International Journal of
AGRICULTURE AND BIOSCIENCES
www.ijagbio.com P-ISSN: 2305-6622 E-ISSN: 2306-3599 editor@ijagbio.com

RESEARCH ARTICLE

Isolation, Screening and Identification of Cellulase Producing Fungi from Rotten Wood

Okonkwo IF and FJC Odibo[1]

Department of Agricultural and Bio-resources Engineering; [1]Department of Applied Microbiology and Brewing, Faculty of Bio-sciences, Nnamdi Azikiwe University, PMB 5025 Awka, Anambra State, Nigeria

ARTICLE INFO

Received: October 10, 2013
Revised: October 29, 2013
Accepted: November 23, 2013

Key words:
Carboxymethylcellulose
Colonies
Isolates
Zone diameter

***Corresponding Address:**
Okonkwo IF
iphyokonkwo@yahoo.com

ABSTRACT

Seven dominant isolates of cellulase producing fungi were isolated from pulverized rotten wood samples obtained from Nnamdi Azikiwe University Awka botanical garden in Anambra state Nigeria. The samples were grown in Czapex Dox agar medium incorporated with carboxymethylcellulose. Clear zones surrounded the colonies with zone diameter measuring 12 to 45 mm respectively, and their cellulase activities ranged from 0.9-2.85 (units/ml) respectively. On the basis of morphological characteristics the isolates were identified as *A. flavus, A. niger A. terreus A. fumigatus, penicillium spp, Trichoderma spp and Fusarium spp.* However, *Aspergillus flavus* gave the highest zone diameter of clearance with a corresponding highest enzyme activity in the preliminary submerged fermentation of the isolates and was more effective than the others. Thus, *A. flavus* cellulose system is recommended for use in biodegradation of cellulosic wastes.

Cite This Article as: Okonkwo IF and FJC Odibo, 2013. Isolation, screening and identification of cellulase producing fungi from rotten wood. Inter J Agri Biosci, 2(6): 333-336. www.ijagbio.com

INTRODUCTION

Fungi are eukaryotic organisms, more structurally complex than prokaryotic bacteria and are much larger in size compared to bacteria. Microscopically, they show either of the two basic growth patterns. Molds produce threadlike filamentous structures called hyphae, while yeasts are typically single-celled organisms which reproduce by budding, although if the buds fail to disarticulate from the parent cell, a hypha-like pseudo-hyphal strand may be produced. Most fungi produce only filamentous or yeast like growth, but some species can produce either filamentous or yeast like growth, usually in response to culture conditions. This phenomenon is referred to as dimorphism. The usual conditions favorable for yeast like growth by a dimorphic organism are growth on a nutrient-rich medium and incubation at 37^0C, while growth on a less nutrient-rich medium at 25^0C favors filamentous growth.

Laboratory identification of fungi, especially filamentous molds, is based to a large extent on morphological characteristics, while identification of morphologically similar yeasts is based on physiological characteristics such as carbohydrate assimilation and extracellular enzyme production.

According to Houbraken and Samson (2011) species of Trichocomaceae occur commonly and are important to both industry and medicine. They are associated with food spoilage and mycotoxin production and can occur in the indoor environment, causing health hazards by the formation of β-glucans, mycotoxins and surface proteins. Some species are opportunistic pathogens, while others are exploited in biotechnology for the production of enzymes, antibiotics and other products**.** However classification of some fungal species has been done by (Frisvad *et al*., 2007).

A diverse spectrum of lignocellulolytic micro-organisms mainly fungi have been isolated, screened and identified over the years Enzymatic hydrolysis of cellulosic materials is achieved by a sequence of reactions with the main components of cellulase complex enzymes, which include FPase, CMCase and β-glucosidase. The characteristics of all these three component of cellulase complex are the main factors that influence the application of enzyme-based bioconversion technology. Therefore, research has been directed to discover new microorganisms that have capability to produce cellulolytic enzymes with high specific activity.

Despite the large collection of fungi which were active against cellulose and other fibres, only a few have been studied for cellulases and hemicellulases. Among the cellulolytic fungi, *Trichoderma* spp. and *Aspergillus* spp.have been widely studied for their ability to secrete high levels of cellulose-degrading enzymes (Baldrian and Gabriel, 2003; Zhou *et al.,* 2008). *Aspergillus* spp. is the major agents of decomposition and decay and as such produce a broad range of enzymes, including cellulase. Cellulase characteristics and production by *Aspergillus*

spp. have been well documented in the literature (Singh *et al*., 1996, Pushalkar and Rao, 1998; Lockington *et al*., 2002; Ong *et al*., 2004; Kang *et al* 2004; Kirchner *et al* 2005; Immanuel *et al* 2006; Wang *et al*., 2006). However, only a few reports are available on the production of cellulase by *Aspergillus flavus* (Solomon *et al*., 1990; Solomon *et al*., 1999 Ojumu *et al*., 2003), and in many cases, have not been studied in depth Generally, cellulases responsible for the hydrolysis of cellulose are composed of a complex mixture of enzyme proteins with different specificities to hydrolyze glycosidic bonds. They can be divided into three activity classes (Rabinovinch *et al.,* 2002a, b). These are endoglucanases or endo β, 1-4 glucanase (EC3.2.1.4), exoglucanases or cellobiohydrolases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21). Consequently, the study was aimed at isolation, screening and identification of cellulase producing fungi often found in rotten wood.

MATERIALS AND METHODS

Study area

The work is carried out at the Department of Microbiology, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria. Awka is the capital city of Anambra state, which lies within the southern part of Nigeria. The geographical coordinates of Awka corresponds to 6.22 North and 7.07 East and falls within the humid tropics of Nigeria. The town Awka was made after clearing much of the tropical grassland, and outskirts of the city are still covered with grassland. It has a moderate climate with a very high temperature during the dry season and average rainfall during the rainy season. Awka has the mean annual temperature and precipitation of 35°C and 1117mm, respectively (NIMET, 2006).

Methods

Experimental Design

Generally, to ensure accuracy, most parameters were measured two times and the mean taken as the value of the parameter. Indices that were measured on graded levels were statistically analyzed using one way analysis of variance (ANOVA) and the differences between treatment means were separated using Duncan's New Multiple Range Test (DNMRT).

Also, data collected were presented in graphs and histograms to increase clarity. Other descriptive statistics such as range, intervals and the like were employed were necessary.

Isolation of Cellulolytic Fungi

Samples of rotten wood and compost were collected from Botanical garden of Nnamdi Azikiwe University, Awka, Anambra State, Nigeria. The samples were pulverized and shaken in distilled water, and filtered using white cloth. A drop of each of the filtrate was placed on Czapex Dox medium to which 1% carboxymethyl-cellulose (CMC) of low viscosity (BDH) was incorporated and spread. It was then incubated for 48 h at room temperature (28-30°C.)

A total of seven dominant colonies were isolated and purified by successive subculture on fresh Czapex Dox medium. The cellulolytic activities of the colonies were determined by point inoculation of each fungal isolate on Czapex Dox- carboxymethyl cellulose medium and incubated for 72 h. After the incubation, zone of clearing which is an indication of cellulolysis was detected by flooding the cultures with 0.5% Congo- red solution for 15 min. and distaining with 1M sodium chloride for 10 min (Teather and Wood, 1982). The zones were measured and result recorded.

Identification of the Fungal Isolates

The colonies with the highest zone of clearance were observed by slide culture technique under the microscope with the aid of methylene cotton blue stain, for the characteristic morphological features using standard reference manuals (Ellis, 1976; Raper and Fennel, 1965).
A loopful of conidia was inoculated into 100 ml of the sterilized medium in a 500 ml flask and incubated at 35^0C on a Stuart orbital shaker model S150 for 7 days at 200 rpm. After the incubation, the broth culture was subjected to centrifugation at 4000 rpm for 20 min using Centurion Centrifuge to remove the mycelia and other insoluble materials. The supernatant was recovered and used for the enzyme assays. The isolate with the highest enzyme activity was then selected and used for further studies.

Screening for Cellulase Production

The selected isolates were cultivated in a Mandel and Weber (1969) medium containing the following in g/l:

$(NH_4)_2S0_4$	1.4
KH_2P0_4	2.0
Urea	0.3
$MgS0_47H_20$	0.3
$CaCl_2$	0.3
$FeS0_47H_20$	0.005
$ZnS0_47H_20$	0.0014
$MnS0_4H_20$	0.0016
$CoCl_2$	0.002
Tween 80	2.0 ml
Carboxymethylcellulose	10.0
pH	6.8

A loopful of conidia was inoculated into 100 ml of the sterilized medium in a 500 ml flask and incubated at 35^0C on a Stuart orbital shaker model S150 for 7 days at 200 rpm. After the incubation, the broth culture was subjected to centrifugation at 4000 rpm for 20 min using Centurion Centrifuge to remove the mycelia and other insoluble materials. The supernatant was recovered and used for the enzyme assays. The isolate with the highest enzyme activity was then selected and used for further studies.

Enzyme Assays

Carboxymethyl Cellulose (CMC) Saccharifying Activity

An appropriately diluted (1:2) enzyme sample (0.5 ml) was mixed with 0.5 ml of 1% CMC dissolved in 0.2 M phosphate buffer (pH 6.8) and incubated for 30 min at 40^0C in a water bath (Memmert). The reducing sugar released was estimated by 3, 5- dinitrosalicylic acid method (Miller, 1959) as follows; at the end of incubation, the enzyme reaction was stopped by adding o.5 ml of 3, 5-dinitrosalicylic acid reagent (BDH). The mixture was placed in boiling water for 10 min, after which it was cooled, and 5 ml distilled water added. The absorbance was then read at 540 nm using the substrate solution

treated in the same way as blank to zero the spectrophotometer (JENWAY), model 6405. One unit (IU) of CMCase activity was defined as the amount of enzyme required to liberate 1 µmol of glucose from the substrate under the assay condition.

Filter Paper Saccharifying Activity

The reaction mixture containing 0.25 ml of diluted enzyme solution, 0.5 ml of 0.2 M phosphate buffer (pH 6.8) and 25 mg of Whatman No 1 filter paper strip was incubated at 40^0C for 1 h as described by Stephen *et al.* (2003). The reducing sugar liberated was determined by 3, 5- dinitrosalicylic acid method (Miller, 1959). One unit (1U) of filter paper activity is defined as the amount of enzyme required to liberate 1 µmole of glucose per 1 h.

Cotton Wool Saccharifying Activity

To a mixture of 1.0 ml of diluted enzyme and 1.0 ml of 0.2 M phosphate buffer (pH 6.8) was added 50mg of absorbent cotton wool and incubated at 40^0C for 24 h. The reducing sugar liberated was determined by the 3, 5-dinitrosalicylic acid method described above. One unit of cotton saccharifying activity was taken to be mg of glucose liberated per 24 h.

RESULTS AND DISCUSSION

Isolation of fungi and screening for cellulase production

Seven dominant isolates were recovered from rotten wood samples, and each yielded substantial amount of cellulase. They were identified as *Aspergillus flavus, Aspergillus niger, Aspergillus* fumigates, Aspergillus terreus, Penicillum Trichoderma spp and Fusarium spp. However, *Aspergillus flavus* gave the highest zone diameter of clearance with a corresponding highest enzyme activity in the preliminary submerged fermentation of the isolates and was more effective than the others as seen in Table 1.

Table 1: Zone diameter and enzyme activity pattern of the isolates

Isolates	Zone diameter (mm)	Enzyme activity U/ml		
		Cmc	Filter paper	Cotton wool
A flavus	45	2.85	2.55	3.0
Trichoderma spp	37	2.15	2.0	1.8
A terreus	35	1.76	1.52	1.3
A fumigates	30	1.4	1.6	1.62
A niger	27	1.23	1.0	0.7
Fusarium spp	25	1,12	1.6	1.4
Penicillium spp	12	0.9	1.4	1.2

The colonies of Aspergillus flavus on Czapex Dox agar were yellowish-green, consisting of a dense felt of conidiophores. The microscopy showed the conidiophores stripes as rough-walled hyaline. The vesicles were spherical.

Aspergillus niger colonies were slightly brown with smooth surface consisting of very rough conidia. The vesicles are biseriate and globose. Aspergillus fumigatus colonies were grayish with smooth surface consisting of smooth conidia. The vesicles are uniseriate and globose. Aspergillus terreus colonies were colorless with smooth surface consisting of smooth conidia. The vesicles are biseriate and spherical. Fusarium spp colonies were brightly colored with a cottony aerial mycelium the microconidia are hyaline with an elongated apical cell hyaline and curved. Penicillium spp colonies consist of a highly branched network of multinucleate septate, colorless hyphae with mycelia bearing many branched conidiospores.

Trichoderma spp colonies were light green, circular as a ring, aerial white as cotton and powdery, the hyphae are hyaline with many branches in the edge. The isolation of 7 dominant fungal species with potentials for lignocellulosic degradation from rotten woods confirmed that organisms that produce cellulase can be isolated from such sources which constitute their natural habitat. This could be because these habitats contain a lot of cellulosic materials that can sustain these fungal species.

The isolates produced substantial cellulase at 35°C but highest cellulase production was observed with *Aspergillus flavus* using the various substrates in the preliminary submerged fermentation of the isolates with a corresponding highest zone diameter of clearance. However other isolates gave varying cellulase yield with respect to the substrates used as shown in the Table1. Results obtained during this study indicated that enzyme activity of the isolated fungi was found relatively higher and comparable to some results of other investigators (Updegraff 2004; Kluczek and Turpeinen *et al*, 2005).

This agreed with the reports of Peig *et al.* (1998) and Boddireddy *et al.* (2011) that *Aspergillus* is one of the well-known efficient cellulase producers. The high cellulase production by *Aspergillus flavus* can be attributed to the fact that the growth and fermentation conditions under which the studies were carried out were more favourable to *Aspergillus flavus* than others. This agreed with the reports by Ulikanli and Digrak (2002), which stated that media constituent and other growth factors such as pH and temperature have either a favorable or deleterious effect on the production of the microbial enzymes.

Fungi are well known agents of decomposition of organic matter in common and of cellulosic substrates in particular (lynd *et al*., 2002). Cellulose is the world's most abundant organic substance (Ruttloff, 1987) and comprises a major form of organic compound and major component of biomass energy (Scott *et al*., 1987). This is so because, a large proportion of wood cellulose are added to soil organic matter. Cellulose has a special significance in the biological cycle of carbon (Lederberg, 1992). Since the production of cellulase is a key factor in the hydrolysis of cellulosic material; it is therefore essential to make the process economically viable and cost effective. Hence further work is required to purify the *Aspergillus flavus* cellulase enzymes and determine their characteristics for use in the industry for various purposes.

REFERENCES

Baldrian T and J Gabriel, 2003. Lignocellulose degradation by *Pleurotus ostreatus* in the presence of cadmium, *FEMS* Microbiol. Lett, 220: 235-240.

Barrow GI and RKA Feltham, 1993. Cowan and Steel's Manual for the Identification of Medical Bacteria, 3rd Edn. Cambridge: Cambridge University Press.

Boddireddy Sridevi and MA Singara Charya, 2011. Isolation, identification and screening of potential cellulase-free xylanase producing fungi African Journal of Biotechnology Vol. 10, pp: 4624-4630.

Ellis MB, 1976. More dematiaceous hypomycetes. Knew, SurveyEngland: CABI; illustrated edition.

Frisvad JC, TO Larsen, R de Vries, M Meijer, J Houbraken, FJ Cabañes, K Ehrlich and RA Samson, 2007. Secondary metabolite profiling, growth profiles and other tools for species recognition and important *Aspergillus mycotoxins*, Stud Mycol, 59: 31-37.

St. Germain Summerbell GR, 1996. Identifying Filamentous Fungi: A clinical Laboratory handbook. 1st Ed Star Publishing Company, Belmont, California.

Houbraken J and RA Samson, 2011. Phylogeny of Penicillium and the segregation of Trichocomaceae into three families, Stud Mycol, 70: 1-51.

Larone DH, 1995. Medically important Fungi: A Guide to Identification, 3rd Ed. ASM, press, Washington, DC.

Mandels M and J Weber, 1969. The Production of cellulases, In: Hajny GJ, Reese, ET (Eds): cellulases and their applications, Am. Chem Soc. Washington DC, pp: 319-414.

Miller GL, 1959. Use of dinitrossalicylic acid reagent for determination of reducing sugars, Anal. Chem., 31, 426-428.

NIMET, 2006. Nigerian meterological Agency, Onitsha, Anambra.

Peij N, MMC Gielkens, RP Verles, K Visser and LH Graff, 1998. The transcriptional activator XinR regulates both xylanolytic endoglucanase gene expressions in *Aspergillus niger*. Appl Environ Microbiol, 64: 3615-3617.

Rabinovich M, M Melnik and A Bolobova, 2002a. Microbial cellulases: A review. Appl Biochem Microbiol, 38: 305-321.

Raper KB and Fennel, 1965. The genes of Aspergillus. Baltimore: Williams and Wilkins.

Ellis MB, 1976. More dematiaceous hypomycetes. Knew, Survey, England: CABI; illustrated edition.

Rabinovich M, M Melnik and A Bolobova, 2002b. The structure and mechanism of action of cellulolytic enzymes, Biochemistry (Moscow), 67: 850-871.

Stephen RD, SA William, J Edward, BV Todd and EH Michael, 2003. Automated Filter paper assay for determination of cellulose activity. Appl Biochem Biotech, 108: 689-703.

Teather RM and PJ Wood, 1982. Use of congo-red polysaccharide interactions in enumerations and characterization of cellulolytic bacteria fropm bovine rumen, Appl Envir Microbiol, 43: 770-780

Ulukanli Z and M Digrak, 2002. Alkaliphilic micro-organisms and habitats, Turk J Biol, 26: 181-191.