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Isolation and screening of lignocellulose hydrolytic saprophytic fungi from dairy manure soil

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ABSTRACT

This study was aimed to screen the lignocellulolytic ability of selected fungi under laboratory conditions. In presence of lignin and hemicellulose will reduce the activity of cellulase. Wild type fungal strains which have cellulolytic activity along with lignolytic activity, hemicellulolytic activity, were isolated from different animal dung manure soils collected from Sri Venkateswara Go Samrakshanasaala, Tirupati. All starins were studied by cultural morphological characteristics and microscopic examinations. Seventy fungal strains were isolated and among which ten isolates found to have lingocellulolytic activity. The isolates namely Trichoderma, Aspergillus, Penicillum, Altrernaria, were found to be highly lingocellulolytic compared to the rest. This study showed that the fungal isolates with appreciable hydrolytic zones have good hydrolytic property of lignocelluloses.

Key words: Isolation and screening, Lignocellulolytic properties, Fungi, Dairy manure, Cellulase activity.

INTRODUCTION

Lignocellulose is the major structural component of woody plants and non-woody plants such as grass and represents a major source of renewable organic matter. Lignocellulose consists of lignin, hemicellulose and cellulose, (Fengel and Wegener, 1989; Eaton and Hale, 1993). Cellulases and hemicellulases have numerous applications and biotechnological potential for various industries including chemicals, fuel, food, brewery and wine, animal feed, textile and laundry, pulp and paper and agriculture (Wong and Saddler, 1992a,b;Bhat, 2000; Sun and Cheng, 2002; Beauchemin *et al*., 2001, 2003). The saccharification process of cellulose waste relies on participation of cellulolytic organisms and their cellulase enzymes (Beguin and Aubert, 1994; Singh and Hayashi, 1994; Lynd *et al* 2002).The hydrolysis of the lingocelluloses to fermentable sugars seems to be the main reason for the high producing cost of cellulolytic

enzymes from lignocellulosic wastes. The presence of lignin in cellulosic substrates and the crystalline nature of cellulose make it in accessible to cellulose. Successful utilization of cellulosic materials as renewable carbon sources is dependent on the development of economically feasible process technologies for cellulase production, and for the enzymatic hydrolysis of cellulosic materials to low molecular weight products such as mono saccharides. Plate assays are powerful tools used in screening fungi for lignocellulose degrading enzyme production. Such tests give a positive or negative indication of enzyme production. They are particularly useful in screening large numbers of fungal isolates for several classes of enzyme, where definitive quantitative data are not required. The reagents required are all commonly available and relatively inexpensive. Cellulases have been essentially utilized for the improvement of nutritional values of animal feed and hence the hyper producing species screened for cellulases can be utilized for enhanced live stock Production. The conversion of cellulosic mass to fermentable sugars through biocatalyst cellulase derived from cellulolytic organisms has been suggested as a feasible process and offers potential to reduce use of fossil fuels and reduce environmental pollution.

MATERIALS AND METHODS

Sample collection:
Samples of Domestic and Wild animal dung manure soils (cow, horse, elephant, and camel) collected from Sri Venkateswara Go Samrakshanasaala, Tirupati, Chittoor District, of Andhra Pradesh, India. Soil samples were air dried and mixed thoroughly to increase homogeneity.

Biological Characters:
Micro flora of manure soil such as fungal populations were enumerated by taking 1gm of soil and serially diluted up to 10^{-1} to 10^{-4} with sterilized distilled water. Diluted suspensions of 0.1ml samples were plated and spread over Czapek dox agar medium. The plates were incubated for 7days at room temperature. After incubation colonies appeared on the Czapek-Dox agar surface were counted by colony counter, and the colony forming units (CFU/g) were calculated (Table 1).

Microorganisms:
Four wild type strains of *Aspergillus sp,* four *Trichoderma sp., one Penicillum sp*., and *one Alternaria sp*., were isolated from animal dung manure soils like cow dung, horse dung, camel dung, deer dung, elephant dung, and maintained on PDA slants and stored at 4^{o} C.

Isolation of cellulolytic fungi:
Different animal dung manure soil samples were collected locally in Sri Go Samrakshanasaala, Tirupati. For the purpose of study potato dextrose agar, Czapeck-Dox agar was used. At the set on set, the agar medium was sterilized using an autoclave. The prepared medium was poured in sterile petriplates. The collected animal dung manures were subjected to serial dilutions and pleated on to sterile medium. With appropriate substrate to observe growth as well as substrate utilization by fungi with lignocellulolytic properties.

The following qualitative methods were adopted to select the lignocellulase producing microorganisms:

a) Cellulose agar clearance :

The medium supplemented with 1% phosphoric acid swollen cellulose with Mandel's medium was used for cellulase agar clearance. Incorporation of the cellulose in to solid agar media results in an opaque substrate due to insolubility of cellulose. Clearance indicates cellulolysis. The diameter of the zone was measured (Figure 1).

b) Dye staining of carboxymethyl cellulose agar :

Czapek Dox medium incorporated with 1% carboxymethyl cellulose was used to grow the fungal isolates. The plates were stained with 1% Congo red solution followed by neutralization with 1M NaCl solution. Formation of yellow color halo zone around the colony after flooding with 1% Congo red solution and washed with 1M NaCl confirmed the production of cellulase. (Figure 2).

c) Dye staining of xylan agar :

The medium is supplemented with 1% Xylan (brichwood). The plates were inoculated with the fungus and flooded with iodine stain containing 1% iodine crystals, 2% potassium iodine. Based on the clearing zone the xylanase producing organisms were screened. Xylan degradation around the colonies appeared as a yellow- opaque area against a blue/reddish purple colour for undegraded xylan. (Figure 3)..

d) Bavendamm test :

The cellulolytic fungal isolates were grown on solid LME (Lignin modified enzyme) medium incorporated with 2ml of 20% glucose and 1ml of 1% tannic acid. The plates were observed for browning of the media due to polyphenoloxidase activity. Based on the browning of the media, this is the test used to detect lignin modified enzymes (LME) (Figure 4). The fungal isolates which are positive for the above preliminary and qualitative screening methods were considered as potent cellulolytic strains and further used for enzyme production to select hyper producing strains.

Growth, production and detection of cellulase enzyme activity:

The fermentation media used was Mary Mandel's mineral salts solution and it was used along with different carbon and nitrogen sources. The medium contained the following (per L) Cellulose,10g ; Peptone,1g ; (NH4)2SO4, 1.4g ; KH2PO4, 2g ; CaCl2, 0.3g ; MgSO4.7H20, 0.3g ; Urea, 0.3g ; *trace metal solution 1 ml (2.5g FeSO4; 0.98g MnSO4.H2O ; 1.76g ZnSO4.H2O ; 1.83g CoCl2.6H2O dissolved in 495ml of distilled water and 5ml of conc. HCl), 1ml ; pH 4.8 (Jeffries, 1996).

All the media mentioned above were prepared separately and dispensed into conical flasks. They were sterilized in the autoclave at 121ºC for 15 minutes. Mineral salts glucose medium was prepared and approximately 2.8×10^6 spores/cells of each culture were inoculated into 250 ml flasks containing 50 ml of medium each. The spores/ cells were counted using a Neubauer counting chamber. The flasks were incubated for 96 hours at 29±1ºC on a Gallenkamp (England) rotary shaker at 250rpm to develop the inoculum. Samples were withdrawn from the culture at 5-day and the supernatant that resulted following centrifugation at 3000 rpm for 15 minutes to remove solids, were assayed.

Total cellulase activity in the culture filtrate was determined according to the method of Mandels et al. (1976). Aliquots of appropriately diluted culture filtrate as enzyme source was added to Whatman No.1 filter paper strip (1 X 6 cm; 50mg) immersed in one millilitre of 0.05 M sodium citrate buffer of pH 5.0. After incubation at 50°C for 1 h, the reducing sugar released was estimated by dinitrosalicylic acid (DNS) method (Miller, 1959). One unit of filter paper (FPU) activity was defined as the amount of enzyme releasing 1mole of reducing sugar from filter paper per ml per min.

RESULTS

The cellulolytic fungi were screened from diverse sources of samples for cellulase production a total of 70 fungal spp. were isolated from manure samples, the colony forming units per gram of sample (CFU/g) were calculated (Table 1).

Table 1: Total Fungal count from different sources of samples.

S. No.	Sample	Total fungal population CFU/g.	% of Cellulase Positivity
1.	Domestic animal dung manure	$3.65X10^5$/g	23.07%
2.	Wild animal dung manure	$3.2X10^5$/g	33.33%

The isolated strains were carefully identified by morphological Characteristics include color of the colony and growth pattern studies. Some of the microscopic characteristics examined under the microscope include spore formation and color.

The isolated fungi were selected by several screening methods by plate assay, to evaluate the lignocellulolytic properties like cellulolytic, xylanolytic, and polyphenoloxidase activity and diameter of hydrolysis zones were measured. Among 70 fungal spp only ten strains consists of *Aspergillus sp., Trichoderma sp.* Possessing lignocellulolytic properties were selected for enzyme production. They were named as HMCD1 to HMCD10. All ten isolate posses lignocellulolytic activitity among them HMCD 2 *Aspergillus* sp and HMCD8 *Trichoderma* sp. were showed the highest diameter of clearing zones in plate assays (Figure: 1 to 4), with the total cellulolytic activity **3.89IU**, **3.88 IU** respectively (Table 2 and Figure 5).

a) Cellulose agar clearance

Figure 1: positive strain (left) of *Trichoderma* sp. showing cellulose clearance in the Mandel's medium, negative strain (right) of *Trichoderma* sp. without cellulose clearance.

b) Dye staining of carboxymethyl cellulose agar

Figure 2: positive strain (left) of *Aspergillus niger* showing clearing zone, negative strain (right) of *Aspergillus niger* without any characteristic observation for CMCase activity.

c) Dye staining of xylan agar :

Figure 3: positive strain (left) of *Aspergillus niger* showing a clear zone against deep violet back ground negative strain (right) of *Aspergillus niger* without any characteristic observation for xylanase activity.

d) Bavendamm test :

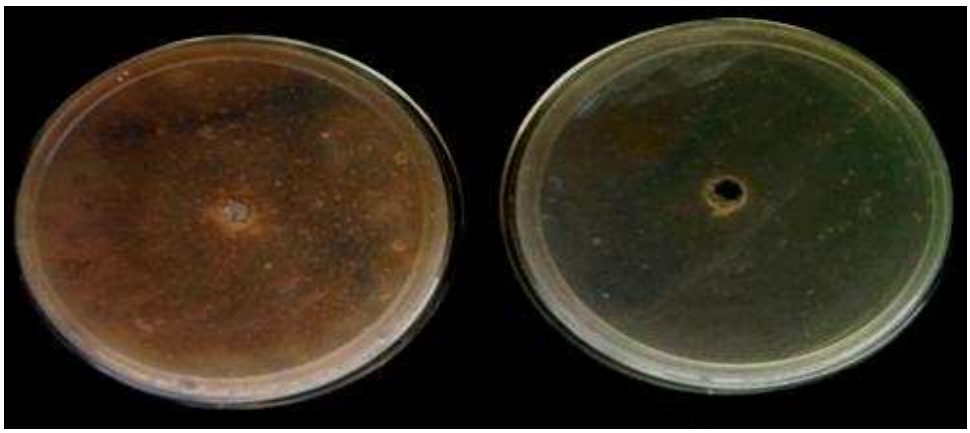

Figure 4: Positive strain (left) of *Trichoderma* sp showing the browning of the medium results the polyphenoloxidase activity, negative strain (right) *Trichoderma* sp without browning of the media.

Table 2: lignocellulolytic activity of selected fungal strains

Strain no	Diameter of zone(mm)			Bavendamm test	Cellulase activity (IU/ml)
	1% Cellulose	1% CMC	1% Xylan		
HMCD 1 *Aspergillus* sp.	25	37	18	Positive	1.77
HMCD 2 *Aspergillus* sp.	40	42	38	Positive	3.89
HMCD3 *Trichoderma* sp.	28	24	26	Positive	1.98
HMCD4 *Penicillium* sp.	22	26	16	Positive	1.29
HMCD5 *Aspergillus* sp.	18	20	14	Positive	1.20
HMCD6 *Aspergillus* sp.	21	38	27	Positive	1.93
HMCD7 *Trichoderma* sp.	24	16	22	Positive	1.36
HMCD8 *Trichoderma* sp.	52	48	42	Positive	3.88
HMCD9 *Penicillium* sp.	29	22	16	Positive	1.29
HMCD10*Alternaria* sp.	13	19	18	Positive	1.22

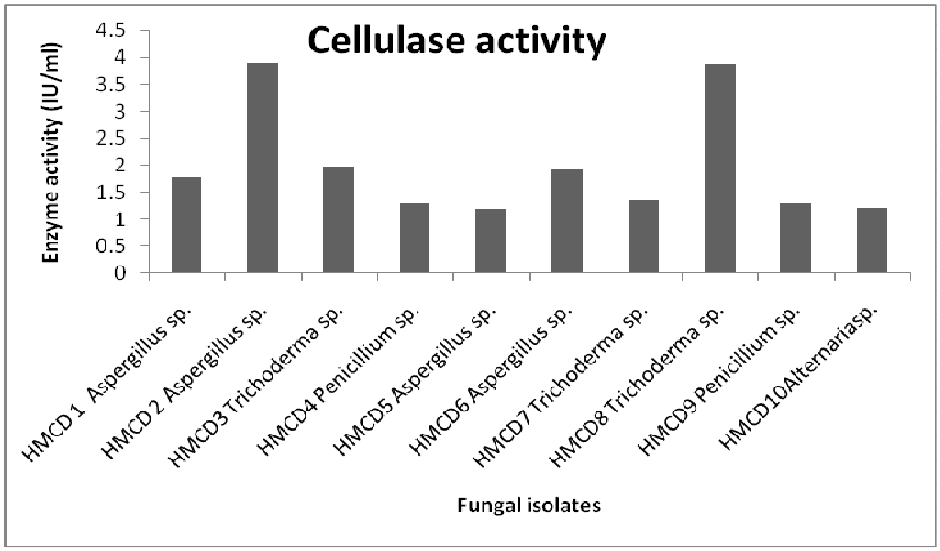

Figure 5. Cellulolytic activity of selected fungal strains.

DISCUSSION

A diverse spectrum of lignocellulolytic microorganisms, mainly fungi (Baldrian and Gabriel, 2003; Falcon et al., 1995) and bacteria (McCarthy, 1987; Zimmermann, 1990; Vicuna, 1988) have been isolated and identified over the years and this list still continues to grow rapidly. In this study we had selected the dairy dung manure as the source of fungi as the ruminants have the ability to degrade the cellulose but not the lignin. Where the animal dung contain partial or non-degraded cellulose after digestion of animal and high content of lignin. The lignin presented in the dung will be degraded by the soil microorganisms and make it into manure soil. The course of fungal lignocelluloses degradation is most readily observable in intact dead organic matter which, despite its complex ultra structure, is actually the simplest and best characterized form of litter. Fungi which were active against cellulose and other insoluble fibers were collected (Mandels and Sternberg, 1976). The results showed the fungal isolates which have the ability to degrade lignin, hemicelluloses simultaneously posses higher cellulolytic activity, where higher content of lignin will blocks the activity of cellulose. Endoglucanase

activity was reported by Boyce and Walsh (2007) for *Rhizomucor mehei* and Moria *et al*. (1984) showed that *cuninnghamella sp*. did not show any cellulolytic activity when cultured on medium containing lignin. *T. reesei* might be a good producer of hemi-and cellulolytic enzymes but is unable to degrade lignin.

The exact mechanism by which lignocelluloses is degraded enzymatically is still not fully understood but significant advances have been made to gain insight into the microorganisms, their lignocellulolytic genes and various enzymes involved in the process. In other study by Darmawal and Gaur, 1991 observed that basal medium containing 1% rice straw yielded high Fpase titres of 0.129 and 0.142 U/ml in *Aspergillus awamori* and *Scerituyn rikfsuu*, respectively. In the present study, along with enzyme production. In our part research work medium amended with !% lignocellyulosic substrate had produced HMCD 2 *Aspergillus* sp (**3.89IU)** and HMCD8 *Trichoderma* sp. (**3.88 IU)** where the two fungal isolates showed higher clearing zone of lignocellulolytic screening assays.

CONCLUSION

Lignocellulosic wastes in the form of cellulose which is the most abundant renewable resources in the biosphere have been shown to be used in the production of valuable products by microorganisms specially fungi. According to clear zone formation of cellulase enzyme activity, ten wild type strains have been the cellulase producers. Among these, HMCD 2 *Aspergillus* sp (**3.89IU)** and HMCD8 *Trichoderma* sp. (**3.88 IU)** were the best producer strain for cellulase in the Mandal's medium supplemented with 1% cellulose.

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