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Endocellulase Production by *Cotylidia pannosa* and its Application in Saccharification of Wheat Bran to Bioethanol

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Abstract

For efficient bioconversion of lignocellulosic materials to bioethanol, the study screened 19 white-rot fungal strains for their endocellulolytic activity and saccharification potential. Preliminary qualitative and quantitative screening revealed *Cotylidia pannosa* to be the most efficient endocellulase producing fungal strain when compared to the standard strain of *Trichoderma reesei* MTCC 164. Ensuing initial screening, the production of endocellulase was further optimized using submerged fermentation to recognize process parameters such as temperature, time, agitation pH, and supplementation of salts in media required for achieving maximum production of endocellulase. The strain *C. pannosa* produced the maximum amount of endocellulase (8.48 U/mL) under submerged fermentation with wheat bran (2%) supplemented yeast extract peptone dextrose (YEPD) medium after an incubation time of 56 h at 30 °C and pH 5.0 at an agitation rate of 120 rpm with a saccharification value of 50.5%. The fermentation of wheat bran hydrolysate with *Saccharomyces cerevisiae* MTCC 174 produced 4.12 g/L of bioethanol after 56 h of incubation at 30 °C. The results obtained from the present investigation establish the potential of white-rot fungus *C. pannosa* for hydrolysis and saccharification of wheat bran to yield fermentable sugars for their subsequent conversion to bioethanol, suggesting its application in efficient bioprocessing of lignocellulosic wastes.

Keywords *Cotylidia pannosa* · Endocellulase · Saccharification · Bioethanol · Lignocellulose

Abbreviations

CMC	Carboxymethyl cellulose
SHF	Separate hydrolysis and fermentation
PAGE	Polyacrylamide gel electrophoresis
YEP	Yeast extract peptone

Introduction

Fossil fuels are an expensive, non-renewable source of energy which are associated with negative influence on the environment. Their high demand has resulted in their diminution at a great pace which has necessitated the search for alternative fuels. For an alternative fuel to be a feasible substitute it should be renewable, sustainable, cost-effective, convenient, and safe for the environment. Bioethanol is one such promising alternative fuel which is renewable and offers several distinct advantages [1]. First generation biofuels involved the production of bioethanol from major food crops such as corn, barley, oats, rice, wheat, sorghum, and sugarcane etc. The birth of second-generation biofuels occurred in an attempt to avoid competitiveness between human food use and industrial use of crops [2]. Like many other countries, the Government of India has launched several initiatives to encourage and support research in the development of second-generation biofuels [3]. Among the various non-food crops employed to produce bioethanol, lignocellulosic biomass is the most profuse and inexpensive source available. A substantial amount of lignocellulosic material is generated as by-products through agricultural practices primarily from various agro-based industries [4]. Regrettably, much of this lignocellulosic biomass used to be recurrently disposed of by burning. But with the acknowledgement of their renewable nature, they have gained

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immense popularity to be used as a source of high value-added products including biofuels, fine chemicals, and cheaper energy source for microbial fermentation and biofuel production [5]. Nevertheless, the lignocellulosic biomass in its native form is quite recalcitrant to hydrolysis owing to the crystalline nature of cellulose and presence of hemicelluloses and lignin [6]. The recalcitrant nature of lignocellulosic biomass thwarts the easy release of simple sugars from them making this a rate limiting/bottleneck step in their commercial application for production of bioethanol and other value-added chemicals [7]. Numerous microbial groups such as bacteria, actinomycetes, and fungi have been isolated which can effectively degrade these lignocellulosic materials [8]. Several members of soft-rot fungi have been extensively investigated for their potential in hydrolyzing lignocellulosic biomass viz. *Fusarium* [9], *Monilia* [10] *Aspergillus sp*. [11], *Neurospora* [12], *Trichoderma* [13], *Rhizopus* [14], *Mucor* [15] etc. The cellulase enzyme system produced by these fungal species has been successfully employed in the saccharification of lignocellulosic wastes to generate simple sugars which are subsequently fermented to produce ethanol.

Separate hydrolysis and fermentation (SHF) technique is considered to be an upgraded method for biofuel production from lignocellulosic biomass as it encompasses carrying out the hydrolysis and fermentation steps sequentially by operating the reactions in separate units [16]. The greatest advantage of SHF is that it allows both the hydrolysis and fermentation processes to be optimized independently of each other.

The majority of cellulolytic fungal studies have been primarily conducted in model organisms such as *Trichoderma viride* and *Phanerochaete chrysosporium* [8]. Therefore, this study aimed to explore the potential of fungal cultures collected from the untapped North-Western Himalayan region for their cellulolytic enzyme activity to identify the most efficient degrader of lignocellulosic biomass. The most competent degrader of lignocellulosic biomass was subjected to further optimizational studies to maximize endocellulase production for effective saccharification of lignocellulosic waste to yield-reducing sugars for subsequent conversion to bioethanol. There are no reports on endocellulolytic activities in *Cotylidia pannosa*, however, laccase and cellulolytic activity has already been reported from our study [17, 18]. This is the first detailed description of the endocellulolytic activity in the white-rot fungi, *C. pannosa*, with respect to its potential in the conversion of lignocellulosic wastes to bioethanol.

Materials and Methods

Collection of Cultures and Their Maintenance

Overall, 19 different fungal strains identified after assessing their morphological characteristics and fruiting bodies were acquired from CSKHPKV (Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya), Palampur, India. The standard fungal culture of *Trichoderma reesei* MTCC 164 and that of the fermenting yeast, *Saccharomyces cerevisiae* MTCC 174 were obtained from IMTech (Institute of Microbial Technology), Chandigarh, India. All the stock cultures were maintained at 4 °C on yeast extract peptone dextrose (YEPD) agar and subcultured routinely every 4–5 week's interval.

Lignocellulosic Substrate

The lignocellulosic substrate, wheat bran was procured from the surrounding areas of Jaypee University of Information Technology, Waknaghat, Solan (H.P.). The amount of cellulose and hemicelluloses present within wheat bran was estimated by the method of Crampton and Maynard [19]; while the amount of lignin present was estimated by the method of Goering and Van Soest [20]. The substrate was air-dried, grounded into a fine powder and sieved using a 0.5-mm mesh.

Screening of Fungal Cultures for Endocellulase Activity

The primary screening of the fungal isolates for endocellulase activity was conducted using both qualitative as well as quantitative methods. Qualitative method entailed agar plate assay while quantitative method involved spectrophotometric assay as given by Mandels et al. [21]. The qualitative assay was conducted on potato dextrose agar containing 0.2% carboxymethyl cellulose (CMC). A loopful of fungal strains and the control strain *T. reesei* MTCC 164, was plated in the center of the petri dish and incubated for 72 h at 30 °C. At the end of incubation, CMC agar plates were flooded with 0.1% Congo red solution and left for 15 min with intermittent shaking. The plates were then initially rinsed with distilled water and finally with 1 M NaCl solution. The diameter of the zone of clearance surrounding the fungal growth was determined for qualitative estimation of endocellulase activity. For the quantitative assay, the positive control *T. reesei* and all the fungal strains were inoculated in different flasks ($n = 3$) containing YEP media (pH 5.0) at 30 °C for 72 h. Following incubation, 5 mL of sample was withdrawn and centrifuged at 4 °C at 6300 g for 15 min. The supernatant obtained was finally filtered through a Whatman No. 1 filter paper to remove any residual matter. The filtrate thus obtained was analyzed for endocellulolytic enzyme activity as mentioned under the section "Enzyme assays." Based on initial screening assays, the strain exhibiting maximum endocellulase activity was selected for further optimizational studies [21].

Enzyme Assays

Total Cellulase Activity (Filter Paper Activity)

Filter paper activity taken as a reference of the cultural supernatant was assessed by the method of Mandels et al. [21]. The test tubes containing 0.8 mL of 0.05 M citrate buffer (pH 4.8), 0.2 mL cultural supernatant and a rolled strip (1 × 6 cm) of Whatman No.1 filter paper were incubated at 50 °C for 1 h 3 mL of DNS was then added to halt the reaction. The tubes were heated in boiling water bath for 10 min, and the absorbance was read at 540 nm. One unit of enzyme activity (U) was defined as the amount of enzyme required to liberate 1 μmol reducing sugar from the appropriate substrate per min under the assay conditions. A blank with no enzyme and a control with inactivated enzyme was also incubated under identical reaction conditions. All the tests were done in triplicates and results were represented as mean ± S.D.

β-Glucosidase Activity

The β-glucosidase activity was measured by the method of Herr [22]. The reaction mixture containing 1 ml of 2 mM *p*-nitrophenyl-β-d-glucopyranoside (pNPG) and 0.1 ml of cell-free supernatant was incubated 50 °C for 5 min. The reaction was stopped by adding 2 ml of 1 M sodium carbonate (Na_2CO_3) solution and the amount of *p*-nitrophenol was determined by absorbance at 405 nm using a spectrophotometer. One unit of β-glucosidase activity (U) was defined as the amount of enzyme liberating 1 μmol of p-nitrophenol per minute under the assay conditions. A blank with no enzyme and a control with inactivated enzyme were also incubated simultaneously under same reaction conditions. All the tests were done in triplicates, and the results were represented as mean ± S.D.

Endocellulase Activity

Endocellulase activity of the enzyme was worked out as suggested by Mandels et al. [21]. The test tubes containing a mixture of 1% CMC solution (0.5 mL) and cultural supernatant (0.5 mL) were incubated at 50 °C for 30 min in 50 mM sodium acetate buffer at pH 5.0. A control with inactivated enzyme was measured simultaneously. After incubation, 3 mL of DNS was then added to stop the reaction and the amount of reducing sugars liberated was then estimated by DNS method according to the protocol of Miller [23]. A blank with no enzyme and a control with inactivated enzyme were also incubated simultaneously under same reaction conditions. The production of reducing sugars was estimated using D-glucose as standards. One unit of enzyme activity (U) was defined as the amount of enzyme required to liberate, under the conditions of the assay, 1 μmol of reducing sugar from substrate per minute. All the tests were done in triplicates, and the results were represented as mean ± S.D.

Optimization of Endocellulase Activity

For optimizational studies, 2% of sieved wheat bran was added to YEP growth medium comprising of 1% yeast extract and 2% peptone and sterilized by autoclaving at 121 °C and 15 psi for 15 min. The sterilized YEP medium flasks were allowed to cool down and inoculated with a spore inoculum of *C. pannosa* (2.9×10^8 fungal spores/mL). Various process parameters affecting endocellulase production in SmF were optimized independently and subsequently optimum conditions were employed in each experimental run. For optimization of incubation time, the YEPD flasks containing 2% wheat bran were adjusted to pH 5.0 and inoculated with 2.9×10^8 fungal spores/mL. The flasks were incubated for a duration of 108 h and 5 mL of sample aliquot was withdrawn at a regular interval of 12 h and analyzed for endocellulase enzyme activity. For studying the effect of pH, the flasks containing growth medium with varying pH (3.0–6.0) were incubated at 30 °C for 56 h following which the endocellulase activity was determined. The effect of temperature of incubation was determined by inoculating the flasks containing growth medium (pH 5.0) and incubating at different temperatures (25–40 °C) for 56 h following which the endocellulase activity was determined. The effect of different salts such as ammonium chloride, magnesium chloride, ammonium sulfate, manganese chloride, and calcium chloride on the endocellulase activity was studied by adding these salts at a concentration of 0.25% to the growth medium (pH 5.0) and incubating at 30 °C for 56 h following which the endocellulase activity was determined. In all the above experiments, the withdrawn sample was centrifuged at 4 °C at 6300 g for 15 min. The supernatant collected was analyzed for endocellulase enzyme activity as described earlier.

Optimization of Saccharification

For estimating the saccharification potential of *C. pannosa*, six sets, each containing three flasks ($n = 3$, for each time point) having YEPD media (pH 5.0) supplemented with 2% of sieved wheat bran was inoculated with a spore inoculum of *C. pannosa* (2.9×10^8 fungal spores/mL) and incubated at 30 °C at 120 rpm. For estimating reducing sugar levels, the hydrolysate obtained from the flasks at the end of their respective predefined incubation time was centrifuged and the cell-free supernatant was estimated for reducing sugar as described before. Similarly, for determining the levels of residual cellulose, wheat bran obtained from each flask was subjected to the method of Crampton and Maynard [19]. The percentage

saccharification of wheat bran was calculated using the equation of Mandels and Sternberg [24] as follows:

$$\%Saccharification = \frac{reducing\ sugars(\mathrm{mg/mL}) \times 0.9 \times 100\%}{initial\ substrate\ concentration\ (\mathrm{mg/mL})}$$

Bioethanol Production from Wheat Bran Hydrolysate

After identification of the optimum saccharification parameters, one set of three flasks ($n = 3$) as described above was taken and incubated under optimal conditions for achieving maximum saccharification. At the end of incubation time, the hydrolysate was collected from each flask and subjected to fermentation separately by *Saccharomyces cerevisiae* MTCC 174 inoculated at the level of 0.25 ($OD_{600nm} = 0.25$) at 30 °C and 120 rpm for 72 h. An aliquot (5 ml) of fermented broth from the flasks was withdrawn at a regular interval of 12 h and estimated for ethanol content using gas chromatography (Agilent technology GC 6820) with FID detector using HP-5MS column (L 30 m, I.D. 0.32 mm, Film 0.25 μ). The FID detector and injector were maintained at a temperature of 220 °C. The oven was programmed to initially maintain a temperature of 40 °C for 2 min and then to steadily increase the temperature at a rate of 5 °C/min till a final temperature of 200 °C was obtained. Helium was used as a carrier gas at a flow rate of 1.5 mL/min [25]. The analytical grade ethanol (SPECTRANAL, ≥ 99.8%, Sigma-Aldrich, Bangalore, India) was used as a standard.

Statistical Analysis

All the experiments were performed in triplicates, and data are presented as mean values ($n = 3$). Comparison of results was done by ANOVA and Tukey's multiple comparison test ($p < 0.05$) with SPSS version 23.0 (SPSS Inc., Chicago, IL, USA).

Results

Screening of Fungal Strains for Endocellulase Activity

The preliminary qualitative and quantitative assays performed for screening of 19 fungal strains for the presence of endocellulase enzyme activity recognized *C. pannosa* as the most potential producer of endocellulase activity. In the qualitative assay, the extent of clear zones developed around the fungal growth was indicative of the presence and the relative amount of endocellulase activity in different fungal isolates. In case of *C. pannosa*, the extent of the clearance zone formed in qualitative assay and endocellulase activity detected in quantitative assay was observed to be 80 mm (data not shown) and 8 U/mL, respectively (Fig. 1), which was comparable to the standard fungal culture of *T. reesei* MTCC 164.

Substrate and Enzyme Activities

The compositional analysis of wheat bran indicated that the cellulose, hemicellulose, and lignin content of wheat bran was 33, 29, and 15%, respectively. The filter paper assay is the key technique for analyzing total cellulase activity. Our strain *C. pannosa* exhibited a total cellulase activity of 1.0 U/mL after 56 h as shown in Fig. 2. The β-glucosidase activity, estimated as PNP released after 30 min of incubation with *β*-PNPGLU at 37 °C indicated that *C. pannosa* produced 1.28 U/mL after 56 h (Fig. 3).

Optimization of Endocellulase Activity

For optimization of endocellulase activity of *C. pannosa*, wheat bran was used as the substrate under submerged fermentation conditions. The optimum incubation time, pH and temperature at which maximum endocellulase activity was detected were identified as 56 h (Fig. 4a), 5.0 (Fig. 4b) and 30 °C (Fig. 4c), respectively. Additional optimizational efforts showed that in comparison to manganese chloride, ammonium sulfate, calcium chloride, and magnesium chloride, the addition of ammonium chloride to the production medium caused an increase in endocellulase activity (Fig. 4d).

Saccharification and Bioethanol Production

In the present study, for the production of bioethanol using *C. pannosa*, the technique of separate hydrolysis and fermentation (SHF) was employed. With a saccharification efficiency of 50.5%, the maximum quantity of fermentable sugar obtained from wheat bran with a cellulose content of 15.6 g/L after 56 h of saccharification was 8.75 g/L (Fig. 5). As detected by gas chromatography, subjecting this fermentable sugar containing broth to fermentation produced a maximum ethanol concentration of 4.12 g/L after 56 h of incubation (Fig. 5). After 56 h, at the culmination of fermentation, there was no substantial detection of reducing sugars indicating an absolute assimilation of reducing sugars released after saccharification.

Discussion

Bioethanol production from lignocellulosic biomass has been proposed to be an effectual, lucrative, and environmentally friendly process which can assist in coping with the world's increasing fuel requirements. Preliminary screening tests

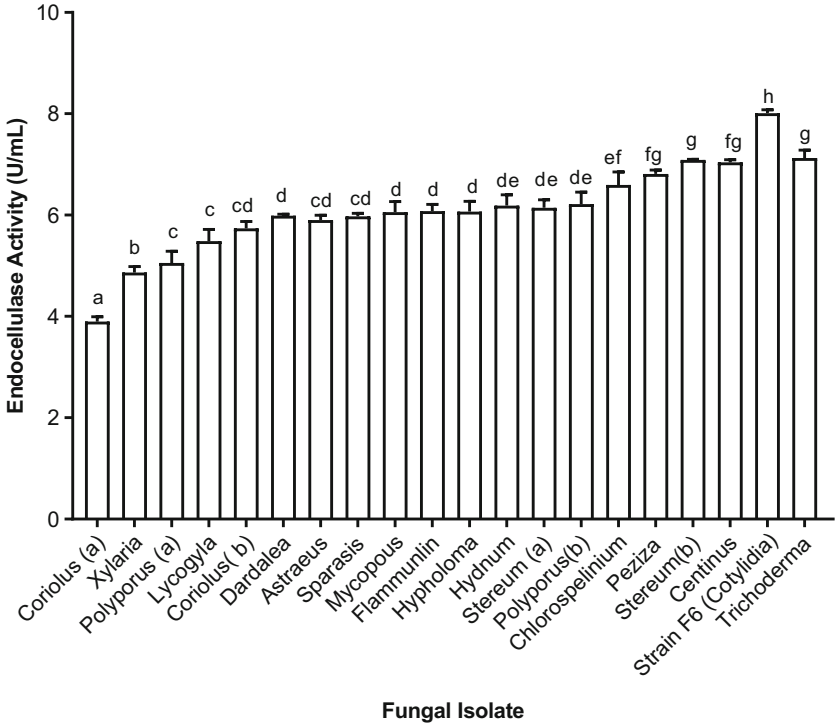

Fig. 1 Screening of 19 different fungal cultures and one standard culture of *T. reesei* for endocellulase activity. A single asterisk (*) indicates that error bars signifies standard deviation, **a**–**g** = bars with same superscript letters are not significantly ($p < 0.05$) different as measured by two-sided Tukey's–post hoc range test between replications

(qualitative and quantitative assays) performed on the 19 different fungal strains, revealed that *C. pannosa* was the most efficient producer of endocellulolytic activity. The endocellulase activity obtained from *C. pannosa* was also found to be greater than that of the positive control strain *T. reesei* MTCC 164 (7.1 U/mL at 30 °C) with wheat bran as the lignocellulosic substrate. Therefore, the selected fungal strain *C. pannosa* was subjected to further optimizational studies to augment the production of endocellulase.

Wheat bran has been used in several studies for production of cellulolytic enzymes as it is considered as an agricultural waste [26]. Compositionally, wheat bran exhibits a well-balanced C/N ratio with a low lignin content of 3–6%, 13–19% of protein, and greater than 30% of hemicellulose [27]. Compared to other substrates which contain higher amounts of cellulose in its crystalline form along with elevated levels of lignin, wheat bran contains high amounts of hemicellulose, and a low amount of lignin, making it a readily degradable substrate. The existence of augmented amounts of hemicelluloses

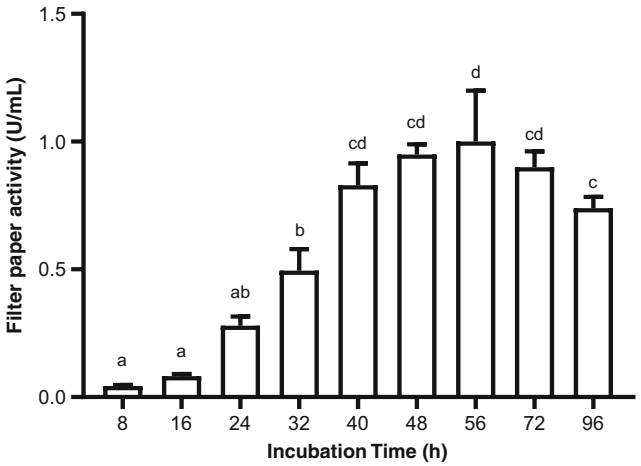

Fig. 2 Filter paper activity (total cellulase activity) of *C. pannosa*. A single asterisk (*) indicates that error bars signifies standard deviation, **a**–**d** = bars with same superscript letters are not significantly ($p < 0.05$) different as measured by two-sided Tukey's–post hoc range test between replications

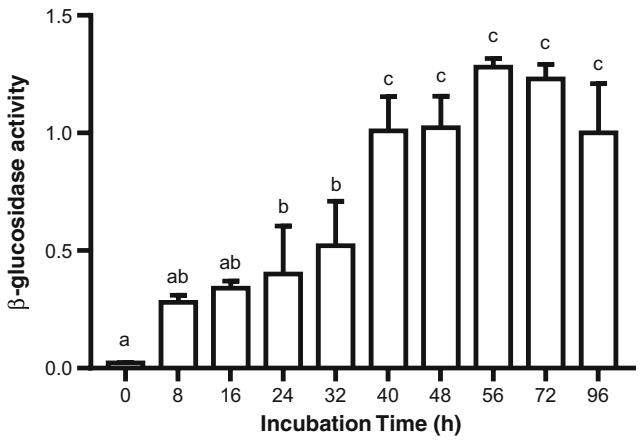

Fig. 3 β-glucosidase activity of *C. pannosa*. A single asterisk (*) indicates that error bars signifies standard deviation, **a**–**c** = bars with same superscript letters are not significantly ($p < 0.05$) different as measured by two-sided Tukey's–post hoc range test between replications

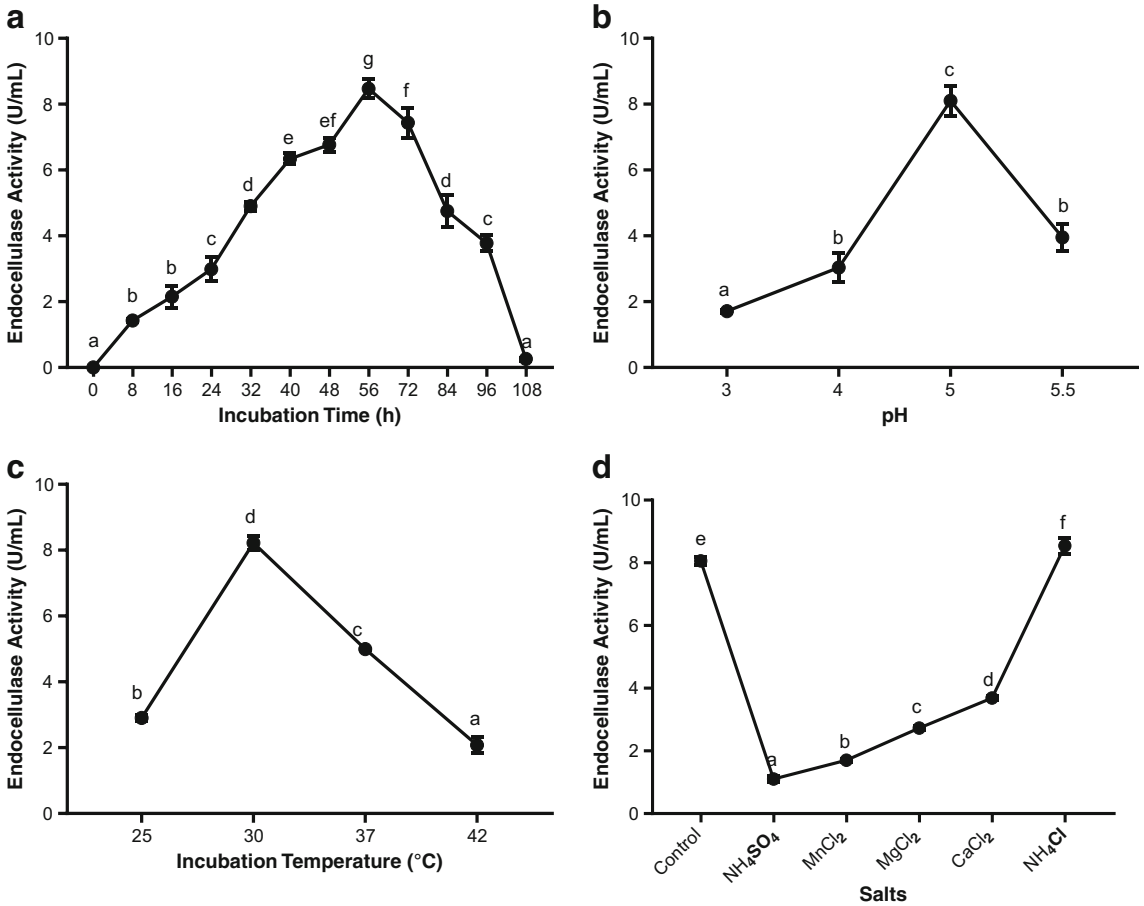

Fig. 4 Effect of **a** incubation time **b** pH, **c** temperature, and **d** addition of salts on the activity of cellulase produced by *C. pannosa*. A single asterisk (*) indicates that error bars signifies standard deviation, **a**–**g** = bars with same superscript letters are not significantly ($p < 0.05$) different as measured by two-sided Tukey's–post hoc range test between replications

provides microorganisms with an easily accessible reservoir of readily metabolizable sugars. Therefore, it has been anticipated that the early degradation of hemicelluloses induces other cell wall degrading enzymes [26]. The comparison of *C. pannosa*'s endocellulolytic activities on wheat bran with those reported for other fungi such as *Aspergillus flavus* (1.23 IU/mL) [28] and, *Penicillium decumbens* (4.7 IU/mL) [27], acknowledges the prospect of using *C. pannosa* for the production of endocellulase enzyme for the subsequent conversion of wheat bran to other by-products.

To compare the efficacy of cellulase activity between microorganisms or their secreted enzymes, techniques for measuring total cellulase activity are required. The total cellulase activity exhibited by *C. pannosa* (1.0 U/mL) was found to be superior or in some cases equivalent when compared with other fungi such as *Neurospora sitophila* (0.142 U/mL) [29],

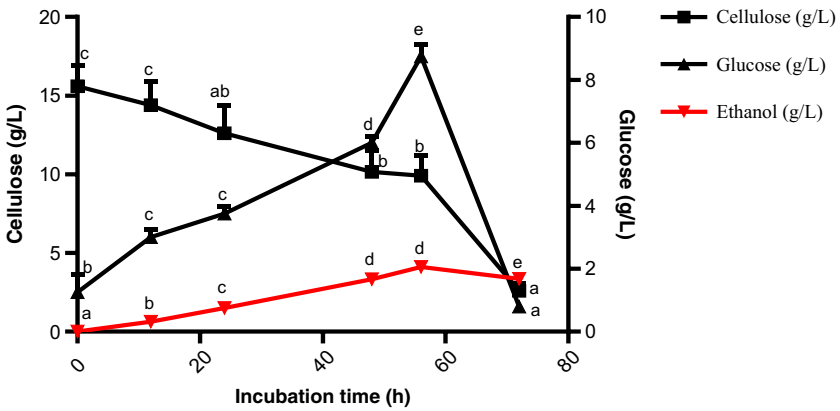

Fig. 5 Amount of remnant cellulose levels in wheat bran, fermentable sugars released, and ethanol produced following saccharification of wheat bran (2%) by *C. pannosa.* A single asterisk (*) indicates that error bars signifies standard deviation, **a**–**e** = bars with same superscript letters are not significantly ($p < 0.05$) different as measured by two-sided Tukey's–post hoc range test between replications

Aesculus wangii (0.96 U/mg) [30], *Irpex lacteus* CD2 (2.5 IU/g) [31], *Cirripectes polyzona* (1.0 ± 0.1 U/mL), *Phaseolus coccineus* (1.0 ± 0.1 U/mL) [32], *Sporotrichum pulverulentum* (0.22 U/mL), and *T. reesei* QM9414 (0.55 U/mL) [33].

Saccharification of complex polymers to simple sugars is carried out by the synergistic action of various hydrolytic enzymes like cellulases (exoglucanase, endoglucanase, β-glucosidase) and hemicellulases. β-glucosidase is regarded as the bottleneck enzyme as it liberates glucose from cellobiose [34]. However, the proportion of β-glucosidase is meager in most of the commercial cellulases and this causes not only accumulation of cellobioses but also feed-back inhibition of the cellulase enzyme complex [35]. Although there is growing interest in the search for new fungal β-glucosidases, the vast majority of studies have been performed with imperfect fungi, and there are scant reports that deal with basidiomycetous fungi, not only for biotechnological purposes but also for physiological research. β-glucosidase activity of *C. pannosa* (1.28 U/mL) was found to be higher in comparison to those of *Fomes fomentarius* (0.026 U/mL) [36], *Pleurotus ostreatus* (0.385 U/mL) [37], *Volvariella volvacea* (0.13 U/mL) [38], *Trametes trogii* (0.25 U/mL) [39], *Flammulina velutipes* (1.5 U/mL) [40], and *F. velutipes* CFK 3111 (1.6 U/mL) [41].

It is always preferable to use a strain which can produce maximum cellulolytic enzyme activity in the shortest possible time as it increases the economic feasibility of the process. Comparing the incubation time of *C. pannosa* (56 h) with those of *Aspergillus awamori* 2B.361 U2/1 (4900 IU/L in 7 days) [42], and *I. lacteus* (0.15 U/mL in 35 days) [43] revealed that *C. pannosa* required significantly shorter time for realizing maximum endocellulase levels.

It has been reported that the fungal cellulolytic activity is highly sensitive to alterations in the pH [44]. A similar observation was made in the present study also. It was observed that as the pH was increased from 3 to 4, there was a gradual upsurge in the enzyme activity. Thereafter, as the pH was increased to 5, the activity increased steeply reaching its peak value (8.1 U/mL). On further increase of pH to 5.5, the activity was observed to sharply decline. Our results are in congruence with those reported for *Aspergillus niger* wherein a maximum endoglucanase activity of 5.57 U/mL has been reported within a pH range of 4 to 5.5 [45].

The temperature of incubation is an indispensable factor which influences the production of endocellulase. *C. pannosa*'s ability to produce endocellulase increased as the temperature was raised to 30 °C where it reached its peak production capacity. Further increase in temperature exhibited a decrease in its endocellulase production potential. It has been reported that at higher incubation temperatures, the membrane composition begins to alter causing catabolism of proteins along with an inhibition of the growth rate of fungi. A similar trend of variation in endocellulase activity with increasing temperature has also been reported for *A. flavus* (0.7 IU/mL at 30 °C) [28], and *T. reesei* QM9419 mutant (1.30 IU/mL at 30 °C) [46]. Review of literature revealed that though the optimum temperature (30 °C) of incubation in numerous similar studies was alike, however, the optimum endocellulase enzyme activity obtained with *C. pannosa* was greater than that reported in these studies.

Various reports dealing with the optimization of enzyme production by fungi suggested that supplementation of the growth medium with different salts could further enhance the enzyme's activity. This could be due to two probable reasons: (a) the presence of a particular type of ion increase the enzyme's production by the strain through enhancing the various enzyme's activity involved in intermediate metabolism [47]or (b) the ion itself acts as a cofactor to enhance the enzyme's activity. As can be seen in Fig. 4d, *C. pannosa's* potential to produce endocellulolytic enzyme received a boost when the production medium was supplemented with ammonium chloride as compared to the control flask which was devoid of any salt supplement. For all the other supplemented salts there was a significant decrease in the activity as compared to the control.

Following saccharification of wheat bran by *C. pannosa* under optimum conditions, the fermentation of released reducing sugars by *S. cerevisiae* MTCC 174 yielded an ethanol content which was found to be higher in comparison to an earlier report where reducing sugars generated through saccharification of pretreated cotton stalk by *P. chrysosporium* gave an ethanol yield of 0.027 g ethanol/g [48]. The ethanol content obtained was also comparable to that achieved via consolidated bioprocessing of wheat bran under submerged fermentation using *Trametes hirsuta* after 96 h of cultivation [49].

Conclusions

This study reports for the first time the saccharification potential of *C. pannosa*, in the hydrolysis of lignocellulosic waste material to yield fermentable sugars for converting into bioethanol by yeast. Additional investigations of the fungus are necessary to explicate the enzymatic machinery essential for the efficient saccharification of lignocellulosic substrates. The collective action of *C. pannosa* (saccharification) and *S. cerevisiae* (fermentation) proved to be an effective collaboration for enhanced bioethanol production from lignocellulosic biomass. Therefore, the current study not only opens the door for the sustainable use of lignocellulosic biomass but also reduces the use of hazardous chemicals required during the pretreatment steps prior to production of bioethanol.

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Compliance with Ethical Standards

Conflict of Interests The authors declare that they have no conflict of interest.

References

1. Brethauer S, Wyman CE (2010) Review: continuous hydrolysis and fermentation for cellulosic ethanol production. Bioresour Technol 101(13):4862–4874. https://doi.org/10.1016/j.biortech.2009.11.009
2. Kim S, Dale BE (2004) Global potential bioethanol production from wasted crops and crop residues. Biomass Bioenergy 26(4):361–375. https://doi.org/10.1016/j.biombioe.2003.08.002
3. Oberoi HS, Vadlani PV, Nanjundaswamy A, Bansal S, Singh S, Kaur S, Babbar N (2011) Enhanced ethanol production from Kinnow mandarin (*Citrus reticulata*) waste via a statistically optimized simultaneous saccharification and fermentation process. Bioresour Technol 102(2):1593–1601. https://doi.org/10.1016/j.biortech.2010.08.111
4. Perez J, Munoz-Dorado J, de la Rubia T, Martinez J (2002) Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. Int Microbiol 5(2):53–63. https://doi.org/10.1007/s10123-002-0062-3
5. Asgher M, Ahmad Z, Iqbal HMN (2013) Alkali and enzymatic delignification of sugarcane bagasse to expose cellulose polymers for saccharification and bio-ethanol production. Ind Crop Prod 44:488–495. https://doi.org/10.1016/j.indcrop.2012.10.005
6. Kumar AK, Sharma S (2017) Recent updates on different methods of pretreatment of lignocellulosic feedstocks: a review. Bioresour Bioprocess 4(1):7. https://doi.org/10.1186/s40643-017-0137-9
7. Zhao X, Zhang L, Liu D (2012) Biomass recalcitrance. Part I: the chemical compositions and physical structures affecting the enzymatic hydrolysis of lignocellulose. Biofuels Bioprod Biorefin 6(4):465–482. https://doi.org/10.1002/bbb.1331
8. Kuhad RC, Singh A, Eriksson KE (1997) Microorganisms and enzymes involved in the degradation of plant fiber cell walls. Adv Biochem Eng Biotechnol 57:45–125
9. Christakopoulos P, Macris BJ, Kekos D (1989) Direct fermentation of cellulose to ethanol by *Fusarium oxysporum*. Enzym Microb Technol 11(4):236–239. https://doi.org/10.1016/0141-0229(89)90098-7
10. Gong CS, Maun CM, Tsao GT (1981) Direct fermentation of cellulose to ethanol by a cellulolytic filamentous fungus, *Monilia* sp. Biotechnol Lett 3(2):77–82. https://doi.org/10.1007/bf00145114
11. Delabona PS, Pirota RDPB, Codima CA, Tremacoldi CR, Rodrigues A, Farinas CS (2012) Using Amazon forest fungi and agricultural residues as a strategy to produce cellulolytic enzymes. Biomass Bioenergy 37:243–250. https://doi.org/10.1016/j.biombioe.2011.12.006
12. Deshpande V, Keskar S, Mishra C, Rao M (1986) Direct conversion of cellulose/hemicellulose to ethanol by *Neurospora crassa*. Enzym Microb Technol 8(3):149–152. https://doi.org/10.1016/0141-0229(86)90103-1
13. Stevenson DM, Weimer PJ (2002) Isolation and characterization of a *Trichoderma* strain capable of fermenting cellulose to ethanol. Appl Microbiol Biotechnol 59(6):721–726. https://doi.org/10.1007/s00253-002-1027-3
14. Millati R, Edebo L, Taherzadeh MJ (2005) Performance of *Rhizopus*, *Rhizomucor*, and *Mucor* in ethanol production from glucose, xylose, and wood hydrolyzates. Enzym Microb Technol 36(2–3):294–300. https://doi.org/10.1016/j.enzmictec.2004.09.007
15. Sues A, Millati R, Edebo L, Taherzadeh MJ (2005) Ethanol production from hexoses, pentoses, and dilute-acid hydrolyzate by *Mucor indicus*. FEMS Yeast Res 5(6–7):669–676. https://doi.org/10.1016/j.femsyr.2004.10.013
16. Öhgren K, Bura R, Lesnicki G, Saddler J, Zacchi G (2007) A comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam-pretreated corn stover. Process Biochem 42(5):834–839. https://doi.org/10.1016/j.procbio.2007.02.003
17. Sharma D, Goel G, Sud A, Chauhan RS (2015) A novel laccase from newly isolated *Cotylidia pannosa* and its application in decolorization of synthetic dyes. Biocatal Agric Biotechnol 4(4):661–666. https://doi.org/10.1016/j.bcab.2015.07.008
18. Sharma D, Garlapat VK, Goel G (2016) Bioprocessing of wheat bran for the production of lignocellulolytic enzyme cocktail by *Cotylidia pannosa* under submerged conditions. Bioengineered 7(2):88–97. https://doi.org/10.1080/21655979.2016.1160190
19. Crampton E, Maynard L (1938) The relation of cellulose and lignin content to the nutritive value of animal feeds. J Nutr 15:383–395
20. Goering H, Van Soest PJ (1970) Forage fiber analyses: apparatus, reagents, procedures, and some applications. Agriculture Handbook. U.S. Agricultural Research Service, Washington
21. Mandel M, Andreotti R, Roche C (1976) Measurement of saccharifying cellulose. Biotechnol Bioeng Symp (6):21-23
22. Herr D (1979) Secretion of cellulase and beta-glucosidase by *Trichoderma viride* ITCC-1433 in submerged culture on different substrates. Biotechnol Bioeng 21(8):1361–1371. https://doi.org/10.1002/bit.260210805
23. Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31(3):426–428. https://doi.org/10.1021/ac60147a030
24. Mandels M, Sternberg D (1976) Recent advances in cellulase technology. J Ferment Technol 54:267–286
25. Pontes H, Guedes de Pinho P, Casal S, Carmo H, Santos A, Magalhães T, Remião F, Carvalho F, Lourdes Bastos M (2009) GC determination of acetone, acetaldehyde, ethanol, and methanol in biological matrices and cell culture. J Chromatogr Sci 47(4):272–278. https://doi.org/10.1093/chromsci/47.4.272
26. Brijwani K, Oberoi HS, Vadlani PV (2010) Production of a cellulolytic enzyme system in mixed-culture solid-state fermentation of soybean hulls supplemented with wheat bran. Process Biochem 45(1):120–128. https://doi.org/10.1016/j.procbio.2009.08.015
27. Sun X, Liu Z, Qu Y, Li X (2008) The effects of wheat bran composition on the production of biomass-hydrolyzing enzymes by *Penicillium decumbens*. Appl Biochem Biotechnol 146(1–3):119–128. https://doi.org/10.1007/s12010-007-8049-3
28. Gomathi D, Muthulakshmi C, Kumar DG, Ravikumar G, Kalaiselvi M, Uma C (2012) Submerged fermentation of wheat bran by *Aspergillus flavus* for production and characterization of carboxy methyl cellulase. Asian Pac J Trop Biomed 2(1):S67–S73. https://doi.org/10.1016/S2221-1691(12)60132-4
29. Oguntimein G, Vlach D, Moo-Young M (1992) Production of cellulolytic enzymes by *Neurospora sitophila* grown on cellulosic materials. Bioresour Technol 39(3):277–283. https://doi.org/10.1016/0960-8524(92)90217-L
30. Wu X, An Q, Dai Y, Si J (2016) Investigating lignocellulose in cornstalk pretreated with *Trametes pubescens* Cui 7571 to improve enzymatic saccharification. Bioresources 11(1):2768–2783
31. Xu C, Ma F, Zhang X (2009) Lignocellulose degradation and enzyme production by *Irpex lacteus* CD2 during solid-state

fermentation of corn stover. J Biosci Bioeng 108(5):372–375. https://doi.org/10.1016/j.jbiosc.2009.04.023
32. Elisashvili V, Kachlishvili E, Penninckx M (2008) Effect of growth substrate, method of fermentation, and nitrogen source on lignocellulose-degrading enzymes production by white-rot basidiomycetes. J Ind Microbiol Biotechnol 35(11):1531–1538. https://doi.org/10.1007/s10295-008-0454-2
33. Eriksson K-E, Johnsrud SC (1983) Mutants of the white-rot fungus *Sporotrichum pulverulentum* with increased cellulase and β-d-glucosidase production. Enzym Microb Technol 5(6):425–429. https://doi.org/10.1016/0141-0229(83)90024-8
34. Sørensen A, Lübeck M, Lübeck PS, Ahring BK (2013) Fungal beta-glucosidases: a bottleneck in industrial use of lignocellulosic materials. Biomol Ther 3(3):612–631. https://doi.org/10.3390/biom3030612
35. Singhania RR, Patel AK, Sukumaran RK, Larroche C, Pandey A (2013) Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production. Bioresour Technol 127:500–507. https://doi.org/10.1016/j.biortech.2012.09.012
36. Větrovský T, Baldrian P, Gabriel J (2013) Extracellular enzymes of the white-rot fungus Fomes fomentarius and purification of 1, 4-β-glucosidase. Appl Biochem Biotechnol 169(1):100–109. https://doi.org/10.1007/s12010-012-9952-9
37. Morais H, Ramos C, Matos N, Forgács E, Cserháti T, Almeida V, Oliveira J, Darwish Y, Illés Z (2002) Liquid chromatographic and electrophoretic characterisation of extracellular β-glucosidase of *Pleurotus ostreatus* grown in organic waste. J Chromatogr B 770(1):111–119. https://doi.org/10.1016/S0378-4347(01)00561-8
38. Cai Y, Buswell J, Chang S (1998) β-Glucosidase components of the cellulolytic system of the edible straw mushroom, *Volvariella volvacea*. Enzym Microb Technol 22(2):122–129. https://doi.org/10.1016/S0141-0229(97)00151-8
39. Levin L, Forchiassin F (1997) Efecto de las condiciones de cultivo sobre la producción de celulasas por *Trametes trogii*. Rev Arg Microbiol 29:16–23
40. Maehara T, Ichinose H, Furukawa T, Ogasawara W, Takabatake K, Kaneko S (2013) Ethanol production from high cellulose concentration by the basidiomycete fungus *Flammulina velutipes*. Fungal Biol 117(3):220–226. https://doi.org/10.1016/j.funbio.2013.02.002
41. Mallerman J, Papinutti L, Levin L (2015) Characterization of β-glucosidase produced by the white rot fungus *Flammulina velutipes*. J Microbiol Biotechnol 25(1):57–65. https://doi.org/10.4014/jmb.1401.01045
42. Gottschalk LMF, Oliveira RA, da Silva Bon EP (2010) Cellulases, xylanases, β-glucosidase and ferulic acid esterase produced by *Trichoderma* and *Aspergillus* act synergistically in the hydrolysis of sugarcane bagasse. Biochem Eng J 51(1):72–78. https://doi.org/10.1016/j.bej.2010.05.003
43. Dias AA, Freitas GS, Marques GS, Sampaio A, Fraga IS, Rodrigues MA, Evtuguin DV, Bezerra RM (2010) Enzymatic saccharification of biologically pre-treated wheat straw with white-rot fungi. Bioresour Technol 101(15):6045–6050. https://doi.org/10.1016/j.biortech.2010.02.110
44. Puntambekar U (1995) Cellulase production by the edible mushroom *Volvariella diplasia*. World J Microbiol Biotechnol 11(6):695–695. https://doi.org/10.1007/BF00361023
45. Farinas CS, Loyo MM, Baraldo A, Tardioli PW, Neto VB, Couri S (2010) Finding stable cellulase and xylanase: evaluation of the synergistic effect of pH and temperature. New Biotechnol 27(6):810–815. https://doi.org/10.1016/j.nbt.2010.10.001
46. Deshpande S, Bhotmange M, Chakrabarti T, Shastri P (2008) Production of cellulase and xylanase by *Trichoderma reesei* (QM 9414 mutant), *Aspergillus niger* and mixed culture by solid state fermentation (SSF) of water hyacinth (*Eichhornia crassipes*). Indian J Chem Technol 15:449
47. Jellison J, Connolly J, Goodell B, Doyle B, Illman B, Fekete F, Ostrofsky A (1997) The role of cations in the biodegradation of wood by the brown rot fungi. Int Biodeterior Biodegrad 39(2–3):165–179. https://doi.org/10.1016/S0964-8305(97)00018-8
48. Shi J, Sharma-Shivappa RR, Chinn M, Howell N (2009) Effect of microbial pretreatment on enzymatic hydrolysis and fermentation of cotton stalks for ethanol production. Biomass Bioenergy 33(1):88–96. https://doi.org/10.1016/j.biombioe.2008.04.016
49. Okamoto K, Nitta Y, Maekawa N, Yanase H (2011) Direct ethanol production from starch, wheat bran and rice straw by the white rot fungus *Trametes hirsuta*. Enzym Microb Technol 48(3):273–277. https://doi.org/10.1016/j.enzmictec.2010.12.001