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Culture, Isolation, Identification, Classification, Characterization, Properties and Kinetics

PANKAJ KUMAR CHAURASIA
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NOVA

In: Research Advances in the Fungal World ISBN: 978-1-53617-197-6
Editors: P. Kumar Chaurasia and S. Lata Bharati

Chapter 3

ISOLATION, IDENTIFICATION AND STUDYING THE POTENTIAL APPLICATION OF TROPICAL FUNGI IN LIGNOCELLULOLYSIS

S. K. Jayasekara, M. Kathirgamanathan and R. R. Ratnayake*
Bioenergy and Soil Ecosystems Research Project,
National Institute of Fundamental Studies, Hantana Road, Kandy, Sri Lanka

ABSTRACT

Biodiversity rich tropical countries like Sri Lanka have an enormous number of microorganisms including bacteria and fungi in their natural environments. The microbial diversity is a consequence of unique climatic conditions which exists in the area. The microbial entities partake in numerous biogeochemical reactions in this tropical environment. Lignocellulolysis is one such process which degrades plant biomass. Both bacteria and fungi participate in this lignocellulolytic reaction by producing an array of protein-based enzymes. Especially, fungi are reported to be far more efficient in producing extracellular lignocellulolytic enzymes including cellulases, hemicellulases and ligninases in order to hydrolyze cellulose, hemicellulose and lignin in plant debris respectively. Exploring the natural environment for these fungi is important because their enzymes have attracted a wide range of industrial applications where lignocellulosic biomass could be utilized as an inexpensive raw material. For instance, biofuel production could be emphasized. The objective of this chapter is to discuss the outcomes of a research conducted to isolate fungi from natural environment of different areas of central Sri Lanka, identify them and study their lignocellulolytic enzyme production potential. Moreover, the chapter includes descriptive information about the research methodology, results obtained and a discussion of the lignocellulolytic enzyme production potential of several filamentous as well as basidiomycetes fungal isolates included in genera *Trichoderma* sp., *Aspergillus* sp., *Penicillium* sp., *Talaromyces* sp.,

* Corresponding Author's Email: Renuka.ra@nifs.ac.lk.

and *Earliella* etc. based on their enzyme activities such as total cellulase, xylanase as well as laccase.

Keywords: lignocellulolysis, lignocellulolytic enzymes, cellulolytic fungi, tropical lignocellulolytic fungi, total cellulase activity, xylanase activity, laccase activity

INTRODUCTION

Fungi are a group of diverse eukaryotic microorganisms that play a pivotal role in balancing the environment. Although usually found in moist, dark and cool environments with a supply of decaying organic matter, they surprisingly colonize and easily survive in diverse habitats in the world. Some of them are unbelievably extreme environments such as hot springs, deserts, areas with high salinity, deep sea vents, spots emitting ionizing radiation and ocean hydrothermal areas (Kamburaet al., 2016). For instance, a type of fungus that lives in the hot springs is called gasteromycetes. Very few are also restricted to the arctic areas where the environment is unbearably cold. According to e-learning portal for arctic biology, fungi which grow on arctic seashores, e.g., *Arrhenia salina*, are ecologically bound to that arctic zone. In general, they can even make their home in decaying logs or fallen branches, nooks and corners of home gardens and biologically diverse tropical regions. Most of the fungi grow in terrestrial environments, though several species live partly or solely in aquatic habitats, such as the chytrid fungus *Batrachochytrium dendrobatidis*, a parasite that has been responsible for a worldwide decline in amphibian populations (Brem and Lips, 2008).

A description about fungi is incomplete without an account of their importance to the world. This is explained by tremendous roles played by them. Secretion of hydrolyzing enzymes as well as oxidizing enzymes for degradation of lignocellulosic biomass is one of the most crucial processes carried out by fungi. Lignocellulosic biomass is an abundant complex polysaccharide present on earth. Its application as a raw material in many industrial processes has become feasible due to enzymatic activities of microorganisms including fungi. For instance, biofuel production using plant cell wall polysaccharides could be mentioned. Biofuel is an attractive ecofriendly alternative to fossil fuels. However, in order to convert the complex polysaccharides in plant materials into fuel, it is essential to have a group of efficient microorganisms with the potential to degrade cellulose, hemicellulose and lignin which form lignocellulosic biomass into fermentable sugars. Lignin degrading enzymes *viz*; laccase, manganese peroxidase (MnP), lignin peroxidase (LiP) are oxidizing enzymes which carryout breaking down of lignin component in plant biomass (Pollegioni, Tonin and Rosini 2015). Prior lignin degradation which is called pretreatment allows easy access of hydrolytic enzymes into cellulose and hemicellulose components in the plant biomass. The hydrolytic enzymes

mainly required for the saccharification of plant polysaccharides are cellulases and xylanase (Bischof, Ramoni and Seiboth 2016). Therefore the pretreatment and saccharification are two important processes carried out by lignocellulolytic enzymes secreted by microorganisms.

The mechanisms behind the hydrolysis of lignocellulosic biomass by lignocellulolytic enzymes have been explained. Lignin is an aromatic, complex heteropolymer of phenyl-propanoid units (aromatic alcohols) which is found in plant cell walls. This polymer is playing a major role as it confers structural rigidity to woody plant tissues while protecting them from possible microbial attack (Higuchi, 1990). As previously mentioned, to depolymerize lignin, extracellular enzyme systems including laccases, peroxidases and oxidases producing H_2O_2 are required. The enzymatic composition of the ligninolytic system depends on the fungal species, with laccase being the common component (Hatakka, 1994). Laccase (EC 1.10.3.2) is an enzyme which contains a copper atom in it which is essential for its biological activity. These enzymes are called cuproenzymes. Laccase enzyme oxidizes various types of phenols and similar aromatic compounds, aromatic amines with the reduction of molecular oxygen to water. The lignin, as it is an aromatic complex will be oxidized by laccase.

Cellulases are the enzymes that hydrolyze β -1, 4 linkages in cellulose chains. In nature, complete cellulose hydrolysis is mediated by a combination of three main types of cellulases: (1) endoglucanases, (2) exoglucanases, including cellobiohydrolases (CBHs) and (3) β–glucosidase. Endoglucanase (1, 4-β-d-glucanohydrolase, EC 3.2.1.4) randomly attacks the internal *O*-glycosidic bonds, resulting in glucan chains of different lengths. Exoglucanase (1, 4-β-d-glucan cellobiohydrolase, EC 3.2.1.91) acts on the ends of the cellulose chain and releases β-cellobiose as the end product. β-Glucosidase (β-d-glucoside glucohydrolase, EC 3.2.1.21) is an enzyme that hydrolyzes terminal, nonreducing β-d-glucosyl residues with release of β-d-glucose (Mojsov, 2017). Xylanase (EC 3.2.1.8) is any of a class of enzymes that degrade the linear polysaccharide xylan into xylose thus breaking down hemicellulose, which is one of the major components of plant cell walls (Beg et al., 2001).

The collective action of all these three enzymes is therefore essential for the complete hydrolysis of lignocellulosic biomass. Furthermore, fungal scavenging of plant and animal debris in the environment releases simple inorganic molecules into the soil. Therefore, in natural ecosystems, fungi are the main decomposers which play an indispensable role in nutrient cycling as saprotrophs and symbionts that degrade organic matter into inorganic molecules (Barea et al., 2005).These nutrients facilitate plant growth.

In addition to replenishing the environment with nutrients, fungi interact directly with other organisms in different beneficial as well as disadvantageous ways. These are symbiotic relationships which usually involve a fungus and another organism. The other organisms might be cyanobacteria, algae, plants or animals. The complex mycorrhizal

associations with the roots of plants are one such mutualistic relationship which is beneficial for both members in the relationship. Fungal hyphae which are in contact with soil act as pathways of providing water and minerals into plants. In exchange, the plants supply their photosynthetic byproducts to carry out the metabolic activities of fungus. Some ants farm fungi as a supply of food. Lichens are another example for a symbiotic relationship between a fungus and a photosynthetic organism such as an alga or a cyanobacterium. The photosynthetic counterpart provides energy derived from light and carbohydrates to the fungus, while the fungus supplies minerals and protection to the photosynthetic organism. Some animals that consume fungi help to disseminate spores over long distant areas.

Fungi are also directly important for humans as food. Especially, some of the basidiomycetes generally known as mushrooms are edible. Besides, they take part in producing cheeses, beer and wine, bread, some cakes and some soya bean products. However, some of them are deadly poisonous that should be avoided consumption. Furthermore, fungi produce different metabolic byproducts that could be utilized for various purposes. For instance, many antibiotics are derived from fungi for medical purposes. One of the major examples is Penicillin produced by *Penicillium* sp.

Fungi also become detrimental by causing diseases to humans, animals as well as crop plants. Aspergillosis is an example for a human infectious disease caused by *Aspergillus* which weakens human immune system. Ringworm infection or Dermatomycosis is another fungal infectious disease. It is a parasitic skin disease of man and animals. Damping-off is an example for a fungal disease occurred in crop plants such as tomato. Pathogenic fungi such as *Fusarium oxysporum, Rhizoctonia solani, Phytophthora* and *Pythium* sp. are responsible for causing this disease. Although there are above disadvantages, fungi are extremely necessary for sustainability of the environment and also for human well-being.

Although biodiversity could be simply explained as different types of life on earth, according to National Wildlife Federation of USA, biodiversity can be evaluated in 3 levels. They are Species Diversity (different varieties of living organisms starting from primitive microorganisms to flowering plants and animals), Ecosystem Diversity (from tropical rainforests, deserts, swamps, tundra to all the ecosystems) and Genetic Diversity (variety of genes within a species which creates variations within species that results in their evolution). The diversity among fungi also might be categorized according to these 3 levels of biodiversity. Fungal diversity also depends on the regions they grow. Tropical regions are considered to be rich with a large biological diversity which always draws the attention of many researchers worldwide.

Tropics, the regions of the earth near to the equator are home for large number of flora and fauna including microorganisms like fungi. There are about 100,000 species of tropical fungi that have been described so far, while estimates for the total number of species go as high as 5.1 million and upwards (Aime and Brearley,2012). Sri Lanka is

enlisted as a biodiversity hotspot by United Nations because of its species richness. Fungal diversity is also playing an indispensable role in it. Especially, tropical rain forest ecosystems in Sri Lanka might contain different species of fungi ranging from microscopic yeast and molds to large mushrooms. Apparently, the records on fungal biodiversity of Sri Lanka are rare. However, a large number of researches are conducted on potential applications of different fungal species in agriculture, environment etc. Therefore, it is necessary to conduct more research to explore diverse ecosystems in the island to record available fungal species. Estimation of the number of fungal species is also important. Moreover, there may be undescribed tropical fungal species still remaining to be discovered. It will contribute greatly to fill the gap in knowledge on tropical fungi. Especially the knowledge of the distribution of tropical endophytic fungi, arbuscular mycorrhizal fungi, and soil fungi which are poorly documented in the tropics, await further studies.

In the current chapter, an account of fungi with the ability to produce lignocellulolytic enzymes which was isolated from central areas of Sri Lanka is presented. Furthermore, the morphological as well as molecular biological identification of the most efficient cellulase enzyme producing microorganisms have also been reported. The major focus of the study was to characterize these fungal isolates based on their ability to produce total cellulase, xylanase and laccase like lignocellulolytic enzymes.

METHODS

Isolation of Fungi from Environment

Soil samples were collected from natural environment *viz;* paddy fields, home gardens and forest areas. Samples were also collected from decaying wood and leaf litter. Clean polythene bags were used to collect and transport the soil to the laboratory.The isolation of fungi was done by preparation of soil dilution series. Diluting of soil was essential in order to obtain discrete fungal colonies on agar plates without overgrowth because the soil is a rich source of microorganisms. The initial 10^{-1} dilution was prepared by suspension of 1g of soil in 9ml of sterile distilled water. It was vortexed in order to get a homogenized `suspension. This initial suspension was serially diluted up to 10^{-7}. Fungi were isolated by pour plate technique on Potato Dextrose Agar (PDA).In order to remove bacterial interferences on fungal growth; chloramphenicol antibiotic (50mg/l) was added into the liquid state PDA medium. The plates were incubated at room temperature for 4-7 days and observed daily. Morphologically different fungal growths were sub cultured repeatedly on fresh PDA plates until the pure isolates were obtained. All these procedures were carried out under aseptic conditions. This method was suitable for isolating filamentous fungi.

The isolation of basidiomycetes was different from isolating filamentous fungi or molds. Basidiomycetes were producing this special structure called basidiocarp which is visible to naked eye. The basidiocarps were observed arising from tree trunks, decaying logs, decaying plant litter. Some basidiocarps were found growing on piles of soil in the environment. The basidiocarps were collected for culturing. The isolation of basidiomycetes on to laboratory culture plates was done using the basidiocarps. First, a piece of tissue was removed from the stipe of the basidiocarp by using a sterile scalpel. It was surface-sterilized with 70% (v/v) ethanol for 60 seconds followed by 3% (v/v) H_2O_2 for 30 seconds. It was then washed with sterile distilled water and placed on a plate of PDA with yeast extract (5 g/l), gentamicin (50 mg/l), chloramphenicol (50 mg/l) and Carbendazim (1 mg/l). The plates were incubated at 25°C for up to 14 days and pure growths of basidiomycetes were sub-cultured on to fresh PDA slopes.

After isolation of fungi, they were grown in special broth culture media to facilitate their lignocellulolytic enzyme production and a series of enzyme assays were also conducted to assess their enzyme production potential as mentioned below.

Enzyme Production by Filamentous Fungi

Usually aerobes are producing extracellular enzymes. Submerged fermentation involves the growth of the microorganism as a suspension in a liquid medium in which various nutrients are either dissolved or suspended as particulate solids in many commercial media (Frost and Moss, 1987).The culture filtrate was directly used as the crude enzyme extract. This concept was applied in producing the enzymes from fungi.

Filamentous fungi were grown on PDA slopes for 10 days to allow for sporulation. Spores were harvested by adding sterile 0.1% (v/v) tween 80 solution and the spore concentrations were estimated by using a Neubauer counting chamber and were adjusted to 10^7-10^8spores per ml. The Tween 80 solution was prepared by dissolving 0.1 ml of Tween 80 in 100 ml of distilled water. One hundred microliter of the suspension was inoculated into 100 ml Erlenmeyer flasks containing 20 ml of fungal cellulase production medium described by Mandels and Weber (1969).The Mandels and Weber medium included (per litre): NH_4SO_4 1.4 g; KH_2PO_4 2.0 g; Urea 0.30 g; $CaCl_2$ 0.30 g; $MgSO_4.7H_2O$ 0.30 g; Tween 80 2.00 ml; yeast extract 0.25 g; cellulose 10.0 g; trace metal stock solution 1.00 ml (which was composed of $FeSO_4.7H_2O$ 4.6 g; $MnCl_2.4H_2O$ 0.89 g; ZnSO4. H_2O 1.78 g; and $CoCl_2.5H_2O$ 1.83 g dissolved in 495 ml distilled water and 5 ml of Conc. HCl).The pH of the medium was adjusted to 5.5.The cultures were incubated at 28°C for 7 days with rotary shaking at 100 rpm. Cultures were centrifuged at 4800 rpm for 15 minutes and culture supernatant was used as crude enzyme extracts in enzyme assays.

Enzyme Production by Basidiomycetes

Cellulase and xylanase activities were evaluated in a medium modified from Peláez, Martínez and Martinez (1995). It contained (per litre): cellulose, 10 g; glycerol, 0.5 g; KNa tartrate tetrahydrate, 3 g; $(NH_4)_2HPO_4$, 1 g; yeast extract, 1 g; KCl, 0.5 g; $MgSO_4.7H_2O$, 0.5 g; $CaCl_2.2H_2O$, 0.3 g; $FeSO_4.7H_2O$, 5 mg; trace element solution, 1 ml. Trace element solution contained [per 100 ml]: $ZnSO_4.7H_2O$, 100 mg; $CuSO_4.5H_2O$, 25 mg; $MnSO_4.H_2O$, 100 mg; $CoCl_2.6H_2O$, 100 mg; $(NH_4)_6Mo_7O_{24}.4H_2O$, 10 mg; $Na_2B_4O_7.10H_2O$, 10 mg. pH of the medium was adjusted to 5.6. Laccase, Manganese Peroxidase (MnP) and Lignin Peroxidase (LiP) activities were evaluated in a medium modified from the afore-mentioned medium by replacing cellulose and glycerol with glucose (10 g/l) because it has been demonstrated that glucose like readily utilizable and efficiently metabolized carbon sources by the microorganism results in high levels of laccase activity (Galhaup and Haltrich, 2001). The basidiomycetes were grown on Potato Dextrose Yeast Extract Agar and circular discs of growth (diameter = 5 mm) were cut from near the edge of each colony by using a sterile Cork Borer. Each disc was radially cut into 8 equal pieces with a sterile scalpel, in order to reduce the pellet size of growth under submerged condition. Growth from three discs from each basidiomycete was inoculated to 20 ml of the medium in 100 ml Erlenmeyer flasks and incubated at 28°C in the dark with shaking at 100 rpm. After 7 days, the cultures were filtered with Whatman No.1 filter paper and the filtrates were used for the enzyme assays.

Enzyme Assays

Total cellulase assay was performed using Whatman No.1 filter paper as the substrate (Mandels, Andreotti and Roche, 1976). Reducing sugars formed were measured by using 3, 5-dinitro salicylic acid (DNS) reagent (Miller, 1959) with glucose standards. The total cellulase activity was expressed as filter paper units per ml (FPU/ml) which is the amount of reducing sugar, in micromoles, released by 1 ml of undiluted enzyme per minute.

Endoglucanases (Endo-β-1, 4-glucanase) activities of fungal isolates were measured by a method modified from protocols described by Mandels, Andreotti and Roche (1976). Carboxy Methyl Cellulose (CMC) solution (2% w/v, 20 mg/ml) was prepared in 50 mM citrate buffer (pH 4.8 for fungi).Hundred microliter of enzyme was added into test tubes and they were kept in 50 ˚C water bath. Then the prepared CMC solution was added into each tube and incubated for 30 minutes. After that the reaction was quenched by addition of DNS reagent and released sugar was estimated by the methods described by Sumner(1921) and Miller (1959).

Exoglucanase activities of fungal isolates were determined to understand their potential of breaking down crystalline cellulose. Microcrystalline cellulose suspension

(2%w/v, 20 mg/ml) was prepared in 50 mM citrate buffer (pH 4.8 for fungi).Two hundred microliter of particular enzyme extract was added into 200 µl of the above suspension. The mixture was incubated at 50 ˚C for 2 hours. Glucose standards were added into separate tubes (400 µl of 2.0, 1.0, 0.5 and 0.25 mg/ml).All the test samples as well as standards were kept in boiling water bath for 5 minutes after addition of 600 µl of DNS reagent. The tubes were cooled after that and they were transferred to microcentrifuge and were centrifuged at 10000 g to deposit remaining cellulose. Six hundred microliter of each supernatant was transferred into fresh test tubes and vortexed after adding 3 ml of distilled water. The absorbance of the resulted solution was measured at 540 nm.

Xylanase activity was measured according to methods described by Gottschalk, Oliveira and Bon (2010) with beech wood xylan (Sigma) as the substrate and the reducing sugars formed were measured by using DNS reagent with xylose standards.

Laccase activity was measured by using 2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulphonate (ABTS) as the substrate (Bourbonnais et al., 1995). The assay mixture (3 ml) contained 0.5 mM ABTS, 100 mM sodium acetate (pH 5.0) and 300 µl of culture supernatant. The oxidation of ABTS was followed by measuring the absorbance at 420 nm at one second intervals for 60 seconds at room temperature. The absorbance readings were plotted against time. If the absorbance readings became non-linear within 60 seconds, then the culture supernatant was diluted in distilled water to obtain tenfold dilutions and the diluted supernatant was used for the assay. The enzyme activity of undiluted supernatant was calculated by multiplying the activity of the diluted supernatant by the dilution factor. The amount of oxidized product formed was calculated by using the Beer-Lambert equation $A = \varepsilon cl$, where A= absorbance, ε= molar absorptivity, c = concentration of the analyte and l= path length of the cuvette. Molar absorptivity of 36 mM^{-1} cm^{-1} (Bourbonnais et al., 1995) was used for the calculations.

Manganese Peroxidase activity was measured by using phenol red as the substrate (Peláez, Martínez and Martinez 1995). The reaction mixture (3 ml) contained 0.1 mM H_2O_2, 0.1 mM $MnSO_4$, 0.1 mg/ml phenol red, 100 mM sodium tartrate buffer (pH 5.0) and 1 ml of crude enzyme. The reaction was stopped by the addition of 120 µl of 2 M NaOH after 30 minutes. The increase in absorption at 610 nm was measured against the corresponding enzyme blank (to which 120 µl of 2 M NaOH was added prior to the addition of the reaction mixture). The amount of phenol red oxidized was calculated by using the Beer-Lambert equation and the molar absorptivity $\varepsilon 610 = 4460$ $M^{-1}cm^{-1}$ (Peláez, Martínez and Martinez 1995).

Lignin Peroxidase activity was measured by using methylene blue as the substrate (Magalhães et al., 1996). The assay mixture (3 ml) contained 0.1 ml of 1.2 mM methylene blue, 0.6 ml of 0.5 M sodium tartrate (pH 4.0) and 2.2 ml of enzyme. Reaction was started by the addition of 0.1 ml of 2.7 mM H_2O_2. Conversion of the dye to Azure C was determined by measuring the absorbance at 664 nm immediately after the addition of

H_2O_2 and again after 30 minutes. Enzyme activity was expressed as the decrease in absorbance at 664 nm per minute.

Identification of Filamentous Fungi

The most efficient cellulolytic fungi were morphologically identified using their macroscopic as well as microscopic characteristics. It was essential to obtain fresh fungal cultures on PDA plates in order to clearly observe macroscopic characteristics regarding colony morphology. For instance, the size and shape of the fungal colonies were considered. Although there are unicellular fungi like yeast, here in the current chapter multicellular forms easily seen with the naked eye (e.g., molds) were studied. The molds form large multicellular aggregates of long branching filaments called hyphae. The characteristics of hyphae were considered in identification. Spore size, shape and structure were also used in the identification of fungi. Furthermore, the form (the basic shape of the colony - circular, filamentous, etc.), size (The diameter of the colony), elevation(the side view of a colony), margin/border (the edge of a colony) and the surface (smooth, glistening, rough, wrinkled, or dull),opacity (transparent, clear, opaque, translucent (like looking through frosted glass), etc.) and colour (pigmentation- white, buff, red, purple, etc.) of the fungal colonies and spores were considered as macroscopic morphological characters.

The microscopic characters of the isolates were observed using microscopic slides prepared by sticky tape method. The prepared slides were observed under oil immersion objective (×100) of light microscope (BioBlue. Lab).Types of hyphae (septate or non-septate),mycelium (clear or dark), color of mycelium (colored or colorless), types of sexual spores, types of asexual spores, characteristics of aerial hyphae or spores, sporangia (size, color, shape and location), conidia and their arrangement (chain, budding, single or masses), shape and arrangement of sterigmata or phialides, arrangement of soprangiophores or conidiophores (simple or branched, type of branching), size and shape of collumela at tip of soprangiophore, single conidiophore or bundle of conidiophore and presence of special structures (stolen, rhizoids, apophysis etc.) were some of the characters considered in recording the microscopic characteristics of the fungal isolates. The identification of the isolates was done by referring the identification keys described in literature (Barnett and Barry1998; Dugan 2006).

The most efficient cellulase enzyme producing isolates were identified using molecular biological techniques. Genomic DNA was extracted from the fungi according to methods described by Cenis (1992) with an additional step of freezing and thawing prior to homogenization of the cells. The PCR amplification was performed with ITS1 and ITS4 primers (White et al., 1990). The sequencing of PCR products was done at Macrogen Inc. (South Korea). Consensus sequences were made from the forward and

reverse sequences and were analyzed for similarity with existing nucleotide sequences in the NCBI and ENA databases by using the BLAST tool.

Identification of the Basidiomycetes

The basidiomycetes were identified based on morphological characteristics and/or DNA sequencing of the ITS region. The same method used for molecular biological identification of filamentous fungi was used for the identification of basidiomycetes as well. Morphological characteristics were not always helpful in determination of basidiomycetes genera.

Statistical Analysis of Data

Statistical analyses were carried out by using Minitab®16 statistical software. Comparisons were made by one-way ANOVA with Tukey's method or by two sample t-test ($\alpha = 0.05$).

RESULTS

Isolation, Identification and Screening of Enzyme Activities of Filamentous Fungi (Molds)

Isolation and Identification of Filamentous Fungi

More than150 filamentous fungi were isolated. They were isolated basically from soil samples collected from Kandy and Kegalle districts of Sri Lanka. According to Department of Meteorology Sri Lanka, the climate of the island is characterized as tropical because it is located between 5˚ 55' to 9˚ 5' North latitude and between 79˚ 42' to 81˚ 53' East longitude. Normal temperature of these areas is usually fluctuating from 24-30˚C. The climatic conditions usually shift from dry season to rainy season throughout the year. However, these areas are getting a significant rainfall throughout the year. Therefore, these tropical climatic conditions have a huge impact on fungal diversity of the area.

The fungal isolates were next screened for their total cellulase, endoglucanase, exoglucanase and xylanase activities. The most efficient cellulolytic filamentous fungi were found to be from 3 main genera *viz; Trichoderma, Penicillium* and *Aspergillus* (Figure 1 and Figure 2).

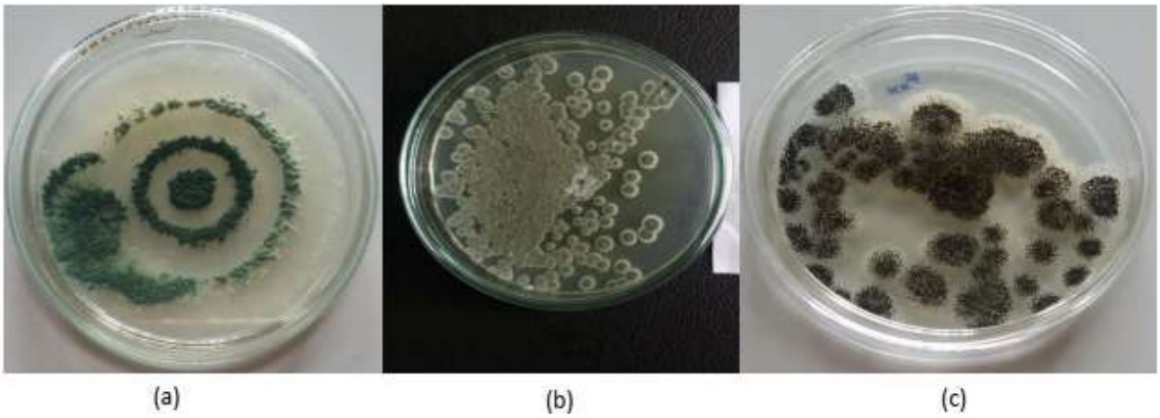

Figure 1. Some of the isolated cellulolytic fungi. The genera which found to be most efficient in cellulolytic enzyme production (figures are not in actual scale). (a) *Trichoderma viridae* (b) *Penicillium* sp. (c) *Aspergillus niger.*

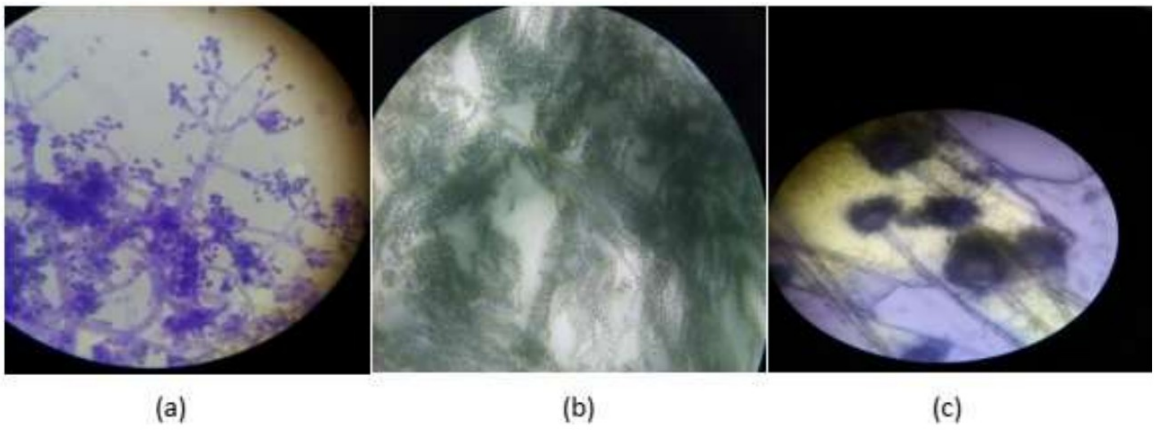

Figure 2. The microscopic observations of main cellulolytic fungal isolates. (a) *Trichoderma viridae* (b) *Penicillium* sp.(c) *Aspergillus niger.*

The morphlogical characteristics of filamentous fungi were very helpful in their identification.The *Trichoderma* isolates showed rapid colony growth.The plate was covered within 4 days. Initially they were hyaline with white filaments appearing gradually.Soon the filaments became whitish to dark green. The middle part of the colony appeareddark green and the edges of the colony appeared white. The colony surface looked rough due to the presence of tufted green conidial areas. Colony margin was filiform. Elevation of the colonies was flat and had a dry texture on the surface.They showed concentric rings of growth on plates.According to microscopic characteristics,conidiophores were hyaline, much branched and were not verticillate. Phialides were single or in groups. Conidia (phialospores) were single-celled hyaline which borne in small terminal clusters. Large conidiophores showed extensive branching.Spores were green in color.Most of the efficient isolates were *T.viridae* isolates (Figure 1.(a)).

The *Aspergillus* isolates which appeared in different colours due to their spore formation (Figure 3) had conidiophores in different shapes (Figure 4) when observed

under microscope. However, the most efficient in cellulolytic activities was the isolates with typical dark brown spores. The spores were observed on the surface of the white mycelium. The surface was flat and dry. Growth was not fast as in *Trichoderma* sp. or no zonation was observed.

The *Penicillium* isolates also were commonly found in soil samples. The frequently observed *Penicillium* colonies were greyish blue green in colour (Figure 1 (b)). The underside was yellowish. Centre of the colony was darker and margin was a white single line. The elevation was flat while entire margins were observed. Almost all the *Penicillium* isolates had the typical conidiophores (Figure 2 (b)). They were elongated, septate, branched conidiophores ending in a group of phialides and then chains of conidia. Light greenishconidiophores arisen from the mycelium singly or less often in synnemata, branched near the apex, penicillate, ending in a group of phialides. Conidia were brightly colored in mass. They were small and 1-celled, globose greenish phialospores (conidia) are present in dry basipetal chains.

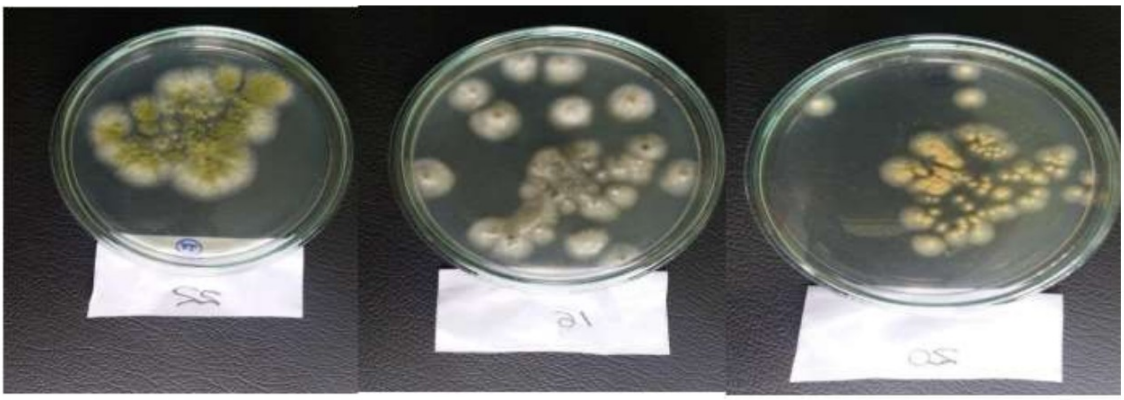

Figure 3. The different *Aspergillus* isolates. (a) *Aspergillus fumigatus*. (b)*Aspergillus clavatus* (c) *Aspergillus* yellow mold.

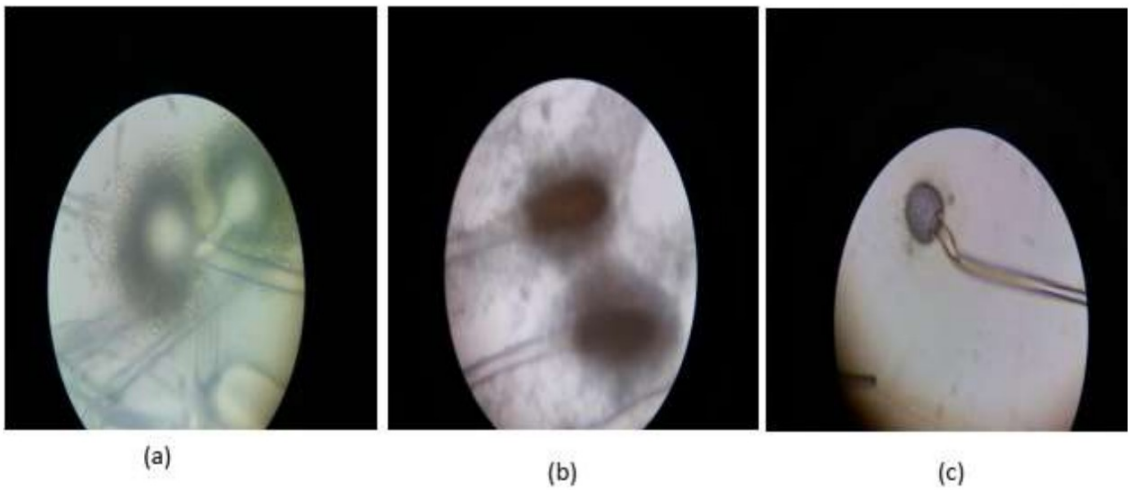

Figure 4. Different conidiophores of *Aspergillus* isolates (a) *Aspergillus fumigatus* conidiophores (b) *Aspergillus clavatus* conidiophores (c) *Aspergillus* yellow mold.

These morphological characteristics are extremely useful in identifying fungal isolates. It was observed that the typical sexual as well as asexual reproductive structures of fungi play a major role in fungal identification. Those typical structures are sometimes capable of predicting the identification up to species level by following the identification keys.

Enzyme Activities of Filamentous Fungi

Filamentous fungi could be considered as preferential sources of industrial enzymes because of their excellent capacity for extracellular protein production. The Figure 5 shows the total cellulase activities of fungal isolates in filter paper units. Most efficient cellulolytic microorganisms recorded were aerobic filamentous fungi. Out of them members of genus *Trcichoderma* were reported as the most efficient cellulase producers. The highest total cellulase activity was observed in *Trichoderma viridae* isolate as 0.937 FPU/ml followed by two other *Trichoderma* isolates giving total cellulase activities 0.775 FPU/ml and 0.74 FPU/ml respectively.

These results gain support from many recent reports on isolating cellulolytic microorganisms from natural environment. One such study identified *Trichoderma* isolates as the most efficient strains in cellulase production out of all the other filamentous fungal genera. However, the highest cellulase activity among them was produced by *T. reesei* (F118) (0.21 FPU/ml) (Mohanan, 2019). In the study reported in the current chapter, the highest cellulase activity was given by a *T. viridae* isolate as 0.937 FPU/ml. It is approximately a more than 4 times increment of cellulase activity than the activity of aforementioned *T. reesei*. This might be resulted by the species variation among the two *Trichoderma* isolates. Different species of the same genus have shown different cellulase activities.

On the other hand, there is proteose peptone 1.0 g/l and Tween 80 2.0 ml/l in Mandels & Weber (1969) medium. In a different study, the effect of Tween 80 and proteose peptone on cellulase production by *Trichoderma* species was studied. Sugar cane pith was used in the medium as carbon source. It was found that Tween 80 increases cellulase production while proteose peptone has influence on enzyme adsorption to the substrate. They also revealed that there is a combined effect of these two ingredientson cellulase production (Hung et al., 1988). The same idea was confirmed in a very recent study as well. According to that study, nitrogen sources had a remarkable effect on enzyme produced by *Trichoderma* sp. The maximum enzyme activities were obtained with yeast extract (1.0 %) which brought about an improvement in all the three cellulase components, including exoglucanase (2.40 U/ml), endoglucanase (2.28 U/ml), and β-glucosidase (1.99 U/ml), where peptone also produced the second most highest cellulose (Gautam et al., 2011).The medium optimized by Mandels contains both yeast extracts and peptone components. Peptone is an efficient nitrogen source for cellulase production.

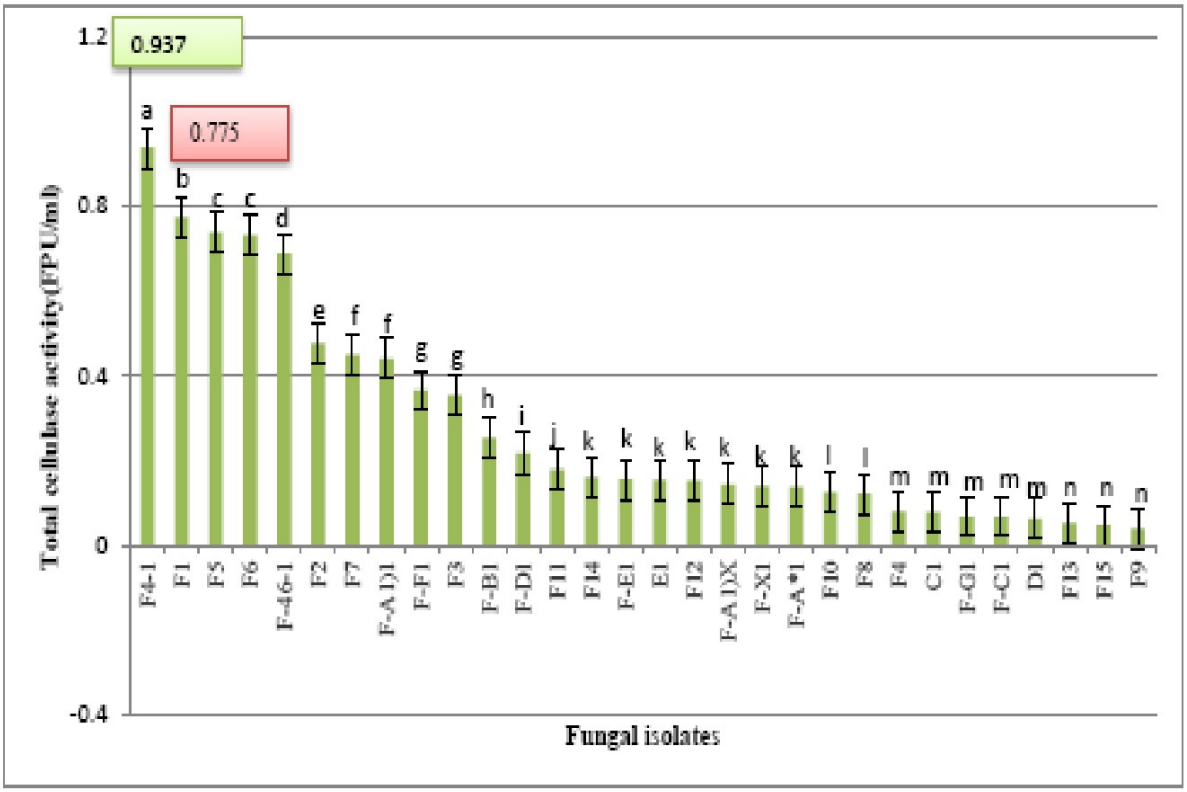

Figure 5. Total cellulase activities of fungal isolates. Error bars indicate the standard errors of the means. Values not sharing the same letter are significantly different ($P < 0.05$).). F4-1, F1, F5 etc. are code names of different fungal isolate.

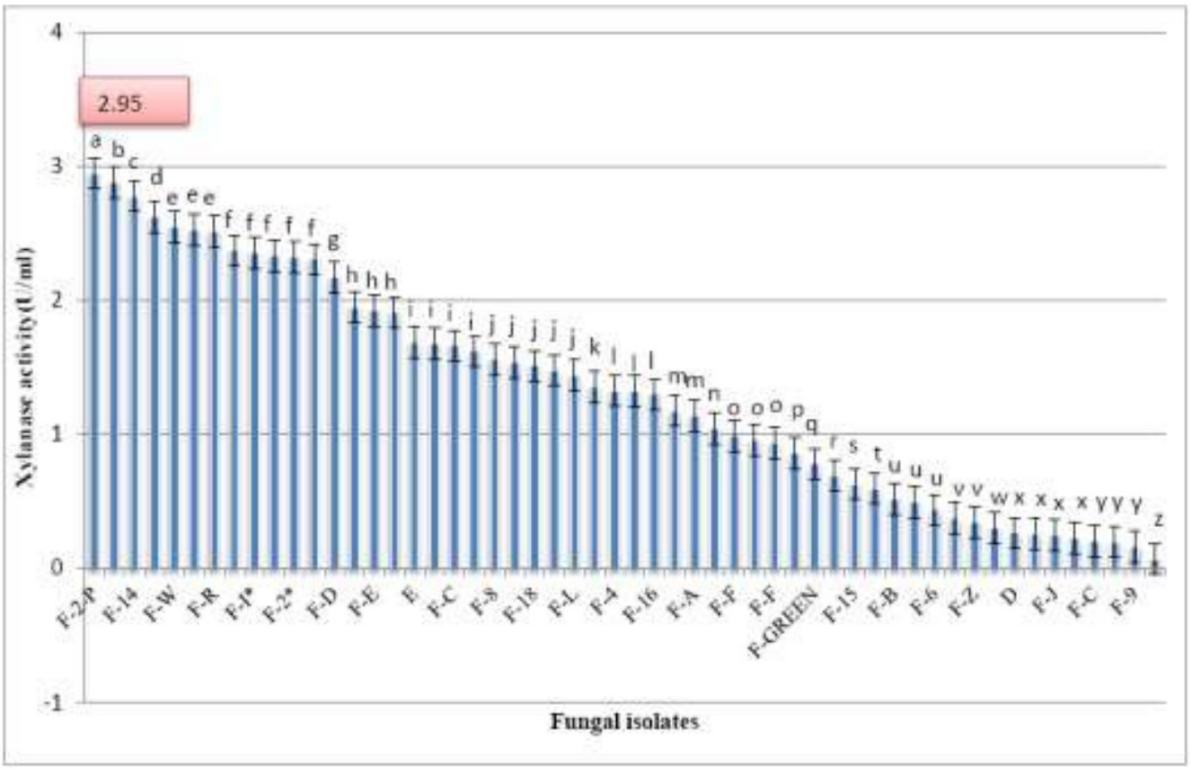

Figure 6. Xylanase activities of fungal isolates. Error bars indicate the standard errors of the means. Values not sharing the same letter are significantly different ($P < 0.05$). F4-1, F1, F5…. etc. are code names of different fungal isolates.

According to results of a study conducted by Pardo (1996), it was revealed that Tween 80 was the best compound for enhancing the production of all of the cellulolytic enzymes of the cellulase complex of *Nectria catalinensis* fungus. It caused an increase of endoglucanase and exoglucanase production in 70% and 72% respectively. It is believed that Tween 80 is a sort of detergent which makes changes in membrane permeability, so that free enzyme in solution is enhanced as a consequence of more excretion(Reese, Lola and Parrish, 1969).But in the above *T. reesei* cellulase production medium there was a slight difference to be noticed. Although tween 80 was present, there is 0.5 g/l of glycerol that has been added to the medium while proteose peptone 1.0 g/l was not added to the medium. Glycerol is an easily utilizable carbon source which could repress the cellulase production (Mandels and Weber, 1969).These reasons might have collectively affected the cellulase activity differences in these *Trichoderma* isolates.

Furthermore, *Aspergillus* as well as *Penicillium* species were recorded to be efficient cellulase producers. Several similar studies have been conducted in India. For example, Sudha and her group conducted a study (2018) about biodiversity of cellulolytic fungi isolated from Kattalagar Kovil, Tamilnadu, India. *Aspergillus niger* was found to produce the highest quantity of cellulase (0.854 ± 0.003 IU/ml). Similarly, Uttamkumar et al., (2014) studied on the fungi isolated from different sources like degrading wood, leaf litter and soil collected from forest nearby Forest Research Institute, Dehradun, India. The microorganisms they identified were *T. viridae*, *A. niger*, *A. fumigatus, A. flavus, Fusarium oxysporum* and species of *Trichoderma, Chaetomium, Curvularia, Penicillium, Alternaria* etc. Among these *A. niger* and *T. viridae* were recorded from all samples. However, only two fungi have exhibited considerable activity to degrade the cellulose, highest activity been recorded for *T. viridae* followed by *A. niger*. India is also a tropical country. The studies mentioned above are proof that the same climatic conditions may share the same genera of fungi.

Figure 6 illustrates the xylanase activities of fugal isolates. The highest xylanase activity recorded is 2.95 U/ml which was shown by a *Trichoderma* isolate. Thirty fungal isolates which showed higher xylanase activities above 0.07 U/ml level have been reported here. Most of the isolates which showed higher xylanase activities belong to genus *Trichoderma*. Xylanase is not an enzyme included in the cellulase enzyme complex. It is a hemicellulase which is required to break down hemicellulose counterpart in lignocellulosic plant material. Therefore, the ability of fungal isolates to produce this enzyme is essential in terms of utilizing cellulosic plant biomass as a substrate.

In Figure 7, the endoglucanase activities of fungal isolates are compared. The highest endoglucanase activity was expressed by a *Penicillium* isolate as 1.51 U/ml. This is an enzyme which attacks the amorphous regions of cellulose polymer complex. The 25 fungal isolates with endoglucanase activities above 0.07 U/ml endoglucanase activity level have been illustrated here.

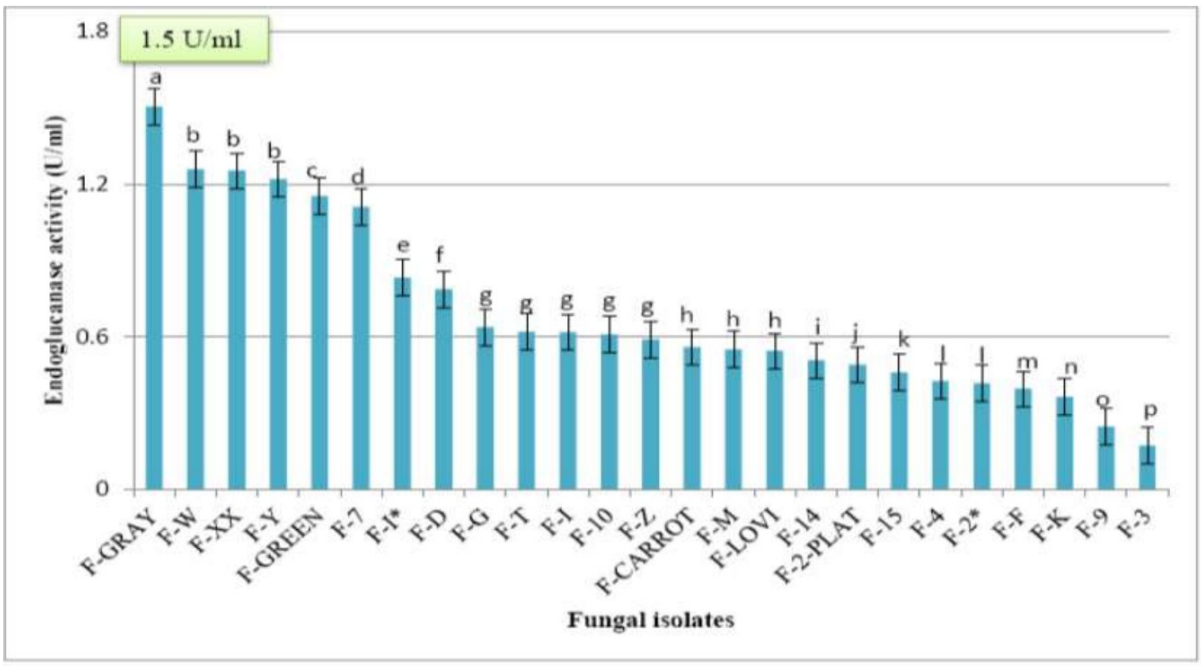

Figure 7. Endoglucanase activity of fungal isolates. Error bars indicate the standard errors of the means. Values not sharing the same letter are significantly different ($P < 0.05$). F4-1, F1, F5…. et care code names of different fungal isolates.

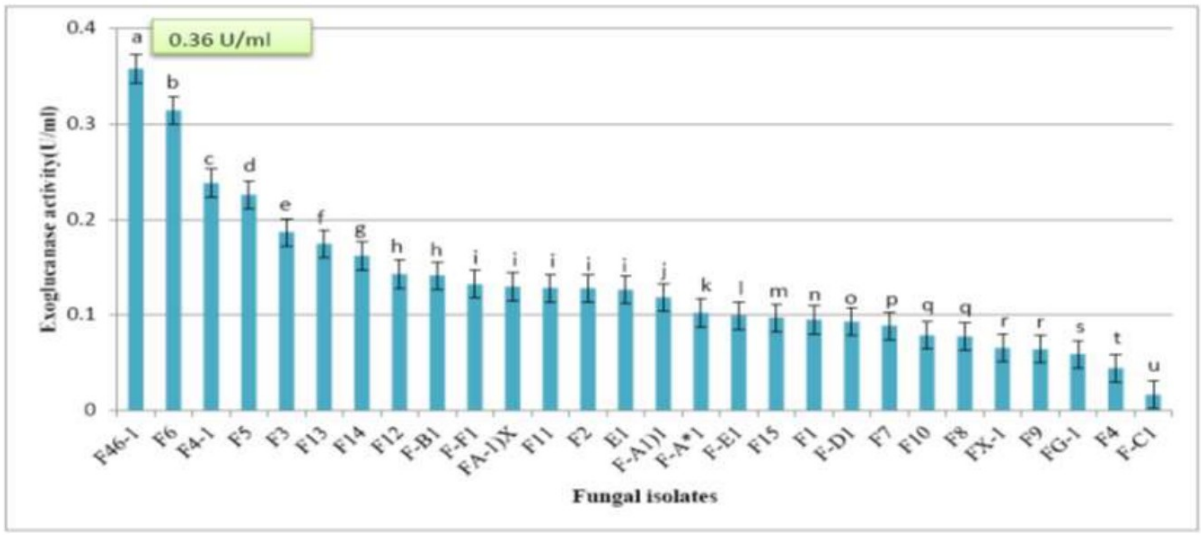

Figure 8. Exoglucanase activities of fungal isolates. Error bars indicate the standard errors of the means. Values not sharing the same letter are significantly different ($P < 0.05$). F4-1, F1, F2, F5…. etc are code names of different fungal isolates.

The Figure 8 illustrates the exoglucanase activities of fungal isolates. The highest activity was observed in a *Trichoderma* isolate as 0.36 U/ml. The 28 fungal isolates which showed exoglucanase activity above 0.015 U/ml have included here. This enzyme is responsible for breaking down the crystalline regions of cellulose.

According to overall results, the *Trichoderma* isolates were the most efficient in producing cellulolytic enzymes. Depending on the species diversity, their enzyme

activities were varying. As complete degradation of cellulose requires the activity of both exoglucanase as well as endoglucanase, the capability of microorganisms to produce these enzymes are essential. However, every individual isolate was not producing all the component enzymes in copious amounts. Moreover, their enzyme production patterns were very diverse. Therefore, it seems to be fairer to select the microbial isolates with highest total cellulase activities as the best isolates in degradation of cellulose because the total cellulase activity of an organisms is a representation of collective cellulase activity of cellulase enzyme complex.

Isolation Identification and Screening of Enzyme Activities of Basidiomycetes

Isolation and Identification of Basidiomycetes

Basidiomycetes are a class in phylum Basidiomycota of kingdom fungi. They are potential sources of lignocellulolytic enzymes. The aim of this section was to provide an account on several basidiomycetes isolated from Central Province, Kandy, Sri Lanka. Those isolates were screened for their lignocellulose degrading enzymes activities, namely cellulase, xylanase, laccase, Manganese peroxidase and lignin peroxidase. The data obtained on enzymatic lignocellulolysis of those basidiomycetes are mainly discussed here.

The isolation of basidiomycetes was challenging and difficult because of two reasons. Firstly, the plates were frequently getting contaminated with filamentous fungi. According to literature, they characteristically grow more slowly in culture than members of other fungal classes such as ascomycetes, zygomycetes and deuteromycetes (Miller, Grand and Tredway, 2011). Most common contaminant was *Trichoderma* species. It rapidly covered the surface of the basidiomycetes inoculated PDA culture plates, completely blocking the basidiomycetes growth. To overcome this problem, a mixture of fungicides was introduced into the medium to suppress the growth of the molds. Secondly, it was noticed that the basidiocarps collected were not live or might be unculturable because they did not produce colonies on culture plates. It was not easy to determine whether they were live or not especially with isolates like *Polyporus* sp. During isolation these information were taken into major concern. Therefore, isolation of basidiomycetes was different from isolating molds.

Identification of wild type basidiomycetes which are generally called mushrooms is basically morphological. The body or thallus of the basidiomycete fungus (the mycelium) is normally hidden within the substrate, and it is generally only the fruiting body or basidiocarp that is visible on the surface. The fruiting body being the most visible part of the basidiomycete with the greatest morphological variation, conventional mycology relies on a number of macroscopic and microscopic features of the fruiting body to distinguish between species (Hood, 2006). This method sometimes leads to

misidentification of the species. Therefore, it is indeed necessary to use molecular approach in order to have accurate and more reliable identification. The morphological characters were first recorded when they were collected from their natural substratum. The recorded information and photos taken were compared with literature to identify the isolates up to genera level. On the culture plates, their growth was mostly limited only to produce a simple white filamentous growth. This might be a thallus of the basidiomycetes. It was not quite different from each isolate. Moreover; basidiomycetes rarely produce sexual structures in culture upon which identification can be based (Miller, Grand and Tredway, 2011).The presence of clamp connections is a special characteristic often used to identify fungal hyphae as a basidiomycete but many basidiomycetes do not form clamp connections (Duncan and Keay, 1990). Therefore, microscopic hyphal characters were inadequate to do a reliable identification. The molecular biological identification with the aid of the DNA sequence of ITS region was essential to confirm the identity of the isolates.

The current chapter specifically includes enzymatic activity data about 18 efficient basidiomycetes in producing lignocellulolytic enzymes (Figure 9). The basidiomycetes and molds that have been described in the chapter are preserved in the Bioenergy Laboratory culture collection of National Institute of Fundamental Studies, Kandy, Sri Lanka. According to their identification, several basidiomycetes isolates belonged to orders Agaricales (*Schizophyllum commune, Coprinopsis cinerea, Marasmius* sp., *Lepiota* sp.), Xylariales *(Annulohypoxylon stygium, Xylaria* sp.*)* and Polyporales (*Trameteshirsuta, Trametes* sp., *Polyporus* sp., *Phlebiopsis* sp., *Pycnoporus* sp., *Microporus* sp., *Fomes* sp., *Ganoderma* sp., *Earliella scabrosa, Lentinus sajor-caju).*

Figure 9. Several basidiomycetes isolates in the culture collection. (a) *E. scabrosa* (M14), (b) *Pycnoporus* sp. (M21), (c) *Trametes hirsuta* (M29), (d) *Schizophyllum commune* (M1).

Some of the isolates including *E. scabrosa, T. hirsuta, L. sajor-caju* and *A. stygium* were collected from the Knuckles Mountain Range (Dumbara Mountain Range) of Sri Lanka which lies in central Sri Lanka, in the districts of Matale and Kandy. This mountain range is famous for collectively representing all the climatic conditions observed in the island. This specific ecosystem harbors a rich biodiversity. Most of the above isolates have been recorded to be wood rotting fungi including white rot fungi as well as brown rot fungi. *Ganoderma* and *Trametes* like isolates are considered to be white rot fungi which are capable of decaying diverse organic compounds besides lignin.

Schizophyllum commune is an example for a brown-rot fungus among these isolates. The brown rot fungi can degrade cellulose and hemicelluloses, but they can only modify lignin, which remains as a polymeric residue in the decaying wood (Arantes and Goodell, 2014).

Enzyme Activities of Basidiomycetes

Enzyme activities observed in basidiomycetes were very diverse (Table1).Total cellulase activity of basidiomycetes was detected from 16 out of the 18 basidiomycetes evaluated. However, xylanase activity was detected in all isolates. *Pycnoporus* sp. produced the highest cellulase activity of 0.23 FPU/ml, which is comparable to the activities produced by the very first wild type filamentous fungus *Trichoderma reesei* QM6a which was isolated and recorded by Mandels (1975).It is the parent strain of mutant strain *Trichoderma reesei* RUT-C30which is currently used for industrial production of cellulases (Peterson and Nevalainen, 2012).Therefore, there is an extensive potential of modifying these basidiomycetes isolates into more efficient cellulase producers. However, according to the results in Table 1, the total cellulase enzyme activities shown by all the basidiomycetes are lower below 0.1 FPU/ml level. As previously mentioned, the highest total cellulase activity recorded by an isolate was 0.937 FPU/ml which was given by a *T. viridae* isolate. In accordance with the calculation, it is approximately more than 4 times higher than the total cellulase activity of above *Pycnoporus* sp. The highest xylanase activity of 5.4 U/ml was produced by *Phlebiopsis* sp. Other isolates that produced high xylanase activities included *Schizophyllum commune* (M1) (5.12 U/ml) and *Pycnoporus* sp. (4.59 U/ml).

Earliella scabrosa, *Polyporus* sp. (M20), *Pycnoporus* sp., *Trametes hirsuta* (M29), *T. hirsuta* (M36) and *T. hirsuta* (M40) produced laccase activities greater than 10 U/l. *Coprinopsis* sp., *E. scabrosa* and *T. hirsuta* (M40)gave Manganese Peroxidase activities above 10 U/l level. The highest laccase (91.2 U/l) and MnP activities (17.5 U/l) were both observed in *E. scabrosa*. However, its total cellulase activity was 0 FPU/ml. Xylanase activity also was very lower when compared with the highest xylanase activity which was recorded in *Phlebiopsis* sp. Lignin Peroxidase activity was not detected from any of the isolates. In a similar study, Peláez, Martínez and Martinez (1995) also found no LiP activity within a larger collection of basidiomycetes. Thus, it appears LiP activity is rare among the basidiomycetes and limited to a few species such as *Phanerochaete chrysosporium* (Januszet al., 2013).These results suggest that basidiomycetes are more efficient in producing laccase like lignin degrading enzymes than the molds. The laccase production by molds was negligible when compared with the basidiomycetes giving negative results by most of the filamentous fungi for laccase, MnP and LiP activity assays. In other words, basidiomycetes are efficient in lignin degradation than other filamentous fungal isolates like molds.

Table 1. A Summary of Enzyme Activities Observed in Basidiomycetes Isolates

Basidiomycete	Cellulase (FPU/ml)	Xylanase (U/ml)	Laccase (U/l)	MnP (U/l)
*Schizophyllum commune** (M1)a	0.025	5.12	0.6	0
Coprinopsis sp.* (M5)	0	0.08	2.7	10.45
Phlebiopsis sp.* (M7)	0.074	5.41	0	0
Marasmius sp. (M12)	0.054	0.7	0.8	0.29
*Earliella scabrosa** (M14)	0	0.16	91.2	17.5
Trametes sp. (M15)	0.082	0.97	0.5	0
Polyporus sp. (M20)	0.024	0.3	79.9	0.91
Pycnoporus sp.* (M21)	0.232	4.59	33.1	0.64
Microporus xanthopus (M25)	0.073	2.6	0	0
*Trametes hirsuta** (M29)b	0.082	0.93	60.4	0.58
*Annulohypoxylon stygium** (M31)	0.033	3.8	0.5	0
*Schizophyllum commune** (M33)a	0.029	1.6	0	0.08
*Trametes hirsuta** (M36)b	0.074	1.67	15.1	0
Lentinus sp.* (M37)	0.018	0.1	0.6	0.16
Polyporus sp. (*M39*)	0.018	0.16	0.2	0.19
*Trametes hirsuta** (M40)	0.059	1.24	69.3	14.87

(*) denotes the code names given to basidiomycetes isolates during initial screening.

According to the summary of enzyme activities, it is understandable that the composition of enzyme complexes produced by the fungal isolates is very diverse. The types of enzymes produced by a certain fungus and the efficiency of its enzymes are likely to depend on the particular fungus itself. These different features reflect the genetic makeup of the particular isolates. The regulation of cellulolytic enzyme production is finely controlled by activation and repression mechanisms that occur in the genes which are responsible for expressing these enzymes. Hence, cellulases are known as inducible enzymes. Only in the presence of the specific substrate these enzymes are induced and repressed whenever easily utilizable sugars are available (Sukumaran, Singhania and Pandey2005). Some isolates produce cellulase that breakdown cellulose whiles some are negative for cellulase production. This might indicate the missing genes of cellulase enzyme in the genome of these isolates. Same phenomenon is observed with their xylanase, laccase, MnP and LiP production as well. Important information that must be considered is the titers of cellulases produced by the fungal isolates. Although cellulolytic activity has been reported in most of the isolates studied here, they have not produced the high titers of cellulases required at industrial scale (Ang et al.,2013).

Moreover, enhancement of lignocellulolytic enzyme production and activities by individual isolates is also very essential. As previously mentioned in the Methods section, all the lignocellulolytic microorganisms described in this chapter have been isolated from natural environment, especially from soil. They were sharing the same environment.

According to enzyme activity results, it is revealed that different microorganisms show unique enzyme production capabilities. Therefore, there is no doubt that these isolates show different efficiencies in degrading lignocellulose. In natural environments also lignocellulolysis is rarely accomplished by the activity of a single microorganism. The process becomes more efficient because of the combined effect of different amounts of enzymes from different microorganisms. It is rather achieved through the sequential order and collective effort of several microorganisms that produce multiple carbohydrate-active enzymes (CAZymes) (Lombard et al., 2013) to degrade the different polymers. Hence, it might be possible to mix lignocellulolytic enzyme extracts from different isolates in different ratios to prepare more efficient lignocellulose deconstructing enzyme mixtures. In other words, to obtain highly efficient enzyme systems, it is possible to formulate enzyme cocktails by mixing the lignocellulolytic enzymes extracted from different microorganisms that are efficient in producing different enzymes. Another approach is to create Genetically Modified Microorganisms (GMM) with genes that express all the essential enzymes to degrade cellulose, hemicellulose and lignin components in lignocellulosic biomass. However, formulation of enzyme cocktails as well as genetic modification of cellulolytic microorganisms to improve their cellulolytic enzyme production has to be done with prior experiments to optimize and stabilize the process. Co-culturing of several cellulolytic fungi together could be utilized as an efficient mode of enhancing cellulase production although mixing the most suitable component fungi is essential in this process (Jayasekara Abayasekara and Ratnayake 2018).

Moreover, cellulolytic enzyme production has been reported to be influenced by various process parameters including pH of the medium, substrate fermentation, and temperature (Sharma and Yazdani, 2016). The lignocellulosic carbon sources utilized for growing the isolates profoundly affect the enzyme production. For instance, a recent study conducted by Bioenergy Laboratory, NIFS found that the laccase activity of *E. scabrosa* isolate to be an average of 764 U/l when it was grown in sugarcane bagasse (Jayasekara, 2019). In another study, the effect of rice bran on laccase production by the same isolate was analyzed. According to the results of that study, laccase production by *E. scabrosa* was significantly higher when rice bran (10 g/l) was used as the main source of carbon. The increase was profound which observed a mean laccase activity of 13060 U/l (Mohanan, 2019). Therefore, it is evident that studying about these fungi and their lignocellulolytic enzyme production potentials is not that simple. However, it is extremely important to isolate, identify and keep records about fungal isolates of this kind from tropical regions. Most importantly, research activities must be expanded to learn about potential applications of enzymes extracted from these isolates in industrial processes. Moreover, it is not good to forget that these fungal isolates are important not only because they are rich sources of lignocellulolytic enzymes but also because they produce wide variety of secondary metabolites for the benefit of mankind. Therefore, collecting data about fungal diversity in diverse climatic regions of the world including

tropical regions is eminently important as it will definitely unravel new microbial species and support to understand evolutionary relationships between those microorganisms.

CONCLUSION

Lignocellulolysis is a natural process which is efficiently taking place in the environment by means of cellulolytic microorganisms. If these microorganisms could be isolated from the environment, cultured and improved under laboratory conditions, there is a huge potential of using them in industrial applications where lignocellulosic material could be utilized as a low-cost raw material. The findings discussed in the current chapter indicate the enormous potential of fungal isolates in lignocellulolysis by means of producing diverse array of enzymes *viz;* cellulase, hemicellulase (xylanase), laccase etc. Both filamentous fungi (molds) *viz; Trichoderma, Penicillium*, *Aspergillus* etc. and basidiomycetes such as *E. scabrosa* have a great potential in industrial applications as they are efficient in producing lignocellulolytic enzymes. *Trichoderma viridae* was found to be the best cellulolytic filamentous fungus among isolates giving 0.937 FPU/mlof total cellulase activity. Among basidiomycetes *E. scabrosa* was the most efficient laccase producer basidiomycete with the highest laccase activity of 91 U/l. *Pycnoporus* sp. produced the highest cellulase activity along with high xylanase activity. The lignocellulolytic potential of these tropical fungal isolates could be further enhanced by means of genetic engineering techniques supported by optimization of their culturing conditions. Preparation of more efficient lignocellulolytic enzyme cocktails by mixing enzyme extracts from different isolates in different ratio could be a more feasible approach of getting more efficient enzymes.

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