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Production of Cellulolytic Enzymes by Endophytic Fungi Isolated from Cameroonian Medicinal Plants

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ABSTRACT

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Cellulose constitutes bulk of the plant cell wall materials and the most abundant and renewable non-fossil carbon source on earth. Cellulose degrading fungi play an important role in recycling of cellulosic materials. Thus, this study was designed to screen endophytic fungi isolated from Cameroonian medicinal plants for their cellulase activity. The plate-clearing assay and enzyme assay methods were used for screening of potent cellulolytic endophytic fungi. The promising was chosen for filter paper cellulase (FPCase) and carboxymethyl cellulase (CMCase) assay. The most potent was *Penicillium* sp 51 (50mm), followed by *P. chermesimum* (30-37.5mm) and *Penicillium* sp (22.5-30mm). The total cellulase activity (FPase) was 0.36 U/mg and 0.44U/mg for avicel and CMC crude enzymes respectively. The optimal pH and temperature for activity of crude cellulases produced by *Penicillium* sp 51 were respectively from 5-6 and 40-50°C. This endophytic fungus can be used for industrial bioconversion of cellulose.

Introduction

Agricultural residues are a great source of lignocellulosic biomass which is renewable, chiefly unexploited, and inexpensive which can be used for the production of a greener energy (Aguiar, 2008). In fact, limited nature of fossil fuels reserves has been depleted at an alarming rate and has created lot of problems for civilized world. These concerns have been replaced to utilize renewable resources for the production of a greener energy replacement

which can meet the high energy demand of the world. The cellulose is initially hydrolyzed into smaller sugar subunits like monomers and dimers. These sugar subunits can be further hydrolyzed to form biofuels or bioethanol (Menezes and Silva, 2009; Aguiar and Ferraz, 2011).

Cellulases which can hydrolyze these cellulose are multi-enzyme system that

consist of three major components: (1) endo- β -1,4-glucanases (EC 3.2.1.4), (2) exo- β -1,4-glucanases (EC 3.2.1.91), and (3) β -glucosidases (EC 3.2.1.21). These three components work together synergistically to hydrolyze cellulose into the sugar. Endoglucanases hydrolyzed internal β -1,4 linkages of cellulose to create new reducing and non-reducing ends.

The exoglucanases acts on the ends of cellulose chain and releases β - cellobiose as the end product. The β -glucosidase acts specifically on the β -cellobiose disaccharides and produce glucose (Silva, 2008). Various forms of these enzymes are produced by different microorganisms to hydrolyze diverse forms of cellulose present in the nature.

Endophytic fungi that live inside the plants without causing any apparent symptoms are one of the most common producers of these cellulases. Like other organisms invading plant tissues, endophytic fungi produce extracellular hydrolases by entering their cells not only as a resistance mechanism against pathogenic invasion but to obtain nutrition from host (Lumyong and Hyde, 2004).

Endophytic fungi use their enzymatic package to hydrolysed plant carbohydrate as a source of energy and therefore represent an underexplored biological resource for screening of cellulases. In fact, Sunitha *et al.* (2013) reported that 32% of the fifty (50) endophytic fungi isolated from *Alpinia calcarata*, *Bixa orellana*, *Calophyllum inophyllum* and *Catharanthus roseus* were able to produced cellulase. Moreover, Devi *et al.*, (2012) reported the cellulase potential of endophytic fungi from *Centella asiatica*. Therefore, this study was carried out to identify potent cellulolytic fungi among endophytic fungi isolated from *C. odorata*, *T. catappa* and *T. mantaly*.

Materials and Methods

Sources of endophytic fungi

Endophytic fungi used in this study were *Fusarium sp* N240, *Chaetomium globosum*, *Fusarium sp* N268, *Nectria rigidiuscula* and *Nigrospora oryzae* isolated from *C. odorata* L (42250/HNC), *Pestalotiopsis spp*, *Penicillium sp*, *Penicillium chermesinum*, *Xylaria sp* N19, *Paraconiothyrium variabile*, *Penicillium sp 51* from *T. catappa* L (51244/HNC) and *Xylaria spp*, *Lasiodiplodia theobromae*, *Cercospora spp*, *Cercospora olivascens*, *Phoma microchlamidospora*, *Fusarium sp*, *Diaporthe sp*, *Phomopsis sp*, *Colletotrichum gloeosporioides* and unknow fungus isolated from *T. mantaly* H (64212/HNC). Their morphology was obtained by wet mount staining and further identified by sequencing of their ITS1-5.8S-ITS2 region as described by Toghueo *et al.*, (2017).

Screening of potential cellulolytic endophytic fungi

Isolated fungi (6mm diameter) were inoculated on malt extract agar (Sigma Aldrich) plates supplemented with 0.5% Na-carboxy-methylcellulose or avicel (Sigma Aldrich). Endophytic fungi were cultured and kept for incubation at 25°C for 3-5 days. After incubation, the plates were flooded with 0.2% aqueous Congo red (Sigma Aldrich) and destained with 1 M NaCl for 15 minutes. Appearance of the clear zone around the colony of each fungus was measured as indicator of cellulase activity. Experiment was performed in duplicate (Hankin and Ananostakis *et al.*, 1975).

Production of extracellular enzymes in broth medium

Four plugs (1cm²) from the edge of the actively growing fungi were inoculated 100ml

Erlenmeyer flasks containing 20ml of malt extract broth medium (malt extract 17g, mycological peptone 3g, (ME; HiMedia) supplemented with 5g/L Na-carboxymethylcellulose or 5g/L avicel. The final pH was adjusted to 5.4±0.2. Fungi were kept for incubation at 25°C for 5 days in static condition. At the end of incubation time, cultures were filter with Whatman N°1 and centrifuged at 7000 rpm for 10 min at 4°C. Supernatants filtered using syringe filters with a 0.45-µm PVDF membrane.

Partial purification of enzyme filtrates with ammonium sulphate

Solid (NH₄)₂SO₄ was added to clarified culture fluid to obtain 30% saturation. This mixture was kept in 4°C overnight. After centrifugation at 10,000g, (4°C, 10min) the supernatant was collected for further analysis. The mixture was again centrifuged, and the precipitate was dissolved in 0.05 M phosphate buffer at pH 7.0 (Macris, 1984). Electrophoresis was used to determine the size of partial purified enzymes.

Gel electrophoresis analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 12% polyacrylamide gel by the method of Laemmli (1970). After electrophoresis, the gel was stained with Coomassie Blue R dye and destained in methanol-acetic acid-water solution (4:1:5, by volume) for 1 h. The molecular weight standards used were from a high molecular-weight calibration kit (Fermentas) (Fig. 3). The concentrated secretomes were assayed for their enzymatic activity.

Enzyme assay

Total cellulase activity was determined by measuring the amount of reducing sugar

formed from filter paper. Endoglucanase (β-1,4-endoglucanase – EC3.2.1.4) activity was assayed by measuring the amount of reducing sugar from carboxymethyl cellulose (CM-cellulose). Enzymes activity was assayed according to the methods recommended by the International Union of Pure and Applied Chemistry (IUPAC) Commission on biotechnology (Ghose, 1987). Endoglucanase (CMCase) activity was determined by incubating 0.5 ml of the supernatant with 0.5 ml of 2% carboxymethyl cellulose (low viscosity CMC, SIGMA) in 0.05 M sodium citrate buffer (pH 4.8) at 50 °C for 30 min. Filter paper degrading activity (FPCase) was determined by incubating 1.0 ml of the supernatant with 1.0 ml 0.05 M of the sodium citrate buffer (pH 4.8) containing Whatman N° 1 filter paper strip – 1.0 × 6.0 (= 50 mg). After incubation for 24 h at 50 °C, the reaction was terminated by adding 3 ml of 3,5-dinitrosalicylic acid (DNS) reagent to 1 ml of the reaction mixture. In these tests, reducing sugars were estimated colorimetrically with 3,5- dinitrosalicylic acid after Miller (1959) and Onsoni *et al.*, (2005), using glucose as standards. The enzymatic activity of total (FP-cellulase) and endoglucanase (CM-cellulase) was defined in the International Units (IU). One unit of enzymatic activity is defined as the amount of enzyme that releases 1 µmol reducing sugars (measured as glucose) per ml per min.

Effect of pH and temperature on the activity of crude enzyme filtrates

Culture filtrate (0.5 ml) was added to 0.5 ml carboxyl methyl cellulose (2 % w/v) in 0.05 M citrate buffer, pH 4.8 in a test tube. Mixture was incubated in water bath at temperatures ranging between 30 to 100°C for 2 h. Endocellulase activity was then determined following the DNSA method recommended by International Union of Pure and Applied Chemistry (Ghose, 1987). The

effect of pH values ranging from pH 4.8 to 11.0 on activity of enzymes was assayed in 0.05 M citrate buffer at pH 4.8 and in 0.05 M phosphate buffer at pH 7 (Macris, 1984). All the tubes were incubated at 50 °C.

Statistical analysis

All data are given as the mean \pm SD of triplicates (n = 3). Analysis of variance (ANOVA) was performed using Microsoft Excel 2010.

Results and Discussion

Screening of endophytic fungi

Twenty six endophytic fungi isolated from *Cananga odorata*, *Terminalia catappa* and *Terminalia mantaly* were analyzed for their cellulases activity using a plate clearing assay on CMC and Avicel supplemented medium. The diameter of clearance ranging from 0 to 50mm in CMC and avicel plates. Among the strains of fungi, the most potent fungus on CMC agar was *Penicillium* sp 51 (50mm) fellow by *P. chermesimum* and *Penicillium* sp (30mm). On avicel agar medium, the most potent was *Penicillium* sp 51 (50mm), fellow by *P. chermesimum* (37.5mm) and *Penicillium* sp (22.5mm). All fungi with cellulase activity were isolated from root of *T. catappa*. Overall, the strong cellulase activity was found with *Penicillium* sp 51 (Table 1).

From table 2, the activity of enzyme produced by *Penicillium* sp 51 in presence of CMC (51c) was higher (0.44U/mg) than enzyme produced in presence of avicel (51a) (0.36U/mg).

In figure 1 the graphical representation above shows the effect of pH on cellulase activity. The optimal pH for each sample is been compared against the absorbance at 540 (nm) wavelength. From figure 1, the optimal pH for

activity of cellulases produced by *Penicillium* sp 51 are 5 and 6 for endoglucanase (51c) and exoglucanase (51a) respectively.

In figure 2 the graphical representation above shows the effect of temperature (°C) on cellulase activity. The optimal pH for each sample is been compared against the absorbance at 540 (nm) wavelength. The determination of optimal temperature of activity for cellulases enzymes produced by *Penicillium* sp 51 shown that 40 and 50°C are optimal for activity of endoglucanase (51c) and exoglucanase (51a) respectively.

The electrophoretic analysis of crude enzyme revealed that they are mixture of forms each of endoglucanase and exoglucanase.

Fungal endophytes are known to produce cell wall degrading enzymes including cellulases, which play an important role in natural biodegradation processes in which plant lignocellulosic materials are efficiently degraded in unit of glucose (Carroll *et al.*, 1983). In industry, these enzymes have found novel applications in the production of fermentable sugars and ethanol (Van Wyk, Mohulatsi, 2003), organic acids (Luo *et al.*, 1997), detergents and other chemicals (Oksanen *et al.*, 1998). They have been used in the pulp and paper industry, e. g., in deinking of fiber surfaces and in improving pulp drainage (Suurnäkki *et al.*, 2004), in the textile industry (Miettinen-Oinonen *et al.*, 2004), animal feed (Ishikuro, 1993), and even in the food industry (Penttilä *et al.*, 2004), for the processing of paper and cellophane, as well as for biotransformation of waste cellulose to fermentable sugars (Van Wyk, Mohulatsi, 2003). The demand for more thermostable, highly active and specific cellulases is on the increase. Therefore, this study was carried out to screened potent cellulolytic endophytic fungi isolated from Cameroonian medicinal plants.

Table.1 Screening and measurement of zone of clearance by endophytic fungi on CMC and Avicel (mm) supplemented medium

Plants species	Fungi	Diameter on clearance on CMC	Diameter on clearance on Avicel
<i>C. odorata</i> (51244/HNC)	<i>Fusarium</i> sp N240	0±0.0	0±0.0
	<i>Chaetomium globosum</i>	0±0.0	11±0.1
	<i>Fusarium</i> sp N268	0±0.0	0±0.0
	<i>Nectria rigidiuscula</i>	0±0.0	0±0.0
	<i>Nigrospora oryzae</i>	0±0.0	0±0.0
<i>T. catappa</i> (64212/HNC)	<i>Pestalotiopsis</i> sp	0±0.0	0±0.0
	<i>Penicillium</i> sp	30±0.0	22.5±0.3
	<i>Penicillium chermesinum</i>	30±0.0	37.5±0.3
	<i>Xylaria</i> sp N19	0±0.0	0±0.0
	<i>Paraconiothyrium variabile</i>	0±0.0	0±0.0
	<i>Penicillium</i> sp 51	50±0.0	50±0.0
	<i>Pestalotiopsis</i> sp	0±0.0	0±0.0
	<i>Paraconiothyrium variabile</i>	0±0.0	0±0.0
<i>T. mantaly</i> (42250/HNC)	<i>Xylaria</i> sp	0±0.0	0±0.0
	<i>Lasiodiplodia theobromae</i>	0±0.0	0±0.0
	<i>Cercospora</i> sp N126	0±0.0	0±0.0
	<i>Cercospora</i> sp N129	0±0.0	0±0.0
	<i>Phoma microchlamidospora</i>	10±0.0	10±0.0
	<i>Xylaria</i> sp N120	0±0.0	0±0.0
	<i>Fusarium</i> sp N138	0±0.0	0±0.0
	<i>Diaporthe</i> sp	10±0.0	10±0.0
	<i>Phomopsis</i> sp	0±0.0	0±0.0
	<i>Colletotrichum gloeosporioides</i>	0±0.0	0±0.0
	<i>Unknow fungus</i>	0±0.0	0±0.0

Table.2 Quantitative analysis of samples

Total cellulase content	Activity (U)	Protein Conc. (mg/ml)	Specific Activity (U/mg)
51a	0.54	1.49	0.36
51c	0.48	1.09	0.44
Endoglucanase Sample	Activity (U)	Protein Conc. (mg/ml)	Specific Activity (U/mg)
51c	0.18	1.15	0.16
Exoglucanase Sample	Activity (U)	Protein Conc. (mg/ml)	Specific Activity (U/mg)
51a	0.21	1.36	0.15

Figure.1 Effect of pH on cellulase activity

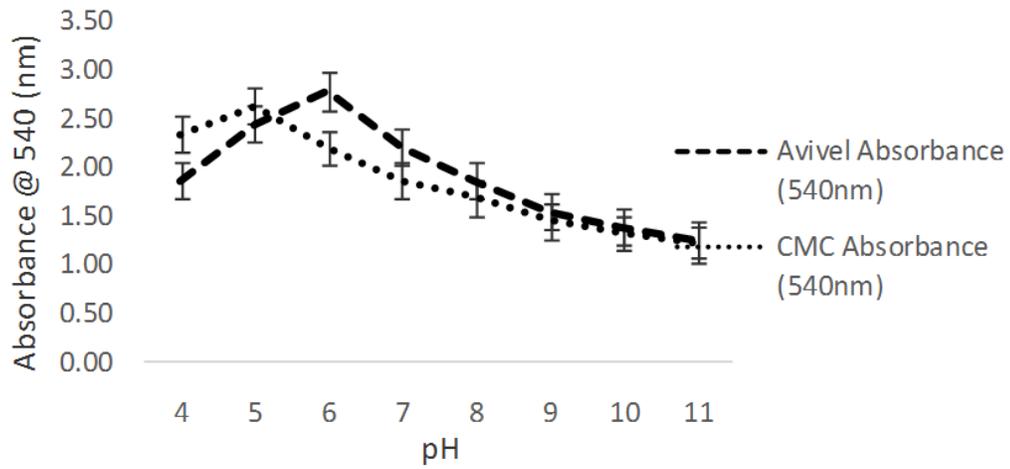


Figure.2 Effect of temperature (°C)

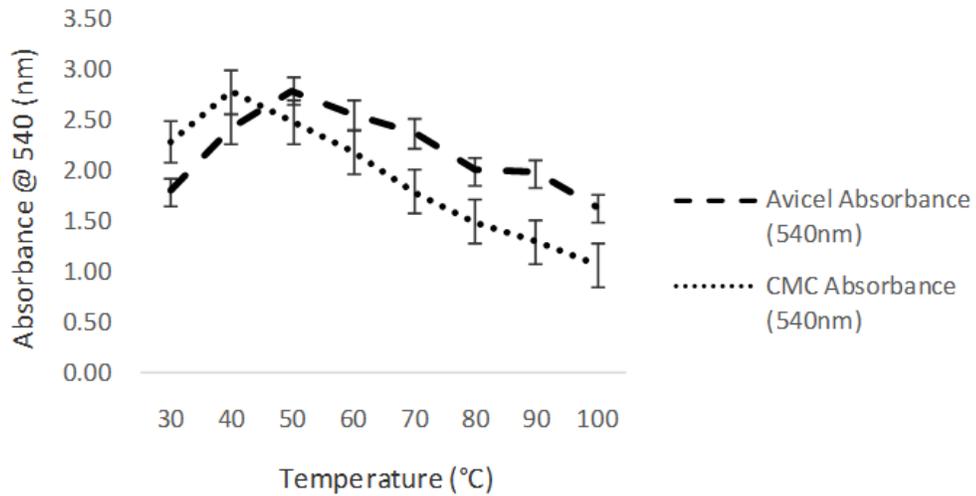
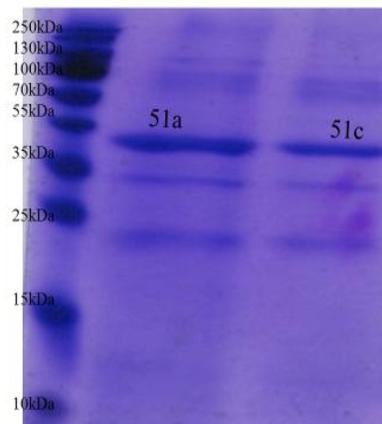


Figure.3 SDS-PAGE of 51a and 51c cellulase samples



From this investigation, among the fungi screened in CMC and Avicel agar plate, the more potent species were all from *Penicillium* spp. Similar to our study, Jahangeer *et al.*, (2005) indicated that majority of *Penicillium* species possess higher cellulolytic activity. In addition, Gusakov and Sinitsyn (2012) reported that *Penicillium* species possess the cellulolytic potential useful in saccharification process of lignocellulosic biomass for biofuel production. *Penicillium* sp 51 screened as potential cellulase producers was investigated in the flask culture using CMC and avicel powder as a carbon source in the culture medium. After 6 days of cultivation, enzymatic activity was measured by the production of sugar reducing end group, which is taken to be an indication of cleavage of cellulose molecules. Three standard substrates were used for the determination of cellulase activity in terms of overall (FPCase), endoglucanase (CMCase) and exoglucanase (Avicel) contents (Ghose, 1987; Wu *et al.*, 2006). From this study, the total cellulase activity (FPase) was 0.36 U/mg and 0.44U/mg for avicel and CMC crude enzymes. The optimal pH and temperature for activity of crude cellulases produced by *Penicillium* sp 51 were respectively from 5-6 and 40-50°C. Lakshmi *et al.*, (2014) also reported maximum FPase activity utilizing CMC as carbon source with stable activity in the range of pH 5-6 and temperature 30°C. Moreover, Ravindran *et al.*, (2010) reported the optimum temperature of 50°C and pH ranged between 5-12 for activity of cellulases produced by *Chaetomium* sp (NIOCC 36). The present finding indicates that this endophytic fungus *Penicillium* sp produced enzymes complexes for the effective hydrolysis of cellulose.

In conclusion, Maximum cellulase enzyme activity was observed by *Penicillium* sp. Further characterization and optimization of the culture condition is needed to suite

industrial application of this potential fungi species for cellulose bioconversion in industries. Hence a further investigation should be carried out in order to maximize the enzyme activity which will open new paradigm for industry.

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