipitis* 3499 were decided based on a literature survey and was obtained from the National Chemical Laboratory (NCL), Pune, Maharashtra. A loopful of culture from 24 h old cultures prepared on MGYP slant (10 g/l glucose; 3 g/l malt extract; 5 g/l yeast extract; 5 g/l peptone and 24 g/l agar) was inoculated in 100 ml MGYP broth (3 g/l malt extract; 10 g/l glucose; 5 g/l yeast extract and 5 g/l peptone) contained in 250 ml flask and incubated at 30 °C on the gyratory shaker for the time period of 12 h. Further, 50 ml medium in a 250 ml flask used to inoculate at the rate of 10% (v/v) til O.D. touched about 0.6. For fermentation of sugar-rich hydrolysate of rice straw, 10 ml of this culture was utilized to make 100 ml inoculum. The sugar-rich hydrolysate of rice straw containing flasks was inoculated with *S. cerevisiae* and *P. stiptis* @10% inoculum, pH was maintained 4.5, all the flasks were incubated at 30 °C, and the culture was left to develop for 72 h. Generated ethanol and residual sugars were estimated in the sample obtained at an interval of 5 h. The fermented material was examined for sugars.

2.6. Estimation of bioethanol production

The bioethanol concentration was analyzed by gas chromatography (Shimadzu GC-14B, Japan) with the specification as Solid phase: polyethylene glycol PEG-20 M, nitrogen as a carrier gas, isothermal packed column (90 °C), injection temperature 160 °C, flame ionization detector (FID) temperature 230 °C; and isopropanol as an internal standard). The conversion efficiency (CE)/yield efficiency (Ey) was measured as $Ey = Yps \times 100/0.51$, where, Yps is ethanol yield (g ethanol per g sugar utilized), and 0.51 is the maximum theoretical ethanol yield of glucose consumption [46]. Experimental data were analyzed as per standard statistical approaches. The results of three replicates were combined and shown as mean value ($n = 3$), and ± standard deviations (SD). SPSS package v.10 was used to interpret the data at $P = 0.05$.

3. Results and discussion

3.1. Composition of rice straw

In the collected rice straw samples, cellulose, hemicellulose, lignin, and ashes content were obtained as 36.2%, 24.2%, 16.1%, and 20.8% respectively. The cellulose and hemicellulose constitute near about 60.4% in rice straw.

3.2. Pretreatment of rice residue

The activity of enzymes on cellulosic fibres have been improved

by several physical, chemical, physico-chemical, and biological pretreatment techniques [16]. Dilute acids (H_2SO_4, HNO_3, or HCl) pretreatment applied to remove hemicellulose segments and open cellulose for enzymatic activity [27]. The ultrastructure of cellulosic fibers could be changed by microwave irradiation [47] to degrade lignin and hemicelluloses in residue and improve the enzymatic suitability [28].

3.2.1. Microwave-assisted acid and alkali pretreatment and sugar recovery

In this study, rice straw was immersed in dilute sulfuric acid (H_2SO_4) (v/v) at the concentration of 1, 2 and 3% at 10% solid loading in a flask with microwave treatment. These samples should keep for a period of 1 and 2 min at 100 °C and 140 °C temperature and 600 W of radiation. After treatment, collected solids were used for enzymatic hydrolysis. The microwave treatment could provide an enhancement in sugar recovery than the control. The results of the study are shown in Table 1. Results indicate that the among the varying concentration of acids, times and temperatures, pretreatment of rice straw by 2% of acid for 1 min at 140 °C showed maximum sugar yield 44.8 ppm followed by (27.4 ppm) 3% of acid for 1 min at 100 °C, and it was minimum (19.4 ppm) with 1% of acid for 2 min at 140 °C in the filtrate. Similarly, when rice straws were immersed in a dilute sodium hydroxide (NaOH) at the same conditions as mentioned in Table 1. Results indicate that the among the varying concentration of alkali, times and temperatures, pretreatment of rice straw by 2% of acid for 2 min at 140 °C showed maximum sugar yield (79.37 ppm) in the filtrate. Compared with acid, alkali pre-treatment seems to be the most effective approach in breaking the bonds between hemicellulose, cellulose, and lignin, avoiding fragmentation of the hemicellulose polymers [48]. Several researchers had also told that the NaOH yielded higher sugars of rice straw than H_2SO_4 pretreatment.

3.3. Screening of cellulase producing microorganisms

Cellulase production by different fungal strains was estimated by using enzyme assays. Full enzymatic hydrolysis of cellulose requires the synergistic performance of different kinds of enzymes, namely, β 1–4 exoglucanase, endoglucanase (CMCase), cellobiohydrolases (β glucosidase) [49,50]. Releases of cellulolytic enzymes are found to drive to the beginning of the attack on cellulosic fragments of lignocelluloses and increase the sugar yield infiltrates. The result of FPase activities by cellulolytic fungal strains from acid and alkali pretreated rice straw are presented in Table 2. The maximum and minimum FPase activities were shown by *T. reesei*, NCIM 1052 (5.45 IU/ml) and *A. niger* ITCC 302 (3.64 IU/ml) respectively at 48 h. The strain *T. reesei* NCIM 1186 showed higher FPase enzyme activity 5.37, IU/ml at 72 h of incubation. The result of FPase activities by cellulolytic fungal strains from alkali pretreated rice straw are presented in Table 2. The highest FPase activity was recorded by *T. reesei,* NCIM 1052, 5.06 IU/ml, followed by *T. viride* ITCC6413 (4.87 IU/ml) and *A. niger* ITCC-302 (4.832 IU/ml) and it was minimum by *A. acculatus* ITCC 5078 (3.475IU/ml) at 48 h of incubation. The result showed that the FPase activities by all cellulolytic fungal strains increased up to 48 h after that it was observed decreased at 72 h.

Table 2
FPase activities by different fungal strains from alkali pretreated rice straw.

Fungal strain	FPase (IU/ml)							
	Acid pretreatment				Alkali pretreatment			
	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
T. reesei ITCC- 4025	0.77	3.76	5.22	4.81	0.34	3.92	4.54	2.13
T. reesei NCIM- 1186	0.05	5.15	4.88	5.37	0.33	3.59	4.23	2.28
T. reesei NCIM- 1052	0.43	5.33	5.45	4.89	0.36	3.88	5.06	2.71
T. reesei NCIM- 992	0.73	3.91	4.27	3.76	0.34	3.60	4.34	1.97
T. viride ITCC- 6413	0.30	3.93	5.07	2.86	0.34	3.98	4.87	2.16
A. acculatus ITCC- 5078	0.42	3.95	4.39	2.66	0.32	3.19	3.48	1.75
A. niger ITCC- 302	0.85	4.82	3.64	4.06	0.34	3.98	4.83	2.16
F. solani ITCC- 6397	0.99	4.98	3.98	3.48	0.31	3.36	4.58	1.83
A. fumigatus ITCC- 4768	0.58	4.41	3.98	3.26	0.33	3.73	4.12	2.03

The result of CMCase activities by cellulolytic fungal strains from acid pretreated rice straw are presented in Table 3. The highest CMCase activity was shown by *A. niger* ITCC 302 (6.29 IU/ml), followed by *F. solani* ITCC 6397 (6.09 IU/ml) and it was recorded

Table 3
CMCase activities by different fungal strains from alkali pretreated rice straw.

Fungal strain	CMCase (IU/ml)							
	Acid pretreatment				Alkali pretreatment			
	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
T. reesei ITCC- 4025	0.58	3.08	3.67	4.21	0.13	4.91	5.82	3.39
T. reesei NCIM- 1186	0.42	3.55	3.71	2.99	0.55	4.10	6.71	3.53
T. reesei NCIM- 1052	0.99	3.55	5.20	3.42	0.43	4.55	5.81	6.40
T. reesei NCIM- 992	0.43	3.49	3.82	2.67	0.16	3.48	5.95	4.14
T. viride ITCC- 6413	0.42	5.04	2.43	2.16	0.67	3.18	3.13	4.74
A. acculatus ITCC- 5078	0.66	3.71	4.21	3.55	0.99	3.49	5.22	5.94
A. niger ITCC- 302	0.34	5.54	6.29	5.20	0.42	3.63	4.39	3.09
F. solani ITCC- 6397	0.51	2.67	6.09	4.21	0.58	3.63	4.25	3.66
A. fumigatus ITCC- 4768	0.17	2.16	3.03	2.43	0.08	4.29	6.22	3.44

Table 1
Effect of microwave-assisted acid pretreatment on sugar yield.

Acid/alkali (%)	Time (minute)	Temperature (°C)	Total sugar (ppm)	
			Acid pretreatment	Alkali pretreatment
1	1	100	24.73^{f}	50.23^{g}
2	1	100	31.47^{e}	57.23^{f}
3	1	100	$28.74e^{f}$	55.81^{f}
1	2	100	32.93^{e}	69.37^{cd}
2	2	100	51.53^{b}	77.37^{a}
3	2	100	48.51^{bc}	73.63^{b}
1	1	140	39.81^{d}	55.13^{f}
2	1	140	31.47^{e}	67.17^{d}
3	1	140	33.07^{e}	60.23^{e}
1	2	140	45.80^{c}	73.56^{b}
2	2	140	55.27^{a}	79.37^{a}
3	2	140	39.82^{d}	71.63^{bc}
LSD (P = 0.05)	–	–	3.45	2.67

Values with different superscripts within column are significantly different at 0.05 level.

minimum by *T. viride* ITCC- 6413 (2.43 IU/ml) at 48 h of incubation. The maximum CMCase activities by all cellulolytic fungal strains were observed at 48 h after that activity decreased at 72 h. The effect of acid and alkali pretreatment of rice straw on CMCase activities by cellulolytic fungal strains is presented in Table 3. The highest CMCase activity shown by *T. reesei, NCIM* 1186 (6.71 IU/ml), followed by *A. fumigatus* ITCC 4768 (6.28 IU/ml) with alkali pretreated rice straw at 48 h of incubation. The higher cellulolytic activity was shown by all the fungal strains with alkali pretreated rice straw as compared to acid pretreated. FPase, CMCase activities from and *T. reesei and A. niger were* in agreement with the reported by other works [50,51].

The result of FPase, CMCase activities, and reducing sugars yields by cellulolytic fungal strains from acid pretreated rice straw are presented in Fig. 1. Though the highest CMCase activity (6.28 IU/ml was showed by *A. niger* ITCC 302 but *T. reesei* NCIM 1052 responded better in terms of FPase, CMCase activities and reducing sugars yields after 48 h incubation (Fig. 1). This may be because of better suitability of *T. reesei* NCIM 1052 with the acidic condition for those activities. The result of FPase, CMCase activities, and reducing sugars yields by cellulolytic fungal strains from alkali pretreated rice straw are presented in Fig. 2. *Trichoderma reesei,* NCIM 1186 responded better in terms of FPase, CMCase, and reducing sugars yield followed by *F solani* at 48 h incubation (Fig. 2). This may be because of better suitability of *T. reesei* with the acidic and alkali condition for those activities. On the basis of recorded observation of enzyme assays i.e., FPase, CMCase activities and reducing sugars yields (Figs. 1 and 2) by cellulolytic fungal strains from acid and alkali pretreated rice straw, out of nine, two efficient fungal strain *Trichoderma reesesi 1052 and Aspergillus niger 302* were taken for further experiments of Saccharification and fermentation study along with control.

3.4. Saccharification and sugar yield from alkali pretreated rice straw

The total reducing sugars liberated from hydrolysis of pretreated rice straw (by 2.0% alkali at 140 °C for 2 min) by the enzymes produced by both fungal strains are presented in Table 4. The highest amount of total reducing sugars was obtained in the hydrolysate of rice straw was 57.7 g/l (*Trichoderma reesesi 1052)* followed by *51*.3 g/l *(Aspergillus niger 302)* after 64 h saccharification. Total reducing sugars liberated from hydrolysis of pretreated rice straw from *T. reesei and A. niger* were in agreement with the reported by other works [50,51].

Observations on saccharification and sugar yields by fungal strain *Trichoderma reesesi 1052 and Aspergillus niger 302* along with control are presented in Fig. 3. Saccharification of rice straw by *T. reesei* NCIM 1052 recorded higher reducing sugar for ethanol production. The concentration of reducing sugar touched 55.6 g/l by saccharification from 100 g rice straw after 48 h, and extended time beyond 48 h helped little in improving the reducing sugars, this may be because of decrease in FPase and CMCase activities (Tables 4 and 5).

3.5. Fermentation of saccharified hydrolysate and ethanol yield

The saccharified reducing sugar-rich hydrolysate was utilized for ethanol generation as the fermentation medium. The results indicated that the *P. stipitis NCIM* 3499 (25.3 g/l) has higher ethanol production potential than the *S. cerevisiae NCIM 3186* (22.6 g/l) from the sugar-rich hydrolysate of rice straw (100 g). Results of ethanol yields by yeast strains *P. stipitis NCIM* 3499 *and S. cerevisiae NCIM 3186* are presented in Fig. 3. It is found that after completion of fermentation (72 h); from 57.7 g/l reducing sugar from 100 g rice straw (filtrate) was fermented by *P. stipitis NCIM* 3499, and found able to produced 25.3 g/l ethanol, equivalent to 86.9% of the theoretical yield, while *S. cerevisiae NCIM 3186* was found competent to produced 22.6 g/L ethanol, equal to 86.9% of the theoretical yield. The kinetic parameters were in agreement with the reported by other works [11,33,38,46]. The ethanol yield (EY), fermentation efficiency (FE) and residual sugar (RS) or sugar consumption, yeast strain *P. stipitis* showed higher values than the *S. cerevisiae*, and this may be because of mixed sugars utilization from rice straw by *P. stipitis* than the *S. cerevisiae*. The high value of ethanol yield conversion and fermentation efficiency also proves the efficiency of the yeast *P. stipitis NCIM* 3499 to ferment biomass, which contains pentose sugars also. The higher possible ethanol conversion and fermentation efficiency have also been reported by Huang et al. [52] using a different *P. stipitis* strain with NaOH-pretreated rice straw

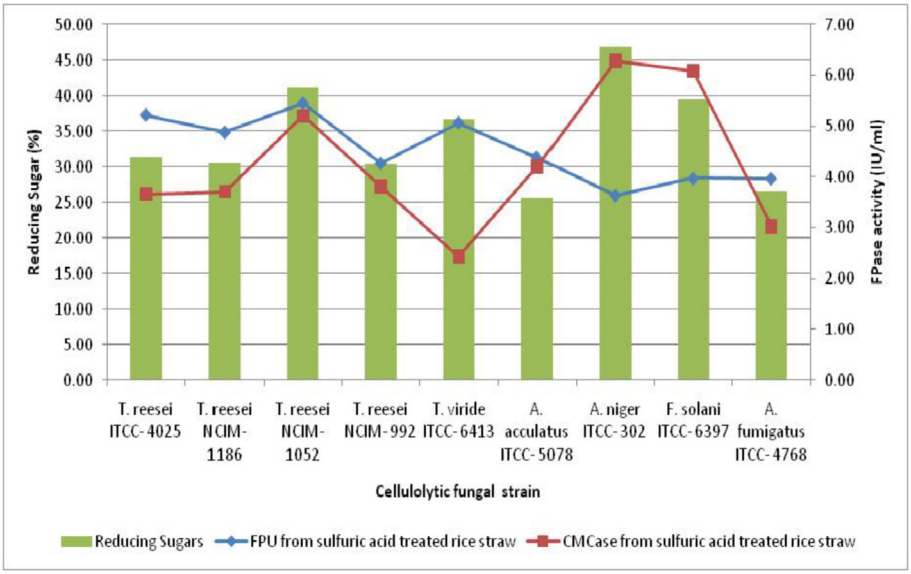

Fig. 1. FPase, and reducing sugars from acid pretreated rice straw.

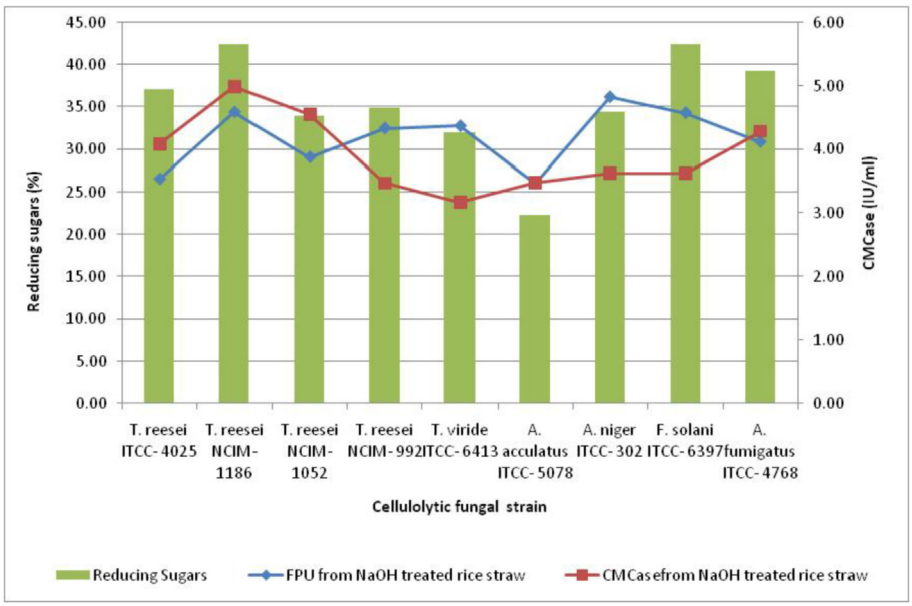

Fig. 2. CMCase and reducing sugars from alkali pretreated rice straw.

Table 4
Saccharification and sugar yield from alkali pretreated rice straw.

Fungal Strains	Total soluble sugars (g/l)			
	16 h.	32 h.	48 h.	64 h.
T. reesei ITCC- 4025	6.36 ± 0.53	42.32 ± 1.39	47.28 ± 1.24	48.63 ± 0.96^{cd}
T. reesei NCIM- 1186	6.90 ± 0.40	41.65 ± 1.53	46.35 ± 1.53	47.35 ± 1.08^{d}
T. reesei NCIM- 1052	9.70 ± 0.79	48.10 ± 1.21	55.62 ± 1.64	57.66 ± 1.28^{a}
T. reesei NCIM- 992	6.94 ± 0.49	41.86 ± 1.10	48.42 ± 0.89	49.73 ± 1.41^{bc}
T. viride ITCC- 6413	7.33 ± 0.57	42.73 ± 1.42	48.44 ± 1.53	50.16 ± 1.43^{bc}
A. acculatus ITCC- 5078	6.06 ± 0.74	42.23 ± 1.03	45.83 ± 1.37	49.58 ± 1.39^{bc}
A. niger ITCC- 302	7.97 ± 0.56	43.73 ± 1.07	49.43 ± 1.51	51.26 ± 1.43^{b}
F. solani ITCC- 6397	6.93 ± 0.59	40.25 ± 1.29	46.35 ± 1.24	50.03 ± 1.25^{bc}
A. fumigatus ITCC- 4768	6.57 ± 0.29	39.66 ± 1.14	47.52 ± 1.51	48.32 ± 1.13^{cd}
Control	1.07 ± 0.27	2.17 ± 0.18	2.55 ± 0.39	2.81 ± 0.42^{e}
LSD ($P = 0.05$)	0.924	1.913	2.269	1.947

Results are expressed as the mean ($n = 3$) and ± standard deviations are given in parentheses and in the last column at the end of process, values with different superscripts are significantly different at 0.05 level.

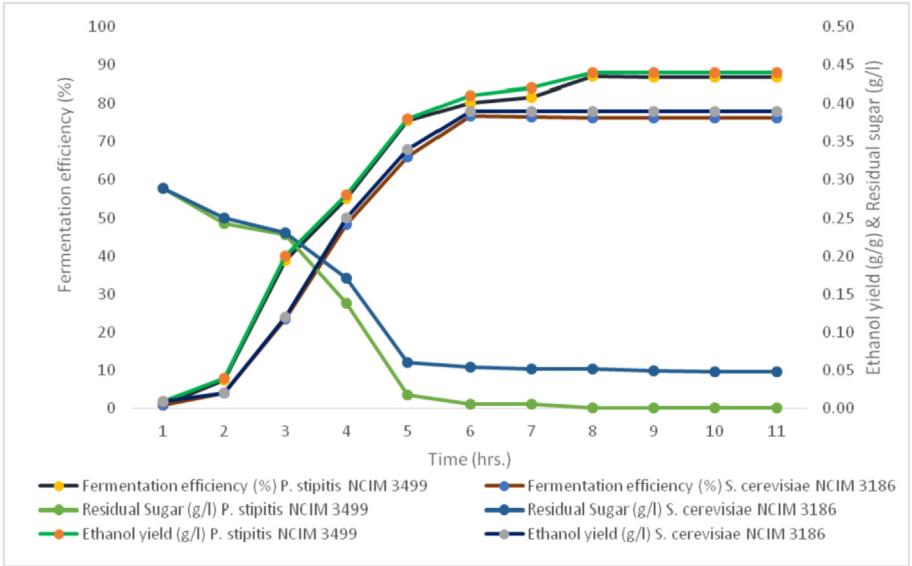

Fig. 3. Fermentation efficiency and ethanol yield by P. stipitis NCIM 3499 and *S. cerevisiae* NCIM 3186.

Table 5
Fermentation efficiency and ethanol yield yields by yeast strains.

Time (hrs.)	Ethanol yield (g/g)		Fermentation efficiency (%)		Residual Sugar (g/l)	
	P. stipitis NCIM 3499	*S. cerevisiae* NCIM 3186	*P. stipitis* NCIM 3499	*S. cerevisiae* NCIM 3186	*P. stipitis* NCIM 3499	*S. cerevisiae* NCIM 3186
0	0.01	0.01	1.02	1.02	57.7	57.7
5	0.04	0.02	7.48	4.08	48.5	50.0
10	0.20	0.12	38.74	23.45	45.7	46.2
15	0.28	0.25	55.05	48.26	27.7	34.2
20	0.38	0.34	75.44	65.93	3.7	12.2
25	0.41	0.39	80.20	76.80	1.3	10.8
30	0.42	0.39	81.56	76.46	1.3	10.5
40	0.44	0.39	86.99	76.12	0.3	10.3
50	0.44	0.39	86.66	76.12	0.3	10.0
60	0.44	0.39	86.66	76.12	0.3	9.8
72	0.44	0.39	86.66	76.12	0.3	9.8

hydrolysate. The observations indicated that the *P. stipitis* has excellent potential for ethanol generation from the sugar-rich hydrolysate of agri-residue.

4. Conclusion

The present investigation revealed that the utilization of microwave aided alkali pretreatment of rice straw for fermentable sugars and its potential for ethanol production would help in the use of ethanol as an alternative fuel. Pretreatment of rice straw significantly affected the saccharification by *T. reesei* NCIM 1052, which had recorded higher FPase and CMCase activities and shown maximum potential for generation of sugar from rice straw. The reducing sugar concentration reached 55.6 g/l by saccharification from 100 g rice straw after 48 h, and extended time beyond 48 h improved little the reducing sugars content. The yeast strain *P. stipites NCIM* 3499 (25.3 g/l) has higher ethanol production potential than the *S. cerevisiae* (22.6 g/l) and could be used for ethanol production from the sugar-rich hydrolysate of rice straw. Overall, rice straw is a viable feedstock for bioethanol generation from both the economic as well as environmental point of view.

4.1. Research gap and future recommendation

During the process of sugar recovery to ethanol production, several bottlenecks appear. Biomass to bioethanol conversion will only be a technical and economically viable option to first-generation bioethanol if suitable solutions are developed. There is a vast assessment required to see the current production difficulties and determine immediate and future research preferences. The optimization of the pretreatment and enzymatic processes could lead to further developments on the yields and efficiencies of the entire process. For improvement of cellulase efficiency and yield under adverse conditions can be maximized by using cellulolytic fungal strains for recovery of ethanol. The ethanol production from lignocellulosic biomass very costly because of the involvement of high enzymatic cost because biological enzymes production is not much efficient. This understanding will furnish a new approach to identify suitable pretreatment and hydrolysis process, viable strain identification to meet the global demand. In the present prospect, there is a need to develop a more efficient and effective cellulolytic strain from nature to reduce the cost as well as more sugar recovery with higher ethanol yield.

Declaration of competing interest

Authors have no conflicts of interest.

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Abbreviations

ADF	Acid Detergent Fiber
ADL	Acid Detergent Lignin
DNSA	2-Hydroxy-3,5-Dinitrobenzoic Acid
EY	Ethanol Yield
FAO	Food and Agriculture Organization
FE	Fermentation Efficiency
IARI	Indian Agricultural Research Institute
ITCC	Indian Type Culture Collection
NCIM	National Collection of Industrial Microorganisms
NDF	Neutral Detergent Fiber
PDA	Potato Dextrose Agar
PDB	Protein Data Bank
RS	Residual Sugar

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