International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Impact Factor (2012): 3.358

Degradation of Timber Wood by Filamentous Fungi

Ashok K. Shukla

Department of Microbiology & Biotechnology, Holy Cross Womens College Ambikapur, Chhattisgarh, India

Abstract: ***A wide range of microfungi has been isolated from the wood sample of Azadiriachta indica (Neem), Mangifera indica (Mango), Ficus benghalensis (Banyan), Dalbergia sissoo (sheesham) and Saraca ascoca (Ashoka) wood. Most of these fungi are filamentous fungi. The fungi dominated in the sample are Aspergillus nidulans, A. fumigatus, A.flavus, Absidia coryambefera., Dictyorythridium quadratum, Emericella nidulans, Fusarium culmorum, Mycelia sterila forms, Pestalotia sps., Theilavia terricola, Torula sps. etc, were isolated on Czapak Dox Agar, Malt Extract Agar & Potato Dextrose Agar plate. Research over the past decade has repeatedly demonstrated the abundance of filamentous fungi in the soil. Evidence for the biodegradative potential of the fungi, in early infestations of wood has been well established, including in situ observations as well as laboratory data on enzymatic activity in tensile strength of timber wood due to degradation of lignocellulosic material. In present investigation fifty eight fungi isolated from various wood samples were tested for extracellular production of α-amylases, gelatinases, pectinase and carboxymethyl cellulases activity. Aspergillus nidulans, Fusarium culmorum and Pestalotia showed highest activity with 3.0 cm, 5.9 cm, 4.9 cm zone of clearance, respectively, in Czapak Dox Agar medium. While Aspergillus nidulans,Fusarium culmorum and Theilevia terricola do not produce gelitinase enzyme. On the other hand, good CMCase activity found with Theilevia terricola, Mycelia sterilia I and Pestalotia species. Overall these fungi associate with different sample appear within 21-30 days after cutting of wood and invade the wood causing reduction in strength of wood qualitatively and quantitavely***

Keywords: Biodegradation, timber wood, filamentous fungi, extracellular enzymes

1. Introduction

Many microbial enzymes are commercially exploited and successfully used on industrial scale to catalyze several chemical processes. These enzymes proved to be better, cheaper and environment friendly compared to the use of chemicals. Recently, enzymes have also been exploited in bioremediation of complex waste substances (Dubey & Pandey, 1996). Therefore, enzyme production now became a multi-billion dollar business (Shukla, 1991). Plant biomass is one of those natural complex materials, containing lignin, cellulose and hemicelluloses, found in abundance and regarded as promising chemical feedstock (Gilman, 1957). Microbial enzymes, involved in the degradation and transformation of plant cell-wall polysaccharides, have found many biotechnological applications (Mohebby, 2003). Ligninases, hemicellulases, cellulases, pectinases and amylases are the enzymes which are required to degrade not only the plant biomass to its completion but also have found application in pharmaceutical preparations (Rai & Shrivastava, 1982).Lignin is acted upon by lignin peroxidase, an enzyme that is not widely distributed among microorganisms. Hemicellulose (xylan and its derivatives), a heterogeneous group of hexoses, pentoses and some other sugars can be converted into fermentable sugars by xylanases (Thompson, 1957). Cellulose is the most abundant biopolymer present on this planet and can be degraded to glucose when different types of cellulases act in synergy (Shukla, 1991). Pectin, the constituent of middle lamella in some plants comprises of galacturonic acid and some other compounds that are frequently degraded by microbial pectinases (Waksman,1932). Starch is a reserve source of glucose in plants and readily hydrolysed by amylases produced by almost all living organisms. However, microbial amylases are of great industrial importance (Shukla, 1991). Beside these carbohydrolases, proteases are another important group of industrial enzymes that is widely used in detergent, baking and some other industries.

Fungi are ecologically involved in the degradation of a variety of complex materials, a property that is attributed to a battery of enzymes produced by these microorganisms. Fungal enzymes have been used in enzyme-technology industries for decades (Shukla, 2014) and hence there is an ever-increasing demand for the isolation and screening of new fungal isolates. Though recombinant DNA technology has provided some new methods to obtain and develop microorganisms of industrial importance nonetheless, classical techniques are still in use (Waxman, 1956). These enzymes can degrade in situ plant cell-mass completely, however, the ecological interactions among these microbial agents and organic matter disintegration are poorly understood. Although, many consortia of microorganisms have been developed in laboratories for the degradation of complex plant materials, still there is and will remain a demand for new microorganisms which can secrete large amount of hydrolytic enzymes to decompose plant biomass like wood. The wood is made up of cellulose (45%), hemi cellulose (20-30%), lignin (25-30%) and other substances (Mohebby, 2003). Biological attack of acetylated wood, PhD thesis submitted to Georg-August University, Goettingen, Germany).Keeping in view the importance of fungal enzymes, present study was initiated to isolate and screen the fungal strains associated with wood and to explore their hydrolytic potential (for 3 enzymes – amylase, Gelatinase and cellulose).

2. Materials and Method

2.1 Sampling

For the collection of sample, various samples of wood such as *Azadiriachta indica* (Neem), *Mangifera indica* (Mango), *Ficus benghalensis* (Banyan), *Dalbergia sissoo* (sheesham) and *Saraca ascoca* (Ashoka), were collected with the help of sterilized spatula in the sterilized polythene bags which were then sealed and brought to laboratory for further analysis. The wood samples were stored at 4°C in refrigerator; to

provide low temperature, low moisture content, low relative humidity until they were analyzed.

2.2 Isolation of fungi

Isolation of fungi was done on pre-poured plates of medium: Czapek Dox Medium ($NaNO_3$ – 3 gm, KH_2PO_4 – 1 gm, $MgSO_4.7H_2O$ - 0.5 gm, KCl – 0.5 gm, $FeSO_4.7H_2O$ – O.O1 gm, sucrose – 30 gm, agar agar - 15 gm, distilled water – 1 L, pH – 7.3) and Malt Extract Agar Medium (Malt Extract – 20 gm, agar agar – 20 gm, distilled water – 1 L). Antibiotics Penicillin and Streptomycin were added in medium at the rate of 30 µg / liter to prevent bacterial growth before pouring of medium (Waksman, 1932, 1956, 1972; and Warcup; 1942)

2.3 Inoculation & Incubation

For inoculation wood sample was converted to powder form with help of sterile mortar pistil and was directly inoculated on plated by sprinkling them (Warcup, 1942) and inoculated on prepoured plate, then incubated at 28°C for 3-4 days. **Identification:** For identification smears of the fungi were observed under microscope and identification was done on the basis of available literature and monograph as described by (Shukla 1991,Gilman 1957), then confirmed by CMI Kew, England UK (Table 1). In present investigation, fifty eight fungi isolated from various wood samples were tested for extracellular production of α – amylase, gelatinase and corboxy methyl cellulase activity.

2.4 Determination of α - amylase activity

Prepoured plates of Czapek Dox Medium (modified) with 0.2% soluble starch ($NaNO_3$ – 3 gm, KH_2PO_4 – 1 gm, $MgSO_4.7H_2O$ - 0.5 gm, KCl – 0.5 gm, $FeSO_4.7H_2O$ – O.O1 gm, soluble starch – 0.2 gm, agar agar - 15 gm, distilled water – 1 L, pH – 7.3), inoculated with a loopful of spore suspention of test fungi contained $2X10^6$ spore per ml in Tween- 80 solution .Then plates were incubated at 28°C for 3-4 days. After incubation plates were flooded with Logus Iodine. (Shukla ,2010, Thompson, 1957 & Yoeh et al.1987).

2.5 Determination of Gelatinase activity

In the present investigation this is performed on tubes of Nutrient Gelatin Medium (peptone – 5 gm, beef extract – 3 gm, gelatin – 120 gm, distilled water 1000 ml).For inoculation, a 6mm disc of isolated fungi was picked up with the help of sterile cork borer and the disc was slowly put deep into broth tubes. (Shukla,1991)The inoculated tubes were then incubated at 28°C for 3-4 days.After incubation, placed the tubes into refrigerator at 4°C for 15 minutes. Then, the refrigerated gelatin tubes to see whether the medium is solid or liquid. (Yoeh et al 1987)

2.6 Determination of CMCase activity

In the present investigation CMCase activity performed on plates of Czapek Mineral Salt Agar Medium ($NaNO_3$ – 2 gm, KH_2PO_4 – 1 gm, $MgSO_4.7H_2O$ - 0.5 gm, KCl – 0.5 gm, Carboxy Methyl Cellulase (CMC) – 5 gm, Peptone – 2 gm, agar agar - 20 gm, distilled water – 1 L). For this, dissolved the agar in 400 ml of hot distilled water by adding in small amounts and stirring with a glass rod; dissolved the magnesium sulphate, potassium chloride, peptone, sodium nitrate in 200 ml of distilled water; dissolved potassium phosphate in 100 ml of distilled water; dissolved CMC in 200 ml of distilled water with heating and mixing; mixed all the solutions and make up to 1000 ml volume; adjusted the PH to 6.5

For inoculation, a 6mm disc of isolated fungi was picked up with the help of sterile cork borer and the disc was slowly placed at the center of the media plate. (Shukla 1991)The inoculated plates were then incubated at 28°C for 3-4 days.The evidence for the microbial utilization of cellulose can be detected using 1 % hexadecyltrimethyl ammonium bromide solution. This reagent precipitates intact CMC in the medium and thus clear zones around a colony in an otherwise opaque medium indicating degradation of CMC. (Yoeh et al 1987)

3. Result and Discussion

About fifty eight isolates belonging to different genera were isolated from various wood sample were used in the present study.

Table 1: Occurrence of fungi associated with test wood samples

S.No.	Name of Organism	CMI No.	Wood Samples				
			Mangifera indica	*Azadiriachta indica*	*Dalbergia sisso*	*Saraca ascoca*	*Ficus benghalensis*
1.	*Pestalotia sp.*	*325144*	+	_	_	+	_
2.	*Dictyoarthridium quadratum*	*325152*	+	+	+	_	+
3.	*Fusarium culmorum*	*325142*	_	+	_	+	_
4.	*Theilavia terricola*	*321218*	_	+	_	+	_
5.	*Emericella nidulans*	*321214*	+	_	+	+	+
6.	*Gilmaniella humicola.*	*321225*	+	_	+	_	_
7.	*Verticillium sp. Section prostrata*	*325139*	+	+	+	+	+
8.	*Aspergillus nidulans*	*317908*	+	_	+	_	+
9.	*Bipolaris australiensis*	*325135*	_	_	+	+	+
10.	*Corynascus sepedonium*	*325134*	_	_	_	+	+

A. The detection of hydrolytic activity **(α – amylase)** is made by performing the starch test to determine the presence or absence of starch in the medium (Table 2). Starch in the presence of iodine will impart a blue black color to the medium, indicating the absence of starch-splitting enzyme and representing a negative result. If the starch has been hydrolyzed, a clear zone of hydrolysis will surround the growth of the organism. This is a positive result.

Table 2: α – amylase activity of isolated fungi associated with wood samples:

S. No.	Name Of Organism	Culture No.	CMI No.	Zone of Clearance (mm dia)
1.	*Pestalotia sp.*	*AKS 52*	*325144*	*49.00*
2.	*Dictyoarthridium quadratum*	*AKS 03*	*325152*	*34.00*
3.	*Fusarium culmorum*	*AKS 13*	*325142*	*59.00*
4.	*Theilavia terricola*	*AKS 47*	*321218*	*35.00*
5.	*Emericella nidulans*	*AKS 19*	*321214*	*30.00*
6.	*Gilmaniella humicola*	*AKS 21*	*321225*	*21.00*
7.	*Verticillium sp. Section Prostrata*	*AKS 49*	*325139*	*22.00*
8.	*Aspergillus nidulans*	*AKS 02*	*317908*	*30.00*
9	*Bipolaris australiensis*	AKS 06	325135	52.00
10	*Corynascus sepedonium*	AKS 07	325134	57.00

The **highest** α amylase activity is seen in *Fusarium culmorum* with a zone of clearance of 59 mm diameter and the **lowest** activity has been seen in *Gilmaniella humicola* with a zone of clearance of 21 mm diameter. In present investigation it is seen that *Fusarium culmorum*, have highest α Amylase activity i.e. have the highest ability to hydrolyze starch by this hydrolytic enzyme, followed by *Pestalotia sp* showing zone of clearance of 59 mm and 49 mm, respectively. *Theilavia terricola, Dictyoarthridium quadratum, Emericella nidulans* and *Aspergillus nidulans* showed a moderate amylolytic activity of 35 mm, 34 mm, 30 mm and 30 mm, respectively they have an average effect on degrading the cellulose present in the wood, while a zone of clearance of 22 mm and 21 mm produced by *Verticillium sp*. Section *Prostrata* and *Gilmaniella humicola*, respectively, shows their least ability in degrading wood's cellulose. Thus, a variety of fungi are inhabitant on wood which have a variation in degree of α amylase activity in the process of wood degradation.

B. Degradation of gelatin occurs in the medium by a proteolytic exoenzyme known as **Gelatinase**; it can be detected by liquefaction of the medium in the tube (Table 3). Deep gelatin inoculated tubes that remain liquefied produce Gelatinase and show positive test for gelatin hydrolysis and those tubes that remain solid demonstrate negative reaction for gelatin hydrolysis.

Table 3: Gelatinlase activity of isolated fungi associated with wood samples

S. No.	*Name Of Organism*	*CMI NO.*	*Liquefaction of medium*	*Gelatinase production*
1.	*Pestalotia sp.*	*325144*	Liquid	Positive
2.	*Dictyoarthridium quadratum*	*325152*	Liquid	Positive
3.	*Fusarium culmorum*	*325142*	Solid	Negative
4.	*Theilavia terricola*	*321218*	Liquid	Positive
5.	*Emericella nidulans*	*321214*	Liquid	Positive
6.	*Gilmaniella humicola*	*321225*	Liquid	Negative
7.	*Verticillium sp. Section Prostrata*	*325139*	Liquid	Positive
8.	*Aspergillus nidulans*	*317908*	Solid	Negative
9.	*Bipolaris australiensis*	*325135*	Liquid	Positive
10.	*Corynascus sepedonium*	*325134*	Liquid	Positive

In present investigation, *Fusarium culmorum,Gilmaniella humicola* and *Aspergillus nidulans* have been found to have no Gelatinase activity indicating absence of proteolytic activity in them, while others have showed a good liquefaction in media due to hydrolysis of gelatin by extracellular Gelatinase produced by them.

C. Wood is composed of about 45% cellulose (a polysaccharide of glucose units in a long linear chain linked together by β-1, 4-glycosidic bonds) which is degrade by an extracellular enzyme **cellulase** (composed of endoglucanase, exoglucanase and β-glycosidase) produced by microorganisms. The cooperative action of these 3 enzymes is necessary for cellulose degradation. The evidence for the microbial utilization of cellulose can be detected using 1 % hexadecyltrimethyl ammonium bromide solution. This reagent precipitates intact CMC in the medium and thus clear zones around a colony in an otherwise opaque medium indicating degradation of CMC.

Table 4: Carboxy methyl cellullase activity of isolated fungi associated with wood samples

S. No.	Name Of Organism	CMI NO.	Zone of Clearance (mm dia)
1.	*Pestalotia sp.*	*325144*	45 mm
2.	*Dictyoarthridium quadratum*	*325152*	27 mm
3.	*Fusarium culmorum*	*325142*	Nil
4.	*Theilavia terricola*	*321218*	42 mm
5.	*Emericella nidulans*	*321214*	23 mm
6.	*Gilmaniella humicola.*	*321225*	Nil
7.	*Verticillium sp.*sec. *prostrata*	*325139*	41 mm
8.	*Aspergillus nidulans*	*317908*	Nil
9.	*Bipolaris australiensis*	*325135*	20 mm
10.	*Corynascus sepedonium*	*325134*	24 mm

In present investigation, *Fusarium culmorum, Gilmaniella humicola* and *Aspergillus nidulans* have been found to have no cellulase activity indicating absence of either of 3 or any or all components of cellulose enzyme. While *Pestalotia sp., Theilavia terricola* and *Verticillium sp. Sec. prostrata* has

capacity to degrade the cellulose well as compared to *Dictyoarthridium quadratum* and *Emericella nidulans.*Thus, comparing all the results it is clear that *Pestalotia sp., Theilavia terricola* and *Mycelia sterilia* have high ability to degrade wood components as compared to *Dictyoarthridium quadratum* and *Emericella nidulans, while Fusarium culmorum, Torula sp.* and *Aspergillus nidulans* are found not to play a major role in wood degradation as they produce only amylases and not Gelatinase and CMCase (Table 4).

Table 5: Extracellure production of α-amylase, gelatinase and cellulase enzyme by some test fungi

S.No.	Name Of Organism	α-amylase production	Gelatinase production	Cellulase production
		Zone of Clearance	Liquefaction of media	Zone of Clearance
1.	*Pestalotia sp.*	+++	+	++
2.	*Dictyoarthridium quadratum*	++	+	+
3.	*Fusarium culmorum*	++++	_	_
4.	*Theilavia terricola*	++	+	++
5.	*Emericella nidulans*	++	+	+
6.	*Gilmaniella humicola*	+	_	_
7.	*Verticillium sp. Sec. prostrata*	+	+	++
8.	*Aspergillus nidulans*	++	_	_
9.	*Bipolaris australiensis*	++	+	++
10	*Corynascus sepedonium*	++	+	++

4. Conclusion

Fungal hydrolytic enzymes share 40% of global enzyme market and have many industrial applications. Cellulases are used in production of biofuels and various other biotechnological processes, pectinases in juice industry, proteases in detergent industry, amylases in starch processing, xylanases in paper and pulp industry. Therefore, there is a need for screening these enzymes for improved characteristics and to be exploited in various new emerging industries.

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