ANNALS OF MULTIDISCIPLINARY RESEARCH, INNOVATION AND TECHNOLOGY (AMRIT)

(A peer-reviewed open access multidisciplinary journal)

www.adtu.in/amrit

RESEARCH ARTICLE

Selection of Fungi with Cellulolytic and Fermentative Capability from Sacred Groves for Consolidated Bioprocessing of Lignocellulose Biomass to Bioethanol

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Article Chronicle: Received: 02/11/2021 Accepted: 31/12/21 Published Online: 12/01/22

Abstract

Consolidated bioprocessing (CBP) of lignocellulosic biomass (LCB) to bioethanol requires selection of suitable strains with cellulolytic and fermentation ability and optimization of process parameters for maximum production of bioethanol. In this study, four selected sacred groves of Manipur, India were explored for isolation and selection of potential fungi for bioprocessing of LCB. Fungal population in the sacred groves ranged from 1.36 ×103 to 1.52 × 104 CFU/g of litter soil sample and a total of 270 fungi were obtained from the population using Congo Red Assay (CRA). Out of the total isolates, 168 isolates exhibited cellulolytic activity on carboxy-methyl cellulose (CMC), among which 26 isolates possessed cellulolytic and fermentative potential. Seven superior cellulolytic and fermentative fungi (CFF) were identified which produced ethanol titre of 0.10 to 4.85 g/L, 0.08 to 0.46 g/L, and 0.12 to 0.22 g/L within 144 hrs of incubation in the medium supplemented with glucose, xylose, and cellulose, respectively. Two fungal strains, identified as *Fusarium oxysporum* HG19 and *Trichoderma koningiopsis* LL28 produced varying level of ethanol titre (0.32 to 1.12 g/L) from four LCB substrates viz. sugarcane bagasse (SB), cassava aerial parts, ficus fruits, and rice straw (untreated and hydrothermally treated). Highest titre of 1.12 and 1.09 g/L of ethanol were produced by strains HG19 and LL28, respectively on hydrothermally pretreated LCB of SB. The resultant ethanol concentration could be attributed to content of reducing sugars in LCB, pretreatment effect and fungal preference for the polymer constituent of LCB. Thus this study showed *F. oxysporum* HG19 and *T. koningiopsis* LL28 as promising fungi from sacred grove to use in production of ethanol by CBP of LCB. Further research on optimization of parameters in CBP of LCB by the isolates may promote enhanced ethanol yield.

Keywords: *Bioethanol, Lignocellulosic biomass, Sacred grove, Cellulolytic activity, Consolidated bioprocessing, Fermentation*

1. Introduction

Lignocellulosic biomass (LCB) is an abundant and inexpensive source of raw material alternative to sugar or starchy crop biomass for production of bioethanol worldwide. Current demand for starch crop based bioethanol jeopardizes adequate food supplies required for ever growing world population through hike in global food price and other ethical issues(1; 2). Successful use of LCB as sources of bioethanol is the need of the hour to eliminate this conflict, encourage national economic growth and also mitigate the negative impacts of climate change(3; 4). However, conversion of LCB sources to bioethanol requires complex steps of pretreatment and scarification to simple sugars followed by their fermentation and these processes have cost and environment implications(5). The conventional system requires separate individual microbial species for saccharification and fermentation. In recent years, a system using a single microbial species

or consortium which is capable of one-step conversion of LCB known as consolidated bioprocessing (CBP) to ethanol, has received significant attention(5; 6; 7; 8). Furthermore, the effective utilization of LCB through CBP requires the fermentation of not only hexoses but also pentose sugars released during saccharification step of LCB. Fermentation of xylose, the primary pentose sugar of the hemicellulose, cannot be accomplished as the most commonly used yeast species, *Saccharomyces cerevisiae*, is unable to ferment xylose and the other yeast species capable of fermenting xylose are few in nature. For instance, *Pachysolen tannophilus*, *Candida spp.*, *Kluyveromyces marxianus* and *Pichia stipitis* have been identified as naturally occurring yeasts capable of xylose fermentation(9; 10; 11; 12; 13). Several other species such as *Monilia sp., Fusarium oxysporum, Neurospora crassa, Paecilomyces lilacinus, Rhizopus oryzae, Aspergillus terreus, Mucor indicus* and *Trametes versicolor* are also able to convert xylose to ethanol(14; 15; 16). Thus, there is a need to enlarge the gene pool of fungal isolates with potential for CBP of LCB through search in diverse habitats. In this study we have selected sacred grove ecosystem of North-East India which falls within Indo-Burma Biodiversity hotspot. A sacred grove is an outcome of traditional values, religious beliefs, taboos, and sociocultural practices which physically represents a forest ecosystem as a common community property and maintained by a community for ecological balance, biodiversity conservation and resource supply in time of economic crisis. These sacred groves are expected to harbour novel fungal species that are capable of both degrading LCB and fermenting the suite of sugars present in LCB to ethanol. In nature, LCB materials occurring in diverse niches such as in the gut of a termite, decomposing wood and in the dirt of a forest are degraded by a consortium of microorganisms. Their remarkable trait is a catabolic activity with respect to both degradation of complex sugar polymers and utilization of C5 sugars (particularly xylose) in the presence of lignin and other inhibitory molecules. In the first part of the present study, we have isolated cellulose degrading fungi (CDF) from litter soil of sacred groves and assessed their ability to ferment different sugars, i.e., glucose, xylose and cellulose to bioethanol which are major components of LCB. In the second part, the superior CDF isolates were evaluated for their potential in bioethanol production by CBP under identical experimental conditions using four LCB viz, sugarcane bagasse (SB), ficus fruits (*Ficus cunia*), cassava aerial parts and rice straw in their native and hydrothermally pretreated state.

2. Materials and Methods

2.1 Soil Samples Collection from Sacred Groves

The litter soil samples from 0-5 cm depth were collected from the four selected sacred groves within Imphal valley, Manipur, India (Table 1). Soil samples were air dried at room temperature for 5 days, grounded and sieved through a sieve of 0.5 mm pore diameter and stored for further analysis.

2.2 Isolation of Fungi

The isolation was based on three methods using Czapek Dox Agar medium; namely soil dilution plating, soil direct plating, and debris plating, as described earlier(17). In the case of dilution technique, three replicate plates were used for each dilution from 10-2 to 10-4. Fungal colonies were counted after 6 days of incubation and purified by subculturing on Potato Dextrose Agar (PDA) medium.

2.3 Preliminary Screening for Cellulolytic and Xylanolytic Activity

All the fungal isolates obtained from the four selected sites were inoculated on carboxy-methyl cellulose (CMC) plates (0.2% NaNO3, 0.10% K2HPO4, 0.05% KCl, 0.05% MgSO4, 0.2% CMC, 0.02% peptone, 1.7% agar and pH 5.2). For the qualitative screening of cellulase activity of fungi, the fungal isolates were cultured on PDA at 28°C for 5 days. A mycelial disc of 4 mm diameter was punched using a sterile cork borer. The fungal mycelial disc punched from the pure culture of each of the isolates was positioned in the centre of the CMC agar plates using a sterile inoculating loop and incubated at 28°C for 5 days. The culture plates with colonies were flooded with 0.1% congo red dye for 15 min and washed with 1 M NaCl and left for 30 min to allow color development. The culture plates were observed for formation of clearing zone around the periphery of the mycelium. The dye only remains attached to regions where there are -1, 4-D-glucanohydrolase bonds(18) Similarly, the screening of isolates for xylanolytic activity was done using 0.2% Birchwood Xylan instead of CMC in the screening media.

2.4 Qualitative Fermentation Test

Fermentation ability of the fungal isolate was tested using the fermentation broth comprised of peptone (10 g/L), sodium chloride (15 g/L), test carbon sources (xylose, glucose and cellulose: 10 g/L) and phenol red (0.018 g/L), with final pH 7.2 in test tubes. Durham's tube was inverted and inserted in the test tube and autoclaved. To avoid their thermal breakdown, both glucose and xylose were added by filter sterilization separately after the autoclaving of the minimal medium. Cellulose was autoclaved for only 3 minutes separately so as to avoid the release of glucose. Single discs of

Table 1: General geographical features of the selected sacred groves for sample collection

Sacred grove site	Area (ha)	Altitude (m)	Aspect and Position	Latitude	Longitude
Konthoujam Lairembi	1.41	711	West Valley	N 24°46'0.6"	E 93°52'32.8"
Mahabali	5.05	710	Central Valley	N 24°47'51.9"	E 93°56'38.8"
Langol Thongak Lairembi	5.05	800	North- West Hill Base	N 24°49'35.2"	E 93°54'5.5"
Heingang Marjing	7.08	834	North- Hill Base	N 24°52'35.2"	E 93°57'1.0"

5 days old fungal growth from PDA plates were removed with a sterilized cork borer and inoculated in the test tube containing the respective test carbon sources. Tubes were incubated at 25°C for 7 days in a static condition. The change in broth colour from red to yellow and formation of gas bubbles in the Durham's tube were recorded. The culture broth was collected aseptically and centrifuged at 10,000x g for 10 min at 4°C (Eppendorf® Centrifuge 5804R). The supernatant was used for qualitative determination of bioethanol using Jones Reagent [K2Cr2O7 + H2SO4](19). A 200 µl sample was taken in a 1.5 ml centrifuge tube, to which 250 µl Jones Reagent was added and then incubated for 20 min at 62.5 °C. Presence of ethanol was ascertained by observation of blue-green color development because of oxidation of ethanol to acetic acid by potassium dichromate in the presence of sulphuric acid(20)

2.5 Cellulase Activity Assay

Cellulase activity was evaluated by Filter Paper Assay (FPA) as per International Union of Pure and Applied Chemistry (IUPAC) protocols(21) using Whatman no. 1 filter paper as the substrate and cellulase activity was expressed in International Unit (IU). The seven fungal isolates which showed fermentation ability in presence of three individual test carbon sources were inoculated separately in 30 ml of Czapek Dox broth with 3% cellulose loadings and incubated in shaking incubator at 25 °C and 120 rpm. Broth cultures after 3 days of incubation period were harvested in 2 ml volume from each replicate culture flask at 24 hour interval consistently up to 8th day to evaluate cellulase activity. The broth samples were centrifuged at 5500 rpm for 10 min at 4°C. The supernatants were used for enzyme assay. At the end of fermentation step of CBP of four LCB by two fungal isolates Fusarium oxysporum HG19 and Trichoderma koningiopsis LL28, separately based on their superior cellulolytic activity and fermentative ability, the contents in the replicate flasks were harvested and centrifuged. In the supernatant, the activity of -glucosidase, -xylosidase, endoglucanase and endoxylanase was determined by following the protocol as described earlier(22).

2.6 Molecular Characterization of Fungal Isolates

Seven fungal isolates viz., MB2, MB63, MB66, HG19, HG69, LL28, and KL45 which showed both cellulase activity and fermentative ability were identified by amplification and sequencing of the ITS1-5.8S-ITS2 rDNA region. Isolates were grown in potato dextrose broth for 48 hours at 28°C. The mycelial mat was pelleted by centrifugation (Eppendorf® Centrifuge 5804R) at 8000 rpm for 10 minutes. Isolation of fungal genomic DNA was performed using rapid extraction method as described by Cenis(23). For molecular characterization of fungal isolates, the ITS1-5.8S-ITS2 rDNA region was amplified by PCR using the primer set: pITS1 (5- TCCGTAGGTGAACCTGCGG-3) and pITS4 (5-TCCTCCGCTTATTGATATGC-3)(24) in a C1000TM Touch Thermal Cycler (Bio-Rad, USA). The cycling condition was programmed with an initial denaturation of 5 min at 95°C, followed by 35 cycles at 95°C for 1 min, 52°C for 1 min and 72°C for 1min, with a final extension of 5 min at 72°C and infinite hold time at 4°C completed the run. The amplified products were verified in 1.5% agarose gel and visualized using ethidium bromide in ChemiDocTM MP Imaging System (Bio-Rad, USA). Amplicons of about 430-580 bp thus obtained were sequenced with an ABI 3130 genetic analyzer (Applied Biosystems) at Merck specialists, Bangalore, India. Consensus sequences were subjected to BLAST analysis(25).

2.7 Quantitative Determination of Fermentation in both pure C-substrates and four LCB substrates

Seven isolates along with *F. oxysporum* MTCC1755 as positive control were grown on PDA plates at 28°C for 7 days. Four discs of 0.3 cm2 pieces of mycelial mat were harvested and transferred aseptically to a 100 ml Erlenmeyer flask individually for each isolates containing 30 ml T1 medium (20 g/L xylose, 1 g/L yeast extract, 10 g/L KH_2PO_4, 2 g/L $(NH_4)SO_4$ and 0.5 g/L $MgSO_4.7H_2O$, pH 5.2) to determine fermentation ability in presence of xylose as source of carbon by incubation at 28°C for 7 days in static condition. For evaluating fermentation ability of the isolates in other carbon substrates, xylose in the T1 medium was replaced by either glucose or cellulose at identical concentration.

Similarly, bioethanol production by two efficient strains *F. oxysporum* HG19 and *T. koningiopsis* LL28 were tested by addition of both native and hydrothermally treated LCB sources, viz. SB, ficus fruits (*F. cunia*), cassava aerial parts and rice straw in substitute of pure C-substrate in three replicates for each substrate.

2.8 Gas Chromatography (GC) Analysis

Extraction procedure
Broth samples (600 µl) from the above experiment containing fungal cells were collected at 24 hours interval up to the 7th day of incubation. The broth was transferred to an Eppendorf tube and centrifuged at 8000 rpm for 2 min at room temperature to sediment the fungal materials. Consequently 500 µl volume of each of the clear supernatants were transferred to a new tube without disturbing the pellets. To it, 1 ml of ethyl acetate and 5 µl of n-butanol (internal standard) were added followed by 30 seconds of vortexing at maximum speed. Finally, the tubes were centrifuged at 5000 rpm for 2 min at room temperature to facilitate phase separation. The sample from the upper phase (organic) was analyzed in a GC (Trace GC Ultra, Thermo ScientificTM).

2.9 Ethanol Standard Solutions and Calibration Curve

Standard solutions of ethanol were prepared in HPLC Grade water in the concentration range of 0.25-1.25 g/L following the same procedure used for extraction and injected. Peak areas of the ethanol were plotted against ethanol concentration (% v/v) and based on the calibration curve, ethanol concentration in the fermentation samples was quantified.

2.9.1 Ethanol Estimation

Ethanol was estimated using Trace GC Ultra, Thermo ScientificTM Chrom-Card Data system, equipped with a TR-V1 wax 30 m column (Thermo Fisher, Id 0.25 mm X 1.40 µm film), on column injector and FID (Flame ionization detector) conditions; 250°C; H2, 25 PSI, equivalent to 25 ml/min; air, 2 PSI, equivalent to 100 ml/min; gain set to 'medium'. Nitrogen (N2) was used as carrier gas with pressure control at 27 ml/min. Oven temperature was initially set at 50°C and then elevated at the rate of 7 °C/min to 100°C, thus giving a total run time of 7 min. Furthermore, 1µl sample was injected manually at time "zero" using a 10µl Hamilton syringe and temperature cycle was started. The syringe was thoroughly washed with ethyl acetate between injections to avoid cross contamination. Each injection was repeated thrice; ethanol routinely came out at retention time equivalent to 65°C.

3. Results and Discussion

3.1 Diversity and preliminary screening of cellulose degrading fungi

A total of 270 fungal isolates obtained from the sacred groves, of which 168 isolates were found to be cellulolytic, based on Congo Red Assay (CRA) results as shown in table 2 and figure 1. The colony morphology of some of the fungal isolates obtained in this study is shown in supplementary figure S1. The CFU/g population of the fungal isolates in cellulolytic fungal media ranged from 1.36 × 103 to 1.52 × 104. Although, the growth of the fungal isolates obtained in Czapek Dox media supplemented with cellulose was an indication of their cellulolytic ability, only 168 isolates could degrade cellulose as evident from formation of clearing zone around their colonies. The effectiveness of CRA in successful detection of cellulase activity was reported for different fungal species by different workers as in Aspergillus species(26), Ophiostoma and Leptographium species(27), Trichoderma species, Penicillium species and Fusarium solani(28).
In the present study also, CRA was found to be effective in the detection of diverse fungal isolates with cellulolytic ability from sacred groves of Manipur, India. Among the different sacred grove sites, Heingang Marjing showed maximum number of cellulolytic fungal isolates compared to the other three sites. Cellulolytic activity was detected after 3 days of incubation and isolates positive for cellulolytic activity were considered as cellulose degraders. The difference in microhabitats of the sacred groves seems to influence the diversity as well as cellulose degrading ability of the isolates. Variations were observed in diameter of clearing zones developed around the colonies due to breakdown of cellulose by cellulase enzyme and this could be related to variation in cellulose degradation ability of the isolates obtained from different sacred groves. Heingang sacred grove site had maximum number and Langol sacred grove site had lowest number of cellulolytic fungi. The pattern of halo zone formation around the periphery of the colonies of the tested isolates varied depending upon type of cellulolytic fungi i.e., sporulating or non-sporulating. The fungal isolates sporulate by diffusing from the main spot of inoculation and forming varied halo zones and therefore the hydrolysis capacity of the sporulating fungi could not be calculated in terms of the ratio of the diameter of clearing zone and colony for accurate comparison among the isolates(29).Yet, this method was used in the preliminary screening employing a large number of isolates for cellulase activity which were subsequently subjected to confirmatory quantitative cellulase activity assay. On the other hand, it was not possible to detect xylanase activity by CRA in our study and our result support earlier results reported(30) and contradict with results

Table 2: Fungal population (CFU/g dry soil) of sacred groves soil on Czapek Dox agar medium and number of cellulolytic and non-cellulolytic isolates obtained

Site	Incubation Temperature (0C)	CFU/g dry soil	Total isolates obtained	Cellulolytic isolates	Non- cellulolytic isolates
Konthoujam Lairembi	30	1.18 X 10^4	69	41	28
Mahabali	30	1.36 X 10^3	73	34	39
Langol Thongak Lairembi	30	3.3 X 10^3	51	26	25
Heingang Marjing	30	1.52 X 10^4	77	67	10
Total			270	168	102

of other authors who could detect xylanase activity by CRA(31; 32).

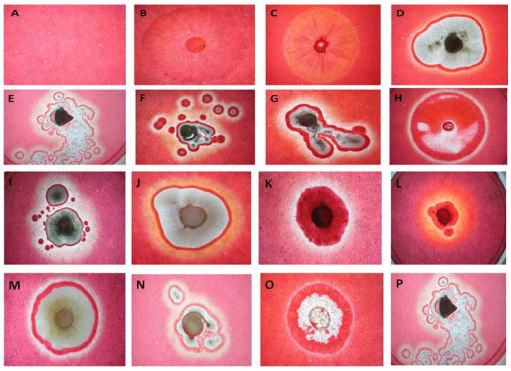

Figure 1: Qualitative cellulase activitythe diverse fungal isolates obtained using congo red assay (CRA) on CMC plates-control (blank), B- negative, C- positive control (MTCC 1755) and D to P- positive fungal isolates

3.2 Preliminary screening of the isolates for their fermentation ability on three carbon sources

The preliminary screening on fermentation ability of the fungal isolates on the three carbon sources viz. glucose, xylose, and cellulose showed interesting results (Figure 2). Seventy-six percent of isolates (207) were unable to ferment any of the carbon sources. Out of the total isolates (270), 12 % were able to ferment glucose and xylose; 10% could ferment all the three carbon sources tested (glucose, xylose, and cellulose) and only 2% could ferment glucose and cellulose. Earlier study(11) reported that the occurrence of microorganisms in nature with ability to ferment both hexose and pentose sugars are rare. We also find that only a very small fraction of cellulolytic fungi of the sacred groves soil samples can use more than one carbon sources for fermentation. It was also observed that the duration of time taken by the isolates to produce alcohol in detectable quantity was also different depending on the type of carbon substrates. For instance, only on the 7th day after incubation, alcohol was detected in cellulose substrate, whereas in xylose and glucose, alcohol was detected on 2nd or 3rd day after incubation by qualitative screening. (Supplementary Table S1, Table S2 and Table S3). Since we were interested in CBP, these seven efficient isolates were selected for the further experiment. Longer duration taken for ethanol production on cellulose substrates could be probably due to longer time taken by the isolates for conversion of cellulose to cellobiose and finally to glucose using cellulase enzymes prior to fermentation. Seven of the 26 isolates with the ability to utilize cellulose as a substrate to produce alcohol also showed high levels of cellulase activity in a preliminary experiment. Thus, the trend in fermentation exhibited by the isolates from the selected carbon sources may be attributed to the relative complexity of carbon sources which has to be hydrolyzed to monomeric sugars and preference of glucose over the other monomeric carbon sources.

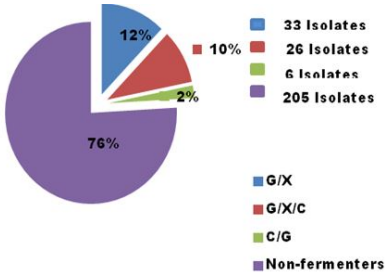

Figure 2: Relative pattern of fungal isolates for their ability to ferment three carbon substrates to bioethanol. G: Glucose; X: Xylose and C: Cellulose

3.3 Identification of selected isolates and their cellulolytic and fermentative potential

The ITS sequences of the seven selected fungal isolates, viz., MB2, MB63, MB66, HG19, HG69, LL28, and KL45 were submitted to GenBank with accession numbers- KR920728, KR920740, KR920737, KR920738, KR920735, KR920726 and KR920724, respectively. The species level identification results of the isolates based on ITS sequences are presented in Table 3. The sequence similarity to most closely related species in the database ranged from 97-100% based on the NCBI blast search. Cellulase activity of seven superior isolates in terms of RCAI and FPase is shown

in Table. 3 and fermentative potential in terms of production of ethanol at different time intervals from three types of carbon sources namely, glucose, xylose, and cellulose in Figure 3A-C. The maximum cellulase activity was observed after the 6th day of incubation at 28˚C (Table 3). Isolate KL45 exhibited highest cellulase activity of 0.041 IU/ml and the other showed activities in the range of 0.028-0.041 IU/ml. The fermentation performance of the seven isolates in terms of ethanol level at three time intervals in T1 medium with substitution of glucose with xylose and cellulose as the sole carbon source at an initial concentration of 20 g/L was different (Figure 3A-C). A maximum titre of 4.85 g/L was reached on 7th day by HG19 (KR920738) with the conversion of 0.24 g/L from glucose fermentation. In the case of xylose and cellulose, the titre was comparatively less where a maximum of 0.46 g/L was attained by MB66 (KR920737) from xylose on the 7th day of incubation. Similarly, a maximum concentration of 0.22 g/L of ethanol was achieved by HG69 (KR920735) from cellulose on the 6th day of fermentation. All the isolates showed a low titre of ethanol yield indicating slow fermentation. As reported in earlier studies, the overall ethanol yield accounted with glucose as sole carbon source was higher than those accounted with any xylose/glucose mixture ratios or from xylose alone(33; 34; 35) and a preference of glucose over other sugars by fermenting microbes for assimilation was always recorded. In comparison with earlier results, our results on fermentation of glucose and xylose substrate (0.24 and 0.02 g/g, respectively) are lower, although the initial substrate concentration used in the earlier published works was higher (40 g/L and 25 g/L) than that of 20 g/L used in the present work.

3.4 Bioethanol production and activity of subfamilies of cellulase enzymes of two fungal isolates on LCB

The result of subsequent study on ethanol production at laboratory scale using 20 g/L as sole carbon source from each of four LCB substrates (untreated and hydrothermally treated) viz. sugarcane bagasse, ficus fruits, cassava aerial parts and rice straw by the two fungal isolates is presented in Figure 4A and 4B, respectively. As observed in Figure 4A, ethanol production by isolate HG19 on hydrothermally treated sugarcane bagasse (BGT) was twice the magnitude of those produced on the untreated material (BGU). The strain did not show any significant difference on ethanol production from the other three substrates between their native and pretreated forms. Similar trend was also observed in the case of isolate LL28 (Figure 4B) on sugarcane bagasse as substrate. Ethanol production by LL28 was reduced when cassava aerial parts was treated hydrothermally (Figure 4B). The apparently higher initial rate of the conversion of untreated substrates, i.e., ficus fruits and cassava aerial parts only compared to their pretreated forms by HG19 and LL28 could be explained due to conversion of available sugars to ethanol apart from those derived from polymer constituents (cellulose and hemicellulose) of the substrates. Hydrothermal pretreatment of cassava aerial parts and ficus fruits might have caused loss of sugars (starch, glucans) present in them possibly due to their degradation. However, from other complex polymers, pretreatment released sugars and therefore, the difference was eliminated as the fermentation advanced. Thus, the varying concentration of ethanol produced could be attributed to inherent level of reducing sugars in LCB, pretreatment effect and fungal isolate preference for the polymer constituent of the particular substrates. Interestingly, a study on thermo chemical pretreatment (1% HCl, 121˚C, 30 min) of brewer's spent grains (BSG) reported reduction in ethanol yield corresponding to the yield of one-third produced from non-pretreated BSG accompanied with prolonged time to achieve maximal ethanol concentrations in 20 days as contradictory to 10 days for the non-pretreated BSG. Both the fungal isolates from our study could produce maximum quantity of ethanol from treated sugarcane bagasse within 144 hrs of incubation. This is also evident from the fact that both strains showed activities of all the sub-families of cellulase enzymes as shown in table 4 but, initially both strains used soluble sugars and released -glucosidase/endoxylanase to further support their metabolic activities. But -glucosidase/endoxylanase activity of HG19 was found to be less and consequently release of soluble sugars could be minimal for enhancement in ethanol production by 144 hrs, whereas LL28 might be less efficient in conversion of C6 sugars to ethanol in spite of its high cellulase activities or diversion of C6 sugars for maintaining other metabolic activities. Similarly, both the fungal isolates showed higher ethanol titer in untreated rice straw in 120 hrs. compared to its hydrothermally treated counterpart which may be attributed to initial available sugars in rice straw along with higher cellulase activities of the fungal isolate on rice straw compared to those on other substrates (Table 4 and Table 5) resulting in release of more sugars for conversion to ethanol. There was a general time trend of increase in ethanol production except in case of untreated ficus fruit (Figure 4A) and cassava aerial parts (Figure 4B).

Table 3: Comparative analysis on cellulolytic activity of selected fungal isolates with their NCBI GenBank accession numbers and ITS sequence similarity retrieved from Database

Isolate Code	GenBank Accession no.	Similarity of species from NCBI database (%)	RCAI (cm)	FPase (IU/ml)
MB 2	KR920728	*Aspergillus awamori* (100)	1.14 ± 0.06	0.032 ± 0.001
MB 63	KR920740	*Aspergillus niger* (100)	1.11 ± 0.06	0.037 ± 0.003
MB 66	KR920737	*Aspergillus foetidus* (100)	1.23 ± 0.07	0.033 ± 0.002
HG 19	KR920738	*Fusarium oxysporum* (99)	1.22 ± 0.08	0.028 ± 0.007
HG 69	KR920735	*Aspergillus tubingenesis* (97)	1.33 ± 0.11	0.029 ± 0.001
LL 28	KR920726	*Trichoderma koningiopsis* (100)	1.15 ± 0.07	0.038 ± 0.004
KL 45	KR920724	*Aspergillus niger* (100)	1.59 ± 0.11	0.041 ± 0.013

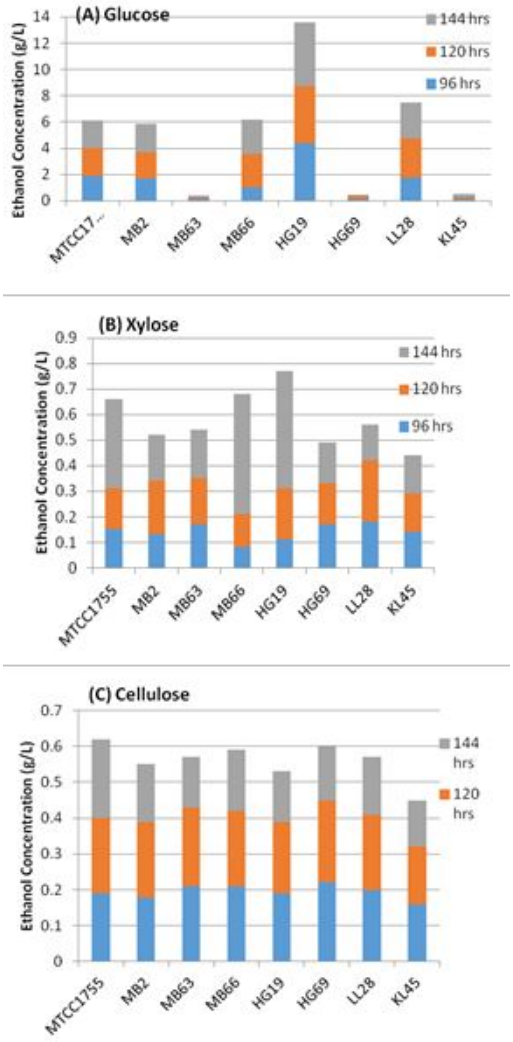

Figure 3: Ethanol production from (A) glucose, (B) xylose and (C) cellulose at three time intervals (hrs) by the selected seven fungal isolates and a positive control MTCC 1755 (*F. oxysporum*)

The fungal isolates initially metabolized and converted glucose/xylose to ethanol and then might subsequently release simple sugars by action of cellulolytic enzymes for observed result of ethanol production at 120 hrs. Cassava aerial parts are rich in pentose sugar and ficus fruit in both glucose and xylose. HG19 did not produce substantial amount of sugars from cellulose (Table 4). On the other hand, LL28 might have produced more xylose by virtue of its high endoxylanase activity (Table 5) and consequently increased ethanol production with time. Hence, it appears that HG19 efficiently utilized reducing sugars of ficus fruit to produce more ethanol compared to LL28 which seems to efficiently utilize reducing sugars of cassava aerial parts to produce ethanol. The reducing sugars were undetected in any supernatant (data not shown) suggesting that liberated sugars from the LCB was likely occurring in very scanty amounts which may have been immediately utilized by the fungus. In a study, cultured Lenzites betulinus IUM 5468 in a medium supplemented with 20 g/L of lignocellulosic materials viz. rice straw and corn stalks separately, resulted in higher ethanol concentration from rice straw compared to corn stalks despite of its high cellulose and hemicellulose contents in rice straw(36). The low titre of ethanol yield in this study may be elucidated in terms of carbon catabolite repression (CCR) exhibited by most microbes since mixed sugars derived from LCB are depleted sequentially. The CCR might have reduced the overall efficacy of the process as catabolism of these pentose sugars is typically suppressed by glucose derived from cellulose(37).Therefore, this selective and consecutive utilization of mixed sugars by most microbes render the fermentation process complex and unpredictable often causing a reduction in yields and productivity from the target biomass(38). The CBP of the selected substrates demonstrated highest ethanol titre of 1.12 g/L and 1.09 g/L in hydrothermally treated sugarcane bagasse which corresponded to ethanol yield of 0.056 g/g and 0.054 g/g of substrate which corresponded to 30.91% and 30.08% of maximum theoretical ethanol yield for both HG19 and LL28, respectively. In other hydrothermally treated substrates, namely cassava aerial parts, ficus fruits and rice straw strain, HG19 achieved maximum ethanol concentrations of 0.70 g/L, 0.70 g/L and 0.67

g/L, respectively (Figure 4A).

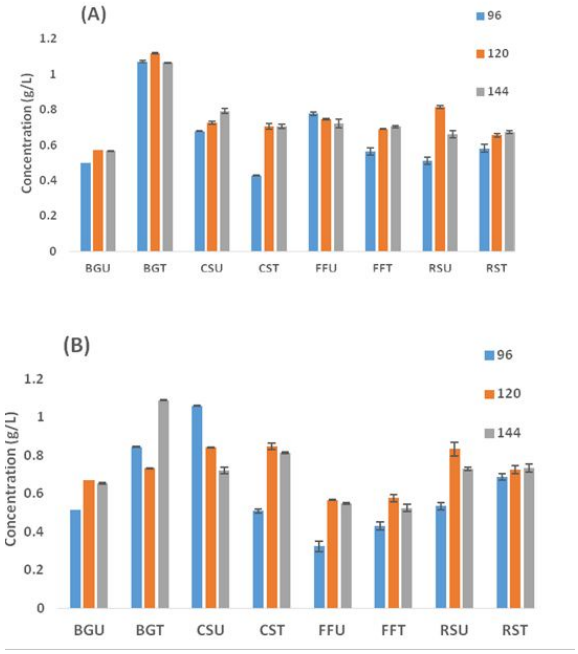

Figure 4: Ethanol production from untreated and hydrothermally pretreated substrates by two superior isolates (A) HG19 and (B) LL28 on four LCB substrates at three time intervals (hrs). BGU: untreated sugarcane bagasse; BGT: hydrothermally pretreated sugarcane bagasse; CSU: untreated cassava aerial parts; CST: hydrothermally pretreated cassava aerial parts; FFU: untreated ficus fruits; FFT: hydrothermally pretreated ficus fruits; RSU: untreated rice straw; RST: hydrothermally pretreated rice straw. Error bars indicate standard deviations of the means from two independent experiments

Similarly, in case of strain LL28, maximum ethanol concentration attained were 0.85 g/L, 0.58 g/L and 0.73 g/L from hydrothermally treated cassava aerial parts, ficus fruits and rice straw, respectively (Figure 4B). Among the untreated biomass, cassava aerial parts attained highest ethanol titre of 1.06 g/L due to LL28 strain followed by rice straw, ficus fruits and sugarcane bagasse within 96 to 144 hours of fermentation (Figure 4B). Cellulolytic activities of the fungal strains recorded after fermentation on various lignocellulosic substrates both untreated as well as hydrothermally pretreated are presented in Table 4 and Table 5. The *T. koningiopsis* strain LL28 had the highest -glucosidase, endoglucanase, endoxylanase and -xylosidase on hydrothermally treated rice straw (1.04, 57.73, 76.78 and 0.24 IU g-1 ricestraw, respectively). Similarly, *F. oxysporum* strain HG19 showed altogether lowest activity of -glucosidase, endoglucanase, endoxylanase and - xylosidase on untreated ficus fruits (0.13, 12.27, 20.21 and 0.04 IU g-1 respectively) among the different substrates. Both the strains HG19 (Table 4) and LL28 (Table 5) showed maximum individual cellulase activities in rice straw, whether untreated or hydrothermally pretreated, among the four substrates experimented. The differences in enzyme activities depending on the substrates could be attributed to heterogeneity in substrate composition and structure(39). Our result provides further evidence that the nature of the carbon source has a significant influence on cellulase enzyme production as observed by earlier workers(40).

The findings of the present study are in the line of the observations as reported by previous workers employing untreated, acid, alkali and oxidative treated agricultural residues for cellulase production, using A. niger(41). The results obtained with combined alkali and acid treated sugarcane bagasse using *Trichoderma* sp. showed maximum cellulase activity of 0.45 U for CM Case; 3.18 U for cellobiase and 0.091 U for FPase, respectively. Efficient conversion of LCB to monosaccharides requires synergistic interaction of cellulolytic β-xylanolytic enzymes. The low β-glucosidases and β-xylosidases of both the fungal strains may be a limiting factor for reduced ethanol yields in the present study as β-glucosidase and β-xylosidase are terminal enzymes which are crucial in LCB bioprocessing from intermediary products (cellobiose, xylobiose and oligosaccharides) to glucose and xylose. Further, productivity enhancements could be achieved by optimizing the SSF relevant parameters, such as temperature, moisture content, aeration, supplementation of the medium. Even though it is tedious to compare enzyme production and ethanol yield values given the different cultivation conditions and microorganisms employed in each study, the values reported here, using fungal strains, demonstrate their ability to produce endoglucanases and endoxylanases from the substrates under study and produced ethanol through CBP.

4. Conclusion

F. oxysporum HG19 and *T. koningiopsis* LL28 are two potential fungal strains obtained in this study; both exhibited fermentative ability on the selected carbon sources and produced both cellulolytic and xylanolytic enzymes for degradation of cellulose present in different LCB. The strains showed maximum individual cellulase activities in rice straw, used in native or hydrothermally pretreated state, among the four substrates tested. Although cellulolytic fungi produce a large repertoire of saccharolytic enzymes to digest LCB efficiently, the development of fungi as CBP organisms still remains a challenge in enhancing ethanol yields and rates of fermentation through assimilation of all available sugars towards conversion to ethanol. Results of this study are encouraging and to utilize LCB for enhanced bioethanol yield through CBP, we suggest optimization of process parameters in future experiments.

Table 4: Activity of the four sub-families of cellulase enzymes of isolate HG19 (*F. oxysporum*) at the end of CBP of four LCB substrates in their native and hydrothermally pretreated states.

Substrate	Enzyme activity (Ug-1 substrate)			
	$\beta - glucosidase$	Endoglucanase	Endoxylanase	$\beta - xylosidase$
	Untreated substrates			
Sugarcane bagasse	0.18 ± 0.01	22.29 ± 1.42	33.25 ± 1.06	0.11 ± 0.02
Cassava aerial parts	0.16 ± 0.06	18.73 ± 2.03	24.47 ± 1.65	0.14 ± 0.05
Ficus fruits	0.13 ± 0.04	12.27 ± 1.01	20.21 ± 1.3	0.04 ± 0.02
Rice straw	0.32 ± 0.02	28.48 ± 0.66	42.26 ± 1.16	0.12 ± 0.04
	Hydrothermally treated states			
Sugarcane bagasse	0.25 ± 0.04	33.47 ± 1.89	46.32 ± 1.31	0.16 ± 0.02
Cassava aerial parts	0.22 ± 0.02	27.89 ± 1.84	33.37 ± 1.2	0.13 ± 0.11
Ficus fruits	0.14 ± 0.04	18.86 ± 0.67	29.28 ± 1.14	0.07 ± 0.01
Rice straw	0.32 ± 0.10	39.35 ± 1.3	55.84 ± 0.43	0.16 ± 0.05
#Values are mean of three replicates ± standard error				

Table 5: Activity of the four sub-families of cellulase enzymes of isolate LL28 (*T. koningiopsis*) at the end of CBP of four LCB substrates using in their native and and hydrothermally pretreated states

Substrate	Enzyme activity (Ug-1 substrate)			
	$\beta - glucosidase$	Endoglucanase	Endoxylanase	$\beta - xylosidase$
	Untreated substrates			
Sugarcane bagasse	0.53 ± 0.03	44.24 ± 1.53	60.22 ± 0.89	0.18 ± 0.03
Cassava aerial parts	0.32 ± 0.04	35.37 ± 1.20	44.24 ± 1.53	0.14 ± 0.04
Ficus fruits	0.13 ± 0.07	21.93 ± 1.60	35.05 ± 0.51	0.07 ± 0.02
Rice straw	0.75 ± 0.10	46.39 ± 1.07	63.62 ± 0.68	0.20 ± 0.02
	Hydrothermally treated substrates			
Sugarcane bagasse	0.86 ± 0.02	55.42 ± 2.59	73.89 ± 0.89	0.26 ± 0.03
Cassava aerial parts	0.60 ± 0.04	47.07 ± 1.66	62.93 ± 1.31	0.21 ± 0.02
Ficus fruits	0.19 ± 0.01	33.86 ± 0.87	47.66 ± 1.21	0.12 ± 0.03
Rice straw	1.05 ± 0.15	57.73 ± 1.21	76.78 ± 1.16	0.24 ± 0.05
#Values are mean of three replicates ± standard error				

Conflict of Interest The authors declare no conflict of Interest in this reported communication.

Acknowledgments The authors deeply acknowledge the anonymous reviewers for improving the manuscript and DBT for the work.

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