Research Article

Assessment of Thermophilic Fungal strains for Cellulase Production using Chemical Pretreated Rice Straw

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Abstract

Three diverse habitats viz. rice cultivated soil, ruminant cud and spent mushroom compost were explored for the isolation of cellulase producing thermophilic fungi. From a total of 47 fungal strains, only two strains viz. CTS1 and CTS2 were found to be cellulolytic and thermophilic in nature. The maximum filter paper, carboxymethyl cellulase and cellobiase activities of 4.44, 9.19 and 3.79 U/g, were reported for *Aspergillus* sp. CTS 2 at 72 h of incubation using sequential acid-autoclave followed by alkali pretreated rice straw as substrate under solid state fermentation. Thus, *Aspergillus* sp. CTS 2 was found to be promising for cellulase production and its further utilization for enhanced saccharification and bioethanol production from rice straw.

Keywords: Thermophilic, Solid state fermentation, Filter paper, Carboxymethyl cellulase, Cellobiase

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Introduction

The most important application of cellulase is considered to be conversion of lignocellulosic biomass to fuel ethanol due to acute shortage of fossil fuels and ever-increasing energy demands [1]. However, the cost of the cellulase enzyme is the major barrier for the biofuel production [2]. The cost of cellulase enzyme production ranges from 25 to 50 per cent of total ethanol production costs based on submerged fermentation (SmF) technology [3]. The use of lower cost feed stocks like agricultural residues especially rice straw for cellulase production can help to reduce the cost of enzyme production required for conversion of lignocellulosic biomass into ethanol [4].Cellulase production by solid state fermentation (SSF) is rapidly gaining interest as a cost-effective technology as the microorganisms, especially fungal cultures produce comparatively high titres of cellulases as the conditions of fermentation shows similarity to the natural environment [5].

There has been more focus on thermophilic cellulases as the tolerance of high temperature improves the enzyme robustness and increase the reaction rate for industrial scale process, thereby decreasing the amount of enzyme needed. The other benefits of using thermostable cellulases are reduced likelihood of culture contamination, improved substrate accessibility and reduced viscosity of feedstock [6]. In the wake of industrial importance of thermostable cellulases, the present research work was planned to isolate and screen cellulase producing thermophilic fungal strains from natural habitats and production of thermostable cellulase using rice straw under solid state fermentation.

Materials and Methods

Isolation of fungi

The cellulase producing thermophilic fungi were isolated from natural habitats viz. rice cultivated soil, ruminant cud, and spent mushroom compost using serial dilution plating technique on potato dextrose agar (PDA) plates containing 10 µg/ml gentamicin. The plates were incubated at 45°C for 3-4 days in a BOD incubator. All morphologically-contrasting colonies were isolated and purified by repeated sub culturing on PDA plates. The pure cultures were maintained on PDA slants at 4°C for subsequent screening and enzymatic studies.

Plate screening of thermophilic cellulase producing fungi

Isolated fungal strains were tested for cellulase production by culturing on carboxymethyl cellulose (CMC) agar medium containing (g/l): $NaNO_3$, 2.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; carboxymethyl cellulose (CMC) sodium salt, 10.0; peptone, 0.2; agar, 20. The plates were incubated at 45°C for 3-4 days. After incubation, the plates were stained with 1.0 per cent congo red solution for 15 minutes and destained with 1.0 M NaCl solution after few minutes. The halo formation around the fungal colony was regarded as positive test for cellulase production. The diameter of halo around each colony was measured. The cellulolytic index (CI) was expressed as the ratio between the diameter of the halo and diameter of the colony [7]. The screened fungal strains were identified by observing their macroscopic (mycelium and spore colour, margins and colony elevation) and microscopic (microstructures) characteristics according to Gilman [8]. Smears of the fungi were prepared in lactophenol cotton blue and examined with 40X objective of a compound binocular microscope for microscopic appearance.

Pretreatment of rice straw

The rice straw was subjected to sequential acid-autoclave followed by alkali pretreatment. The acid-autoclave pretreatment was carried out by the method of Kocher *et al.* [9] with a slight modification. Five gram rice straw was soaked overnight in 75 ml of 1.0 per cent (v/v) H_2SO_4 and autoclaved at 15 psi, 121°C for 90 min. The solid residue was separated from the hydrolysate using filter paper (Whatmann No. 5), washed thoroughly with water and subsequently dried at 60°C in an oven. The dried solid residue was further soaked overnight in 2.0 per cent (w/v) NaOH (solid to liquid ratio, 1:15) and kept at 60°C for 60 min in a water bath. The acid/alkali pretreated residue was separated, washed thoroughly with water and dried overnight at 60°C in an oven. The chemical composition of the pretreated residue was analysed by the method of Goering and Van Soest [10].

Cellulase production under solid state fermentation

Solid state fermentation was carried out using untreated as well as pretreated rice straw and wheat bran in the ratio 4:1 (w/w). The substrate was moistened with Mandel Weber (MW) medium to attain moisture content of 80 per cent. The composition (g/l) of MW medium is as follows: $CaCl_2.2H_20$, 0.3; KH_2PO_4, 2.0; $MgSO_4.7H_20$, 0.3; $(NH_4)_2SO_4$, 1.4; $FeSO_4.\ 7H_20$, 0.005, $MnSO_4.7H_20$, 0.016; $ZnSO_4.\ 7H_20$, 0.014; $CoCl_2.\ 6H_2O$, 0.002; peptone, 1.0; Tween- 80, 1.0 ml and pH of the medium adjusted to 5.0. The flasks were inoculated with fungal spore suspension ($1x10^8$ spores/ ml) and incubated at 45°C for 96 h. The enzyme extraction was carried out by mixing the contents of inoculated flask with 0.1 M sodium citrate buffer (pH 4.8). The suspension was stirred at 100 rpm for 30 min in an orbital shaking incubator. The extract was filtered through double layered muslin cloth and centrifuged at 6,000 x g for 15 min. The supernatant, thus, obtained was considered as crude enzyme extract and used for cellulolytic enzyme assay.

Enzyme assays

Filter paper activity was determined by the method of Mandels [11] using Whatman No.1 filter paper strip (6 cm ×1 cm) dipped in 1.0 ml of sodium citrate buffer (pH 4.8) and 0.5 ml of crude enzyme extract. The tubes were vortexed to coil filter paper at the bottom of the tube and incubated in a water bath at 50°C for 1 h. The quantification of reducing sugars released was carried out by the DNS (3,5-dinitrosalicylic acid) method [12] using glucose standard curve. Carboxymethyl cellulase (CMC) activity was assayed in a reaction mixture containing 0.1 ml of crude enzyme and 0.9 ml of sodium citrate buffer (pH 4.8) containing 1.0 per cent CMC. The reaction mixture was incubated at 50°C for 30 min [11] and reducing sugars were estimated as described above. Cellobiase activity was determined by the method of Srivastava [13]. The reaction mixture contained 0.5 ml of enzyme extract and 0.5 ml of 0.05 per cent cellobiose solution. The reaction mixture was incubated at 50°C for 2 h and reducing sugars were estimated as described above. One unit of each enzyme activity was defined as amount of enzyme required to release 1μmol of reducing sugars (glucose equivalent) per minute under standard assay conditions and expressed as units per gram dry substrate (U/g).

Statistical analysis

All the experiments were carried out in triplicates with mean and standard deviation (SD) values calculated using MS Excel program.

Results and Discussion

Sequential pretreatment of rice straw

Sequential pretreatment of rice straw viz. acid-autoclave followed by alkali resulted in a decrease in lignin content from 11.5 per cent in untreated rice straw to 2.0 per cent in pretreated rice straw, while hemicellulose content decreased from 23.3 to 9.5 per cent. However, cellulose content increased from 39.5 per cent in untreated rice straw to 62.3 per cent in pretreated rice straw (**Table 1**). Dilute acid pretreatment solubilises hemicelluloses to xylan as well as xylose, a monomeric pentose sugar [14] while alkali pretreatment effectively removes lignin from biomass [15, 16]. Thus, sequential dilute acid and alkali pretreatment effectively removed hemicellulose and lignin with high cellulose content per gram of the straw. Weerasai *et al.* [17] similarly reported the solubilisation of hemicellulose by treating rice straw with 1.0 per cent (w/v) H_2SO_4 at 125°C for 10 min. The delignification was achieved by sequentially subjecting the straw to alkali treatment [1.25% (w/v) NaOH at 90° C for 10 min]. The sequential pretreatment resulted in enhanced digestibility of straw for bioethanol production. Sun *et al.* [18] obtained cellulose-enriched substrate to produce glucose for ethanol production by subjecting the rice straw to a combination of dilute acid (0.25–1.0 % aqueous H_2SO_4, 100–150 °C, 0.5–3.0 h) and alkali (1.5 % aqueous NaOH, 80 °C, 3 h) treatments. Similar studies on sequential acid and alkali preatment of the lignocellulosic biomass have been reported by many researchers [19-21].

Table 1 Chemical composition of chemical pretreated rice straw

Rice straw	Chemical composition (w/w, %)		
	Cellulose	Hemicellulose	Lignin
Untreated straw	39.5±0.20	23.3±0.21	11.5±0.17
Pretreated straw	62.3±3.31	9.5±0.52	2.0±0.11

Isolation and screening of cellulase producing thermophilic fungi

Forty seven (47) fungal strains were isolated from diverse habitats such as rice cultivated soil, ruminant cud and spent mushroom compost on PDA plates. The cellulolytic potential of these strains was identified by congo red plate assay. Among 47 fungal strains, only two strains viz. CTS1 and CTS2 were found to be cellulolytic and thermophilic in nature. As depicted in **Figure 1**, maximum cellulolytic index value of 1.7 was reported for fungal strain CTS 2 followed by fungal strain CTS1 with CI value of 1.5. The standard cultures of *Aspergillus fumigatus* MTCC 5862 and *Aspergillus terreus* MTCC 11778 were reported to have CI values 1.5 and 1.2, respectively. Jamroo *et al.* [22] screened cellulase producing thermophilic fungal isolates at different temperatures. Among 26 isolates, 8 were reported to be cellulase producing on the basis of cellulolytic index. For SW1 isolate, maximum cellulolytic index of 2.93 and 2.67 was observed at 37 ˚C and 50˚C, respectively.

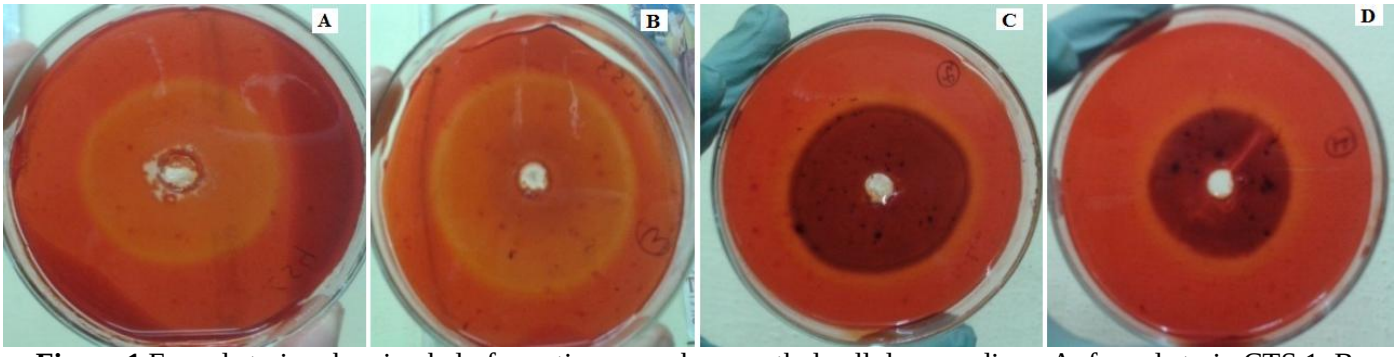

Figure 1 Fungal strains showing halo formation on carboxymethyl cellulose medium, A: fungal strain CTS 1; B: fungal strain CTS 2; C: *Aspergillus fumigatus* MTCC 5862; D: *Aspergillus terreus* MTCC 11778

The results of macroscopic and microscopic characteristics of fungal strains (CTS 1 and CTS 2) and the standard cultures (*Aspergillus fumigatus* MTCC 5862 and *Aspergillus terreus* MTCC 11778) are presented in **Table 2**. The fungal strain CTS 1 had white mycelium, upholstered with dull green spores, light yellow reverse side with prominent streaks and umbonate colony elevations. Light micrographs revealed branched septate hyphae, columnar conidial head and conidiogenous cells composed of biseriate philades covering globose shaped vesicle. The fungal strain CTS 2 had white mycelium, dull grey spores, yellow reverse side with tightened striations, and raised colony elevations.

The hyphae were branched septate, columnar conidial head and uniseriate philades covering dome shaped vesicle. On the basis of macroscopic and microscopic characteristics, two fungal strains were identified as *Aspergillus* sp.

Table 2 Morphological characterization of fungal strains

Fungal strains	Mycelium colour	Spore colour	Colony diameter (mm)	Halo diameter (mm)	Cellulolytic index (CI)	Colony characteristics	Microscopic characteristics
CTS 1	White	Dull green	42	64	1.5	Margins- entire, Reverse side- light yellow with streaks, Elevations- umbonate	Hyphae-branced septate, Conidial head- columnar, Vesicle–globose shaped, Philades- biseriate
CTS 2	White	Dull grey	37	64	1.7	Margins- entire, Reverse side- yellow with tightened striations, Elevations-raised	Hyphae-branced septate, Conidial head-columnar, Vesicle–dome shaped, Philades uniseriate
Aspergillus fumigatus MTCC 5862	White	Dull green	32	48	1.5	Margins- entire Reverse side- yellow, Elevations- umbonate	Hyphae-branced septate, Conidial head-columnar, Vesicle-dome shaped, Philades- uniseriate
Aspergillus terreus MTCC 11778	White	Golden yellow	50	60	1.2	Margins-entire, Reverse side- yellow, Elevations- umbonate	Hyphae-branced septate, Conidial head-compact columnar, Vesicle –globose shaped, Philades- biseriate

CTS 1 and CTS 2 are the abbreviations for cellulolytic thermophilic soil strains

The data pertaining to cellulase production by fungal strains (*Aspergillus* sp. CTS 1 and *Aspergillus* sp. CTS 2) and standard cultures (*Aspergillus fumigatus* MTCC 5862 and *Aspergillus terreus* MTCC 11778) under solid state fermentation using untreated and pretreated rice straw as substrate at different incubation periods are presented in **Table 3**. For all fungal strains, an increase in filter paper, carboxymethy cellulase and cellobiase activity using untreated rice straw as substrate was observed from 24 h up to 72 h of incubation and declined thereafter. The decline in enzyme activity after 72 h was explained on the basis of consumption of hydrolytic enzymes by fungi following the depletion of readily available sugars [23]. The maximum filter paper, carboxymethyl cellulase and cellobiase activity of 1.10, 2.06 and 0.82 U/g was reported by *Aspergillus* sp. CTS 2 followed by *Aspergillus* sp. CTS1 (0.96; 1.92 and 0.53 U/g filter paper, carboxymethy cellulase and cellobiase activity, respectively). The filter paper, carboxymethy cellulase and cellobiase activity of standard cultures of *Aspergillus fumigatus* was reported to be 0.98, 2.03 and 0.58 U/g, while for *Aspergillus terreus*, the respective activities were found to be 0.60, 1.92, 0.49 U/g at 72 h of incubation.

Similarily, for all fungal strains, an increase in filter paper activity, carboxymethy cellulase and cellobiase activity on pretreated rice straw was observed from 24 h up to 72 h of incubation and declined thereafter. The maximum filter paper, carboxymethyl cellulase and cellobiase activity of 4.44; 9.19, and 3.79 U/g was reported by *Aspergillus* sp. CTS 2 followed by *Aspergillus* sp. CTS 1 (4.10, 5.82 and 2.70 U/g of filter paper, carboxymethy cellulase and cellobiase activity, respectively). The filter paper, carboxymethy cellulase and cellobiase activity of standard cultures of *Aspergillus fumigatus* were reported to be 4.21; 8.69 and 3.68 U/g, while for *Aspergillus terreus*, the respective activities were found to be 3.50; 5.12 and 2.58 at 72 h of incubation. An improvement in enzyme activities (filter paper, carboxymethy cellulase and cellobiase) was observed using pretreated rice straw as substrate under solid state fermentation. This can be explained on the basis of alteration in complex structure of rice straw as a result of pretreatment that eventually supported the growth of fungal biomass and facilitated the enzyme production. Salihu *et al.* [24] subjected eleven agricultural residues to three pretreatments viz. acid, alkali and oxidative methods and

studied growth of *Aspergillus niger* on the pretreated residues up to 96 h of incubation. A maximum carboxymethyl cellulase, filter paper and β-glucosidase yields of 9.91, 6.20 and 5.69 U/g, respectively was reported on alkali-treated soybean hulls.

Table 3 Cellulase production by fungal strains on untreated and pretreated rice straw at different incubation times

Fungal strains	Incubation time (h)	Enzyme activity (U/g)					
		Filter paper		Carboxymethyl cellulase		Cellobiase	
		Untreated	Pretreated	Untreated	Pretreated	Untreated	Pretreated
Aspergillus sp. CTS 1	24	0.12±0.006	2.14±0.118	0.93±0.051	4.51±0.248	0.42±0.023	2.50±0.136
	48	0.31±0.017	2.78±0.153	1.82±0.100	4.86±0.267	0.46±0.025	2.53±0.139
	72	0.96±0.053	4.10±0.226	1.92±0.106	5.82±0.320	0.53±0.029	2.70±0.149
	96	0.43±0.024	1.38±0.076	1.05±0.058	4.23±0.233	0.26±0.014	2.10±0.116
Aspergillus sp. CTS 2	24	0.19±0.01	2.46±0.135	1.82±0.100	5.47±0.300	0.49±0.027	2.18±0.120
	48	0.55±0.03	3.14±0.173	1.94±0.107	7.67±0.422	0.71±0.039	2.88±0.158
	72	1.10±0.061	4.44±0.244	2.06±0.113	9.19±0.505	0.82±0.045	3.79±0.208
	96	0.80±0.044	2.01±0.111	1.15±0.063	4.83±0.266	0.34±0.019	2.29±0.126
Aspergillus fumigatus MTCC 5862	24	0.16±0.009	2.34±0.129	1.00±0.055	5.24±0.289	0.41±0.023	2.44±0.134
	48	0.43±0.024	3.42±0.189	1.56±0.086	7.31±0.402	0.48±0.026	2.54±0.139
	72	0.98±0.054	4.21±0.232	2.03±0.112	8.69±0.478	0.58±0.032	3.68±0.202
	96	0.71±0.039	1.81±0.996	1.10±0.061	5.34±0.289	0.32±0.018	2.22±0.122
Aspergillus terreus MTCC 11778	24	0.10±0.006	1.35±0.074	1.16±0.064	4.25±0.234	0.31±0.017	2.38±0.131
	48	0.26±0.014	2.21±0.121	1.78±0.098	4.78±0.263	0.46±0.025	2.50±0.138
	72	0.60±0.033	3.50±0.193	1.92±0.106	5.12±0.286	0.49±0.027	2.58±0.142
	96	0.48±0.026	1.30±0.072	1.04±0.057	3.77±0.207	0.31±0.014	2.10±0.116

Conclusion

The present study resulted in the selection of two efficient cellulase producing thermophilic fungal strains which are being standardized for saccharification and fermentation parameters to find their future potential for bioethanol production.

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