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Method and Materials

Collection of Sample

Soil samples were collect from the different places and soil samples were collected in sterile polyethylene bags.

Culture Media

Two media were used, Emerson Yss Agar medium containing (g/l) yeast extract – 4 , soluble starch – 15 , K2HPO4 – 1 , MgSO4 . 7H2O – 1 , agar – 20, distilled water – 1000, adjust pH to 7.0 ± 0.2. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. 1% chloramphenicol solution was added to the medium before pouring in to petri-plates for preventing bacteria growth. The petri dishes were then incubated at 28+2 ˚C in dark. Sabouraud Dextrose (SD) broth medium containing (g/l) : dextrose – 40, peptone – 10 , distilled water 1000ml and final pH 5.6 ± 0.2 at 25 ˚C were prepared.

Isolation of fungi

Isolation of fungi from soil samples collected from different places by using serial dilution method . One gram of soil sample was dissolved in 100ml of sterilized distilled water and then diluted up to $1/10^4$ times, from which ,0.5 ml of volumes were pipetted onto Yss agar and incubated at 30 ºC for 3 days. Fungi were isolated from each plate and subcultured on Yss agar. Subculturing was continued until a pure isolate was obtained. Stock cultures were maintained on Yss agar at 4 ºC.

Identification of Isolated Fungi

The technique of James and Natalie (2001) was adopted for identification of the unknown isolated fungi using cotton blue in lactophenol stain. The identification was achieved by placing a drop of the stain on clean slide with the aid of a mounting needle, where a small portion of the mycelium from the fungal cultures was removed and placed in a drop of lactophenol. The mycelium was spread very well on the slide with the aid of the needle. A cover slip was gently applied with little pressure to eliminate air bubbles. The slide was then mounted and observed with x10 and x40 objective lenses respectively. The species encountered were identified in accordance with Cheesbrough (2000).

Molecular identification of fungal isolate

Identification of the fungal isolates was also done based on molecular characterization by sequencing 18S rRN sequencing by sending cuture samples..

Screening of cellulolytic fungi

The isolated fungal cultures were screened for their ability to produce cellulases complex following the method of Teather & Wood (1982). Czapek-Dox medium used in this method contained (g/l): sucrose – 30, NaNO3 – 2,

K2HPO4 - 1, MgSO4 – 0.05, Kcl – 0.5, FeSO4 – 0.01, carboxy- methyl cellulose – 1%, Agar agar - 20. pH of the medium was adjusted to 5. After autoclaving at 121°C and 15 lbs. pressure, the medium was poured into Petri plates and allowed to solidify. Cavities of 6 mm size were made in the solidified medium and inoculated with 0.1 ml of fungal suspension prepared from 7 day old culture plates in Sabouraud Dextrose broth medium . The plates were incubated at room temperature (28 ± 2 °C) for three days to allow fungal growth, then again incubated for 18 h at 50 °C which is the optimum temperature for cellulases activity. After incubation, 10 ml of 1% Congo - Red staining solution was added to the plates that were shaken at 50 rev/ min for 15 min. The Congo - Red staining solution was then discarded, 10 ml of 1 N NaOH was added to the plates for 15 minutes. Finally 1 N NaOH was also discarded and the staining of the plates was analyzed by noticing the formation of clear zones around the fungal spore inoculated wells.

The Esculin Gel Diffusion Assay

One hundred millilitres of 4% agar in 0.2M Na-acetate buffer pH 5 was autoclaved and kept hot at 50 ◦C. One hundred millilitres of 0.2% esculin and 6ml of 1% FeCl3 were freshly prepared and heated up to 50 ◦C in the water bath. All the reagents were mixed and immediately poured over a 23cm×23 cm plastic assay plate. The gel was allowed to solidify and wells of 0.6 cm diameter were made at appropriated distances from each other. Eighteen microlitres of the sample was poured into each well. The plates were sealed with adhesive tape to stop excessive evaporation and placed at 37 ◦C for incubation for 5 h. After the incubation period, the plates were placed on ice to slow both diffusion and further enzymatic activity. This step ensured stabilization of the otherwise slowly expanding zones. Zone diameters were measured with Mitutoyo® dial calipers.

Hemicellulotic (Xylanolytic) enzyme assay

Each medium contained the following constituents (g/L): 0.05 g MgSO4. 7H20, 0.005 g CaCl2, 0.005 g NaNo3,0.009 g FeS04.7H20,0.002 g ZnSO4, 0.012 g MnSO4, 0.23 g KCl, 0.23 g KH2PO4, 2 g peptone, 19 g Agar . After seven days of incubating the plates at 28±2°C, each was flooded with 0.4% Congo red for 10 min and then destained with 1 M NaCl. The hydrolysis zones on the plate media were measured and the relative enzyme activity of each isolate was determined using the following formula: Relative enzyme activity = diameter of clearing (mm) / diameter of growth (mm) . Isolates with wide clear zones (high relative enzyme activities) were selected for further work. Pure cultures of the selected fungal isolates were regularly sub-cultured onto fresh sterile Potato Dextrose Agar (PDA), slants every 2 to 3 weeks to maintain viability and kept in refrigerator at 4°C.

Plate assay for lignin degradation

The ability of the fungi to degrade lignin was tested using alkaline lignin (Sigma Aldrich) as the lignin source, using the same method as described by Tekere *et al.* (2001b). The test medium were comprised of 5 g/*l* glucose, 5 g/*l* Ammonium tartrate, 1g/*l* malt extract, 0.5 g/*l* $MgSO_4.H_2O$, 0.01 g/*l* $CaCl_2.2H_2O$, 0.1 g/*l* NaCl, 0.01 g/*l* $FeCl_3$, 1 mg/*l* thiamine and 20 g/*l* agar. The 0.25 % (w/v) of alkali lignin was added in the medium before autoclaved. Sabouraud Dextrose (SD) broth medium were prepared from seven days old pure culture was inoculated 18 microlitre poured onto tested media in a 9 cm sterilized plastic Petri dishes and then incubated in dark at room temperature (25-27°C) for seven days. After seven days, the mycelia were scraped out from the media plate and then poured with 1% of $FeCl_3$ and $K_3[Fe(CN)_6]$ aqueous solution. The diameter of clear zone which indicated the degradation zone of lignin was measured.

Laccase assays

Isolated fungi were tested for their abilities to produce laccase by laccase plate assay, in which they were grown on guaiacol-agar medium (0.02% w/v guaiacol, 1% w/v yeast extract, and 2% w/v agar agar). Eighteen microlitre

sample culture was poured into well and the plates were then incubated at 37 ºC for 7 days to get sufficient growth of the cultures on the medium.