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RESEARCH PAPER

Isolation of cellulolytic fungi from the litter of *Phyllostachys bambusoides* from Ziro Valley, Arunachal Pradesh, Eastern Himalaya

Tapi Taka[1], Tonlong Wangpan[1], Luk Bahadur Chetry[2], PhongamTesia[1], Jentu Giba[1], SumpamTangjang[1]*
[1]Department of Botany, Rajiv Gandhi University, Rono Hills, Doimukh-791112, Arunachal Pradesh
[2]Department of Zoology, Rajiv Gandhi University, Rono Hills, Doimukh-791112, Arunachal Pradesh

ABSTRACT

Several substrates present in nature, such as leaf litter, compost soil, decayed wood and lignocellulosic waste helps in the growth of fungi. Cellulose, considered the most abundant biomass is considered to have the extremely great potential to be used as bioenergy. In this study, an attempt was made to isolate the cellulolytic fungi from the leaf litter of *Phyllostachys bambusoides* Siebold & Zucc. from Ziro valley, Arunachal Pradesh, Northeast India. Leaf litter of *P. bambusoide*s were collected from the bamboo groves and fungi was isolated, identified and screened for cellulolytic activity following standard methodology. A total of nine fungal species belonging to five genera were isolated of which *Aspergillus flavus* was the most dominant one while *Curvularia* sp. showed the highest amount of cellulolytic activity.

KEYWORDS: Litter degradation, Micro-fungi, Cellulose, Cellulase.

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Introduction

Cellulose is known to be the most abundant biomass on the planet and it is considered to have the extremely great potential to be used as bioenergy to meet the growing energy demand (Sanchez et al., 2008; Kim et al., 2008). It is made up of several glucose monosaccharides linked by β-1, 4-glycosidic bonds to make a linear polysaccharide (Gupta et al., 2012).

In recent times, there has been a considerable increase in the use of enzymes such as cellulases, hemicellulases and pectinases, especially in the textile industry (Uhlig et al., 1998; Galante et al., 1998), brewery and wine industry (Galante et al., 1998), food and fodder industry (Chesson, 1987) and in paper and pulp industry (Pere et al., 1996; Akhtar et al., 2006; Blanchette et al., 1991). The industrially utilized enzymes have about 20% share of the world enzyme market (Bhat, 2000).

Cellulase plays a vital role in bioremediation where the bioconversion of plant based cellulosic and lignocellulosic waste can open a new avenue for the inexhaustible source of energy in the form of renewable biofuel (Gautam et al., 2012; Petre et al., 1999). Bioethanol can be generated by using fungal cellulases (Okeke et al., 2015). Generally, the cost of enzyme production is very high and it accounts for almost half of the total expenditure of cellulose hydrolysis process. In this regard, several researchers are emphasized to suppress the production cost of enzymes (Howard et al., 2003). Isolation and characterization of cellulase producing microbes render a good initiative to discover several economically useful enzymes. Therefore, several research works are targeted to obtain new cellulolytic microbes with higher specific activities (Rathnan et al., 2012). Cellulase is produced in nature largely by microorganisms such as fungi and bacteria. Fungi are, however, the main producers of extracellular cellulases. There are many cellulase producing fungi which belong to different species such as *Trichoderma* (Atanasova et al., 2010) *Aspergillus, Eurotium, Penicillium, Rhodotorula* (Herculano et al., 2011) and *Neurospora* (Hildebrand et al., 2015). Use of fungi is advantageous as it is cost-effective, faster production, have higher productivity in amenable modified enzymes. The extracellular enzymes produced by fungi can be obtained from the culture media where they are grown and can be further quantified (Vishwanatha et al., 2010; de Souza et al., 2015). Fungi are by nature saprophytes which feed on organic matters and

decompose it (Lynd et al., 2002). There are several substrates present in nature which helps in the growth of fungi such as leaf litter, compost soil, decayed wood and lignocellulosic waste. In this study, an attempt was made to isolate cellulolytic fungi from the leaf litters of *Phyllostachys bambusoides* Siebold & Zucc. from Ziro valley, the land of the Apatani tribe of Arunachal Pradesh, Northeast India.

Materials and method

Collection of Sample

The Ziro valley of Arunachal Pradesh, northeast India was selected for the study. The leaf litters of *Phyllostachys bambusoides* Siebold & Zucc. were collected from the bamboo groves of different villages of Ziro valley brought to the laboratory for the isolation of micro-fungi.

Isolation and identification of fungi

Initially, the surface sterilization (Suryanarayan et al., 1998) of leaf litters was carried out where the samples were washed with 70% alcohol for 5 seconds, followed by 4% sodium hypochloride for 90 seconds and lastly by sterile distilled water for 10 seconds. Then the isolation of fungi was done using a moist chamber technique (Cannon and Sutton, 2004) and washed disk method (Pandey, 1990). The sterilized litters were cut into segments (approx. 0.5 cm^2) using sterilized scissors from the lamina portion. The tissue segments were incubated in Petri dishes of 9 cm diameter, each of which contains three layers of filter papers moistened with sterile water. A total of nine tissue segments was put on each plate and were sealed using parafilm. The petri dishes were then incubated in a light chamber with a 12 hr light and 12 hr dark cycle at 26±2 °C (Suryanarayanan, 1992) for 30 days. After 3 days of incubation, daily three litter segments were observed under a microscope to view the sporulation. The selected leaf litter segment was placed on a glass slide and pulverized, using sterile water and a scalpel, stained with lactophenol and observed under a bright field microscope for the presence of fungal fruiting bodies and spores. The cut segments were also plated on Potato Dextrose Agar (PDA) media added by 0.01 % (w/v) Chloramphenicol and incubated for 3-4 days at 25±2 °C. Fungi were identified based on their colony morphology and microscopic characteristics (Domsch et al., 1981).

The percentage of Relative Dominance (RD) of the isolated fungi was calculated by the following equation:

$$\% \text{ Relative Dominance (RD)} = \frac{\text{No. of colony of species A}}{\text{No. of colony of all species}} \times 100$$

Screening of cellulolytic fungi

The qualitative screening of the fungi for the production of cellulase enzymes was carried out following the method of Rohrmann and Molitoris (1992) and Kumaresan et al. (2002). Yeast-Peptone (Yeast extract 0.1g, Peptone 0.5g, distilled water 1 litre) medium containing Na-carboxy-methylcellulose (0.5%) supplemented with 15% agar was used to grow the desired fungi. Na-carboxy-methylcellulose acts as the substrate for the growth of fungi. After 3-5 days of colony formation, the plates were flooded with 0.2% aqueous Congo red solution and allowed to stand for 15 minutes and de-stained with M NaCl again for 15 minutes. Repeat the process 3 times after which the appearance of clear zone or halo zone around the fungal colony indicates the cellulase activity. The colony diameters of all the isolates were also recorded. The solubilizing index (SI) of every isolate was calculated using the diameter of halo zone and the diameter of the fungal colony (Edi-Premono et al., 1996).

$$\text{Solubilizing Index (SI)} = \frac{\text{Diameter of colony + Diameter of halo}}{\text{Diameter of colony}}$$

Results and Discussion

Altogether, nine fungi were isolated belonging to five different genera. Five of them belong to the genus *Aspergillus*, while the rest belongs to the genus *Trichoderma, Curvularia, Fusarium* and *Penicillium*.

Identifying characters of fungi

Aspergillus

Theconidiophores terminating in an apical vesicle and, at the opposite end, in a basal foot cell inserted into the supporting hyphae. Phialides are attached directly to the vesicle (uniserate) or an intervening cell called a metula (biseriate); these structures may cover the entire surface (columnar head); conidia in chains. Septate hyphae (2.5-8.0 µm in diameter); an unbranched conidiophore arises from a specialized foot cell. The conidiophore is enlarged at the tip, forming a swollen vesicle. Vesicles are completely or partially covered with flask-shaped phialides (formerly referred to as sterigmata) which may develop directly on the vesicle or be supported by a cell known as a metula. The phialides produce chains of mostly round, sometimes rough, conidia (2-5 µm in diameter). The isolated fungal species *Aspergillus niger*, *A. flavus*, *A. fumigatus*, *A. pseudoelegans* and *Aspergillus* sp. are shown in Fig. 1 and 2.

Trichoderma

The colonies grow rapidly and mature in 5 days. From the front, the color is white (wool-like) with scattered blue-green or yellow-green conidia in patches. The patches formed concentric rings. The reverse is pale, tan or yellowish. Phialides are hyaline, flask-shaped, and inflated at the base. They are attached to the conidiophores at right angles. The phialides may be solitary or arranged in clusters. Conidia (3 µm in diameter, average) are one-celled

and round or ellipsoidal in shape. They are smooth- or rough-walled and grouped in sticky heads at the tips of the phialides. The color of the conidia is mostly green (Fig. 1 and 2).

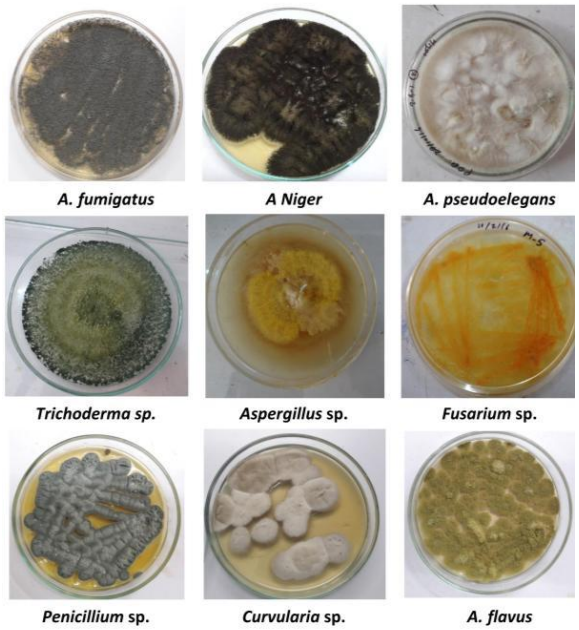

Figure 1. Colony morphology of fungal isolates from the litter of *P. bambusoides* from Ziro Valley, Arunachal Pradesh, Eastern Himalaya.

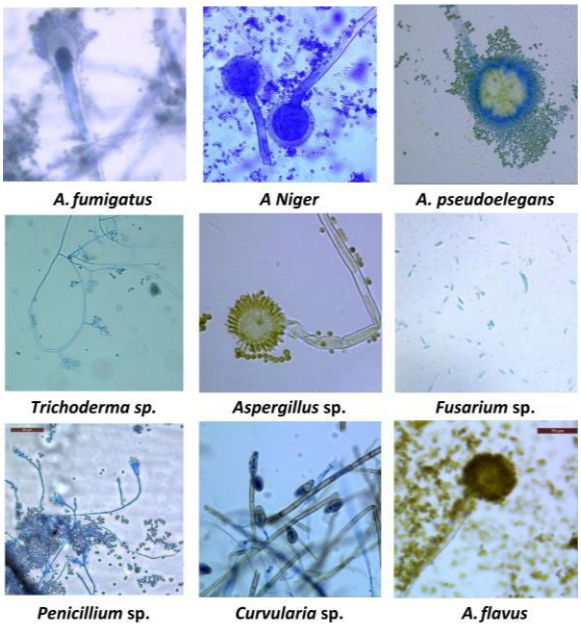

Figure 2. Fungal spores (stained with lactophenol cotton blue) isolated from the litter of *P. bambusoides* from Ziro Valley, Arunachal Pradesh, Eastern Himalaya.

Curvularia

The fungus grows rapidly and gives wool-like colonies which are white to pinkish gray initially and turns to olive brown or black from the front. The reverse of the colony is dark brown to black in color. The hyphae are septate and brown in color and it bears brown conidiophores. The conidiophores may be simple or branched and these are bent at the points where the conidia are born. The conidia (8-14 x 21-35 µm) are multi-septate where the transverse septa divide each conidium into multiple cells. The conidium is straight or pyriform in shape and brown in color. The central cell is generally darker and enlarged as compared to the end cells in the conidium (Fig. 1 and 2).

Penicillium

The fungus grows rapidly and sporulates in 3-5 days with greenish-blue conidia. When observed under a microscope, the conidia are arranged in single-celled chains borne on phialide in a basipetal succession. In *Penicillium,* phialides are produced in groups on branched metulae, giving a brush-like appearance to the conidiophore. The branching pattern is simple (non-branched or monoverticillate), one-stage branched (biverticillate-symmetrical) and two-stage branched (biverticillate-asymmetrical). Phialides are usually flask-shaped, consisting of a cylindrical basal part and lanceolate neck which bear globose and ellipsoidal conidia (Fig.1 and 2).

Fusarium

Colonies are woolly, cottony, flat, spreading and white to cream in color. From the reverse, it may be colorless and tan. When observed under a microscope, the hyphae is hyaline and septate. Macroconidia, and microconidia are observed. Macroconidia (4-8 µm) two or more celled, thick-walled, smooth, and cylindrical or sickle-shaped and have a distinct basal foot cell and pointed distal ends. They tend to accumulate in balls or rafts. Microconidia (2-4 µm), on the other hand, are formed on long or short simple conidiophores. They are 1-celled (occasionally 2- or 3-celled), smooth, hyaline, ovoid to cylindrical, and arranged in balls (occasionally occurring in chains) (Fig 1 and 2).

Percentage of dominance and cellulolytic activity of fungi

Aspergillus spp. was ubiquitously present in the bamboo litters collected from different locations; of which, *Aspergillusflavus* was the most dominant and *Aspergillus* sp. was the least. The availability of the substrate such as carbon source may be an important factor for the growth of fungi (Crowther et al., 2012). The fungi utilize their cellulases to degrade the biological cellulose to release the carbon back into the nature. All the fungi isolated above revealed the cellulolytic activity, but with varying intensities *Aspergillus niger* and *Aspergillus* sp. displayed the highest

cellulolytic activity, producing greater clear zone or halo around the colony (Table 1 and Fig. 3).

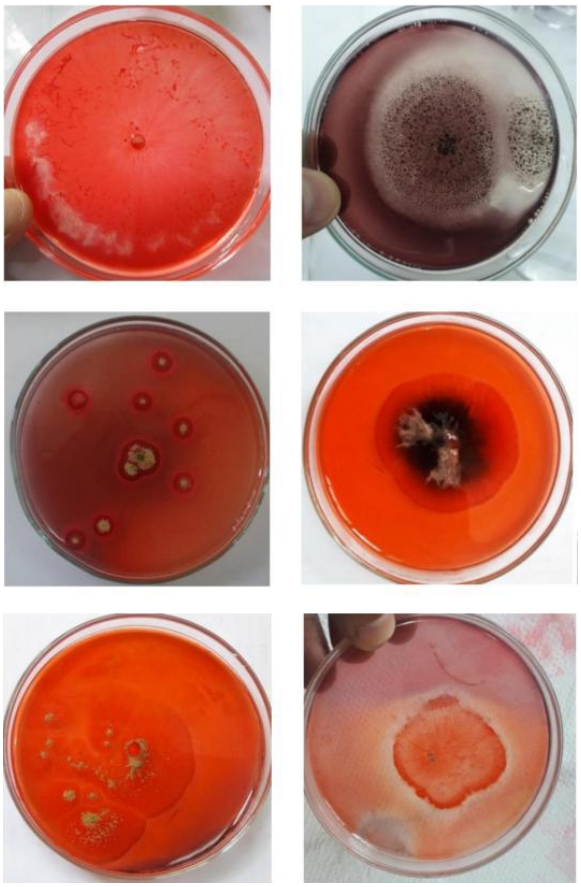

Figure 3. Cellulolytic assay of different fungus from the Litter of *P. bambusoides* from Ziro Valley, Arunachal Pradesh, Eastern Himalaya.

Based on the recorded SI value, *Aspergillus* sp. was found to have produced highest extracellular cellulase activity while it has the lowest dominance as shown in the graph (Fig. 4). Likewise, there are several other fungi (*Aspergillus niger, Aspergillus flavus, Trichoderma sp. and Emericella nidulans*) which produce a good amount of extracellular cellulases (Reddy et al., 2014, Manju et al., 2016). *Aspergillus flavus* showed the highest percentage of relative dominance of 33.9% and *Aspergillus* sp. the least with 0.65% (Table 1 and Fig. 4). Two species of *Aspergillus* (*A. niger* and *Aspergillus* sp.) produces the largest diameter of clear zone or the halo of 34.75 mm each. *Curvularia* sp. which has the percentage of relative dominance of 1.31 showed the lowest solubilizing index (SI) of 1.08 (Table 1 and Fig. 4). Hence, as per this study *Aspergillus* sp. with the highest value of SI could be a potent source of economically usable cellulase.

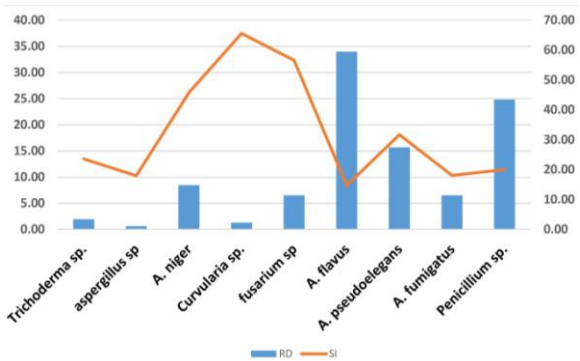

Figure 4. Graph representing the relation between percentages of relative dominance (RD) and solubilizing index (SI) of fungi isolated from the litter of *P. bambusoides* from Ziro Valley, Arunachal Pradesh, Eastern Himalaya

Table 1. Showing colony diameter, zone of hydrolysis, dominance and solubilizing index (SI).

Fungus	CD	ZH	CA	CFU	RD	SI
Trichoderma sp.	23.25	8.5	+	3	1.96	1.37
Aspergillus sp.	15.75	34.75	+++	1	0.65	3.21
A. niger	45	34.75	++	13	8.50	1.77
Curvularia sp.	65.5	5	+	2	1.31	1.08
*fusarium*sp	56.5	5	+	10	6.54	1.09
A. flavus	14.5	3.25	+	52	33.99	1.22
A. pseudoelegans	31.5	5.75	+	24	15.69	1.18
A. fumigatus	17.75	6.25	+	10	6.54	1.35
Penicillium sp.	19.5	10.25	++	38	24.84	1.53

CD= Colony diameter (in mm), ZH= Zone of Hydrolysis (in mm), CA= cellulolytic activity, CFU= Colony Forming Unit, %RD= Percentage of Relative Dominance, SI= Solubilizing Index. '+' denotes intensity of cellulolytic activity.

Therefore, it is a need of the moment to carry out research studies in this region to find the new and potent fungi or other micro-organisms which could lead to the discovery of novel enzymes with great industrial values.

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