Isolation and screening of potential cellulolytic fungi from Areca nut husk waste

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Abstract

The present study was aimed to isolate and screen the ability of cellulolytic fungal strains from areca nut husk waste to produce cellulolytic enzymes for use in the bioconversion of waste products into valuable biomass products. Out of twenty eight fungal culture isolates from areca nut husk waste, twenty four fungi were able to degrade the cellulose. The highest cellulolytic activity was detected in only ten isolates viz. Aspergillus niger (16±0.6 mm), Aspergillus terreus (15±0.5 mm), Trichoderma viride (13±0.3 mm), Aspergillus flavus (12±0.3 mm), Fusarium chlamydosporum (10±0.04 mm), Aspergillus fumigatus (9.5±0.2 mm), Aspergillus clavatus (9.0±0.05 mm), Paecilomyces carneus (8.0±0.2 mm), Penicillium chrysogenum (7.2±0.3 mm) and Aspergillus wentii (7.0±0.2 mm) then compared to other fungi. While Aspergillus sp 2, Penicillium sp 3, Heteroconium chaetospira and Staphylotrichum coccoporum were not produce cellulase enzymes. These fungal strains are very important for degradation of agricultural wastes, municipal solid wastes and some other organic wastes.

Keywords: screening, fungi, cellulolytic activity, areca nut husk waste

Introduction

The large volumes of cellulosic waste generated annually from forestry commercial, agricultural and industrial activities are difficult to degrade and cause imbalances in the ecosystem. These residues encompass material such as agricultural residue, municipal solid waste (MSW), cellulosic material (35-45%) found in MSW (Kader et al., 1999; Gautam et al., 2010). Cellulose is commonly degraded by an enzyme called cellulase. Cellulases are consortia of complex hydrolytic enzymes capable of hydrolyzing these materials to smaller sugar components like glucose units. This enzyme is produced by several microorganisms, mainly by bacteria and fungi (Bahkali, 1996; Mangelli and Forchiassin, 1999; Shin et al., 2000; Immanael et al., 2006). Although, a large number of microorganisms are capable of degrading cellulose, only few of these produce significant quantities of cell free enzymes capable of completely hydrolyzing crystalline cellulose in vitro. Fungi are the main cellulase producing microorganisms, although a few bacteria and actinomycetes have also been reported to yield cellulase activity (Sharma et al., 1991; Sridevi and Charya, 2011). Several studies were carried out to produce cellulolytic enzymes from bio-waste degradation process by many microorganisms including fungi such as Trichoderma sp, Penicillium sp, Aspergillus sp. etc. (Miller, 1972; Mandels and Reese, 1985; Lakshmikant and Mathur, 1990). Similarly cellulolytic property of bacterial species like Pseudomonas sp, Cellulomonas sp, Bacillus sp, Micrococcus sp, Cellovibrio sp and Sporosphytophaga sp. were also reported (Immanael
et al., 2006). The specific cellulolytic activity shown by the bacterial species is found to be depending on the source of occurrence (Saxena et al., 1993).

Some features of natural cellulosic materials are known to inhibit their degradation/bioconversion (Solomon et al., 1999). These factors are the degree of crystallinity, levels lignification and the capillary structure of cellulose cellulolytic enzymes and other hydrolytic agents. However, many physical, chemical and microbial pre-treatment methods for enhancing bioconversion of cellulosic materials have been reported (Nazareth and Mavinkurve, 1987; Kanosh et al., 1999; Pothiraj et al., 2006). Areca nut (Areca catechu L.) popularly known as betelnut or supari, is one of the most important plantation crops of India. It is cultivated in India covering an area of about 2.6 lakh ha with an annual production of 3.13 lakh tonnes. Areca nut is a most important commercial plantation crop in Shivamogga district of Karnataka state, cultivated in 26,725 ha with an annual production of 37,458 tonnes (Narayanamurthy et al., 2008). The areca nut husk fibers are predominantly composed of cellulose and varying proportions of hemicellulose (35-64.8%), lignin (13.0-26.0%), pectin and protopection. The total hemicellulose content varies with the development and maturity, the mature husk containing less hemicellulose than the immature ones. The lignin content proportionately increases with the development until maturity (Rajan et al., 2005; Mohankumar, 2008). The present study was initiated to isolate and screen the cellulolytic indigenous fungal strains and to explore their hydrolytic potential for their possible future application.

Materials and Methods

Collection of Areca nut husk waste

Areca nut husk waste was collected from the local farmer in Shivamogga region, Karnataka, India during the 2011-2012. The samples were taken by means of sterilized spatulas and collected in sterile sealed polythene bags. The sample was brought to the laboratory and was maintained at room temperature for microbiological study.

Isolation of fungi by serial dilution method

One gram of areca nut husk waste sample was taken in a conical flask containing nine milliliters of sterile distilled water and shaken well in vortex mixer for 30 mins. From this stock, various dilutions were prepared from 10⁻¹ to 10⁻⁷, using sterile distilled water. One milliliters of the diluted sample was poured into petriplates containing the Martin Rose Bengal agar medium and Potato Dextrose agar medium. Streptomycin was added to the molten medium after autoclave and the plates were incubated at 28 ± 2°C for 4 to 5 days to identify the fungi. Distinct fungal colonies grown on Martin Rose Bengal agar medium and Potato Dextrose agar medium were isolated from repeated plating (Aneja, 2001; Paul and Daniel, 2007; Naveenkumar et al., 2011).

Isolation of fungi by war cup soil plate method

Areca nut husk waste sample was collected in sterilized polythene bag. Then, 0.15 g of soil sample was added to sterile plates with the help of a sterilized cooled loop or transfer needle. Then, 15-20 ml of melted, cooled (45°C) sabouraud’s agar media was added supplemented with Streptopenicillin and Rose Bengal, to each soil inoculated petriplate. Dispense the soil particles throughout the medium by gentle rotation of the Petri dishes and allowed the plates to solidify. Plates were incubated at room temperature (28°C) in an inverted position for 15 days. After incubation, observed the different fungal colonies grown on sabouraud’s agar media (Aneja, 2001; Naveenkumar et al., 2011).
Identification of fungi

Fungal morphology was studied macroscopically by observing colony features (colour and surfaces) under stereo binocular microscope (Magnus BQ0004) and microscopically by staining with lacto phenol cotton blue and observe under binocular compound microscope (LABOMED Vision 2000) for the conidia, conidiophores and arrangement of spores (Fundar, 1961; Booth, 1971; Barnett, 1975; Domsch et al., 1980; Subramanian, 1983; Aneja, 2001; Naveenkumar et al., 2012).

Screening of more efficient cellulolytic fungal species

Primary screening

Isolated fungi they were inoculated on potato dextrose agar plates supplemented with 2% Carboxymethyl cellulose sodium salt (CMC) (HiMedia) as a carbon source and Tetracycline (10-25 µg/mL) to control the bacterial contamination (pH 7.0), incubation was done at 28 ± 1ºC for 5-7 days. After incubation fungal species were purified and sub cultured on Carboxymethyl cellulose agar plates and used for the subsequent purpose (Gautam et al., 2010; Bankar et al., 2012).

Secondary screening

Secondary screening was done for the selection of more potent colonies for the production of extracellular cellulase by culturing on Carboxymethyl cellulose agar plates. Actively growing mycelium (3 days old) were removed from the growing edge of the fungal isolates by using sterile cork borer of 6 mm dia., the discs were inoculated to the pre-welled CMC agar (pH 7.0) plates and incubated at 28±1ºC for 5-7 days. After incubation plates were flood with Congo red solution (1 mg/ml in distilled water) for 20 min, decant the dye and flooded with 5 M NaCl for 20-30 min and decanted it. Carboxymethyl cellulase (CMCase) producing colonies were seem to be surrounded by the pale orange to clear against the background (Teather and Wood, 1982; Bankar et al., 2012).

Statistical analysis

Data presented on the average of three replicates as means ± standard error obtained from independent experiments.

Results

In the present study, mainly focus on cellulolytic fungi isolated from the areca nut husk waste. Twenty eight different isolates were screened from areca nut husk waste for their ability to produce extracellular cellulases. The cellulolytic activity of the fungal strains was determined according to their ability to grow and form clear zones around fungal colonies in 2% carboxymethyl cellulose sodium salt (CMC) (HiMedia) medium. Twenty four isolates were able to synthesize extracellular cellulase. Highest cellulolytic activity was detected in only ten isolates viz. Aspergillus niger (16±0.6 mm), Aspergillus terreus (15±0.5 mm), Trichoderma viride (13±0.3 mm), Aspergillus flavus (12±0.3 mm), Fusarium chlamydosporum (10±0.04 mm), Aspergillus fumigatus (9.5±0.2 mm), Aspergillus clavatus (9.0±0.05 mm), Paecilomyces carneus (8.0±0.2 mm), Penicillium chrysogenum (7.2±0.3 mm) and Aspergillus wentii (7.0±0.2 mm) (Table 1), (Figures 1 and 4). The cellulolytic activity was significantly medium in other fungi such as Exophiala jeaneselmei (6.6±0.1 mm), Penicillium brevicompactum (5.0±0.2 mm), Rhizopus stolonifer (4.8±0.1 mm), Gibberella avenacea (4.0±0.1 mm),...
Table 1. Screening and measurement of zone of clearance by fungal isolates

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Zone of Clearance (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus niger (Van Tiegham 1867)</td>
<td>16±0.6</td>
</tr>
<tr>
<td>Aspergillus terreus (Thom 1918)</td>
<td>15±0.5</td>
</tr>
<tr>
<td>Aspergillus fumigatus (Fres.1863)</td>
<td>9.5±0.2</td>
</tr>
<tr>
<td>Aspergillus flavus (Link ex Gray 1821)</td>
<td>12±0.3</td>
</tr>
<tr>
<td>Aspergillus clavatus (Desm 1834)</td>
<td>9.0±0.05</td>
</tr>
<tr>
<td>Aspergillus wentii (Wehmer 1869)</td>
<td>7.0±0.2</td>
</tr>
<tr>
<td>Aspergillus candidus (Link ex Link 1824)</td>
<td>3.0±0.03</td>
</tr>
<tr>
<td>Aspergillus sp1.</td>
<td>0.9±0.1</td>
</tr>
<tr>
<td>Aspergillus sp2.</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Penicillium chrysogenum (Thom 1910)</td>
<td>7.2±0.3</td>
</tr>
<tr>
<td>Penicillium brevicompactum (Dierckx 1901)</td>
<td>5.0±0.2</td>
</tr>
<tr>
<td>Penicillium canescens (Sopp1912)</td>
<td>3.2±0.1</td>
</tr>
<tr>
<td>Penicillium sp1.</td>
<td>1.1±0.2</td>
</tr>
<tr>
<td>Penicillium sp2.</td>
<td>0.8±0.04</td>
</tr>
<tr>
<td>Penicillium sp3.</td>
<td>0.04±0.0</td>
</tr>
<tr>
<td>Gibberella avacacea (R.J. Cook, 1967)</td>
<td>4.0±0.1</td>
</tr>
<tr>
<td>Fusarium chlamydosporum (Wollenw and Reink, 1925)</td>
<td>10±0.04</td>
</tr>
<tr>
<td>Rhizopus stolonifer (Ehrenb.ex Link) Lind 1913</td>
<td>4.8±0.1</td>
</tr>
<tr>
<td>Paecilomyces carneus (Duche and Hein), A.H.S. Brown and G. Sm., 1957</td>
<td>8.0±0.2</td>
</tr>
<tr>
<td>Trichoderma viride (Pers.ex Gray 1821)</td>
<td>13.0±0.3</td>
</tr>
<tr>
<td>Humicola fuscoatra (Traena 1914 var.fuscoatra)</td>
<td>1.0±0.1</td>
</tr>
<tr>
<td>Staphylocitrinum coccosporum (J. Meyer and Nicot 1957)</td>
<td>0.04±0.0</td>
</tr>
<tr>
<td>Pentasporium batista (Batista, 1957)</td>
<td>1.9±0.2</td>
</tr>
<tr>
<td>Exophiala jeansi (McGinnis and Padhye, 1977)</td>
<td>6.6±0.1</td>
</tr>
<tr>
<td>Cheiromyces stellatus (Damon, 1950)</td>
<td>1.0±0.1</td>
</tr>
<tr>
<td>Gliocladium viride Matr. 1893</td>
<td>2.0±0.06</td>
</tr>
<tr>
<td>Lacellina graminicola (Subramanian, 1952)</td>
<td>1.5±0.1</td>
</tr>
<tr>
<td>Heterocormium chaetospira (Grove) M.B. Ellis 1976</td>
<td>0.0±0.0</td>
</tr>
</tbody>
</table>

Penicillium canescens (3.2±0.1 mm), Aspergillus candidus (3.0±0.03 mm) and Gliocladium viride (2.0±0.06 mm) (Table 1), (Fig. 2). The cellulolytic activity was considerably low in other fungi such as Pentasporium batista (1.9±0.2 mm), Lacellina graminicola (1.5±0.1 mm), Penicillium sp 1 (1.1±0.2 mm), Humicola fuscoatra (1.0±0.1 mm), Cheiromyces stellatus (1.0±0.1 mm), Aspergillus sp 1 (0.9±0.1 mm) and Penicillium sp 2 (0.8±0.04 mm). While Aspergillus sp 2, Penicillium sp 3, Heterocornium chaetospira and Staphylocitrinum coccospormus were not produce cellulase enzymes. Aspergillus sp. occurrence was also predominantly higher among other fungal strains followed by Fusarium sp., Penicillium sp., Rhizopus sp. and Trichoderma sp (Table 1; Fig. 3).

Fig. 1. The graphical representation of highest cellulolytic activity showed by fungal species isolated from areca nut husk waste.
Species of the fungal genus *Trichoderma* sp., *Fusarium* sp. and *Aspergillus* sp. have been broadly studied, particularly due to their ability to secrete cellulose degrading enzyme the most common and most potent cellulase producers are *Aspergillus niger* (16±0.6 mm), *Aspergillus terreus* (15±0.5 mm), *Trichoderma viride* (13±0.3 mm), *Fusarium chlamydomosporum* (10±0.04 mm), *Aspergillus fumigatus* (9.5±0.2 mm), *Penicillium chrysogenum* (7.2±0.3 mm) and *Paecilomyces carneus* (8.0±0.2 mm) showed excellent cellulose producing results in the screening. These fungi were potential cellulose degrader and useful for the management of municipal solid waste, agricultural residues and areca nut husk waste.

**Discussion**

This study mainly concentrates on isolate and screens the cellulytic indigenous fungal strains from areca nut husk waste and to explore their hydrolytic potential enzymes for their possible future application. The cellulase production ability of fungi assessed by estimating zone around the colony formed due to ability of fungal isolates to hydrolyse cellulose. Cellulases are a group of hydrolytic enzymes capable of hydrolysing cellulose to smaller sugar components like glucose units. Cellulolytic enzymes play an important role in nature's biodegradation processes where plant lignocellulosic materials are efficiently degraded by cellulolytic fungi. Lynd et al. (2002) reported that the screened fungal strains will be useful for further studies by enzyme producers. Fungi are known agents of decomposition of organic matter in general and of cellulosic substrate. In our study, clearly represents that *Aspergillus niger* (16±0.6 mm), *Aspergillus terreus* (15±0.5 mm), *Trichoderma viride* (13±0.3 mm), *Fusarium chlamydomosporum* (10±0.04 mm), *Paecilomyces carneus* (8.0±0.2 mm), *Penicillium chrysogenum* (7.2±0.3 mm) showed excellent cellulose producing results in the screening. Morederate cellulose producers were also recorded among other fungi. Gautam et al. (2010) have been reported that to screen the highest cellulolytic ability of fungi *Aspergillus fumigatus* and very low cellulase activity showed by *Humicola* sp., *Torula* sp., these fungi isolated from municipal solid waste.

In this study, *Aspergillus* sp., *Penicillium* sp. and *Trichoderma viride*, which is the most extensively studied cellulase producer, isolated from areca nut husk waste. *Aspergillus niger* showed excellent cellulose producing results in the screening. Reese and Levinson (1952) reported that a few studies have been conducted earlier with
Fig. 4. Shows highest cellulolytic activity of fungal species. a. Aspergillus niger, b. Aspergillus terreus, c. Trichoderma viride, d. Aspergillus flavus, e. Fusarium chlamydosporum, f. Aspergillus fumigatus, g. Aspergillus clavatus, h. Paecilomyces carneus, i. Penicillium chrysogenum

Aspergillus niger and Trichoderma sp. to investigate their cellulolytic ability. This may be due to a possibility that the growth of Trichoderma sp. was suppressed by rapidly growing fungi like Aspergillus sp. Isolates were found to possess cellulolytic activity and produce extracellular cellulases. Aspergillus niger and Trichoderma sp. is well known among the cellulolytic fungi for their potential to degrade areca nut husk waste. Gautam et al. (2010) reported that the Trichoderma sp. is well known among the cellulolytic fungi for their potential to degrade organic municipal solid waste. A few studies have been conducted earlier with Trichoderma sp. to investigate their cellulolytic ability.

Doolotkeldieva and Bobusheva (2011) have been reported the soft-rot fungi, Trichoderma viride and Trichoderma reesei are the most extensively studied cellulolytic fungi. During the past few years, investigations of these fungi have made significant progress towards elucidating the enzymology of cellulose degradation. Screening of different fungal genera species and finding the suitable cellulolytic enzyme producer from areca nut husk waste. In some genera like Trichoderma sp., Fusarium sp., Aspergillus sp., Penicillium sp. and Paecilomyces sp. showed some differences in their cellulase activity. The present finding indicates that the cellulose system of these fungal forms contain enzymes complexes for the effective hydrolysis of
The present study indicates that number of cellulolytic fungi isolated from areca nut husk waste.

**Conclusion**

The present study concluded that to isolate and screened the cellulolytic indigenous fungal strains from areca nut husk waste and to explore their hydrolytic potential for their possible future application. These fungal strains are very essential for degradation of agricultural wastes, municipal solid wastes and some other organic wastes. Cellulolytic enzymes play an important role in nature's biodegradation processes where plant lignocellulosic materials are efficiently degraded by cellulolytic fungi.

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**Reference**


