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Production and characterization of cellulase from mushroom (*Pleurotus ostreatus*) for effective degradation of cellulose

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Abstract

Cellulases are a group of hydrolytic enzymes capable of hydrolyzing the most abundant organic polymer that means cellulose to smaller sugar components including glucose subunits. The aim of this study was to screen cellulase producing oyster mushroom collected from Eucalyptus tree bark to evaluate the in vitro production of cellulase by *Pleurotus ostreatus* using different lignocellulosic substrates, and to characterize the cellulase produced with respect to changes in pH, temperature, and concentration of substrates. A total of ten mushroom specimens were randomly collected from Eucalyptus tree bark in the premise of Holetta Agricultural Research Center campus. All of the collected mushroom specimens were identified morphologically and biochemically as *Pleurotus ostreatus* and also screened for their ability to produce cellulase by detecting and measuring zone of hydrolysis on commercial media containing Carbxymethyl Cellulose (CMC) as the sole carbon source. These mushroom specimens were cultivated using both solid state fermentation and submerged fermentation systems supplemented with different lignocellulosic substrates (wheat straw, teff straw, bean straw, wood fiber and Eucalyptus tree bark) to identify the most suitable medium for the production of cellulase. The highest enzyme production was obtained on bean straw and wheat straw which resulted in 0.191 U/ml, 0.868 U/ml and 0.389 U/ml; and 0.216 U/ml, 0.444 U/ml, and 0.245 U/ml of FPase, CMCase, and β-glucosidase in solid state fermentation. The lowest values were, however, obtained in media containing wood fiber in both solid state fermentation and submerged fermentation. Comparison of the lignocellulosic substrates revealed that wheat straw was selected for further growth parameter optimization. The production of cellulase was higher at the 5th day of incubation period, and the optimum pH and incubation temperature required for maximum cellulase production were 4 and 30°C, respectively. Sucrose and Yeast extract at 1% concentration were found to be the most preferred carbon and nitrogen sources for cellulase production by *Pleurotus ostreatus*. The optimum pH and temperature for cell_free cellulase activity on were found to be 4 and 50°C, respectively. Generally the cellulases produced by *Pleurotus ostreatus* were stable and active at temperatures ranging from 20-50°C. These characteristics hopefully would make this enzyme potentially attractive in a variety of industrial applications including animal feed treatments. There was a linear relationship between cellulase and its substrate concentration for there was an increase in activity with increase in substrate concentration. The relationship between rate of reaction and substrate concentration depended on the affinity of the enzyme for its substrate. Finally the cellulase was tested for its ability to saccharify agricultural wastes and the results showed the highest release of sugars from wheat straw.

Keywords: Cellulose; *Pleurotus ostreatus*; Saccharification; Solid state fermentation; Wheat straw

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1. Introduction

Cellulases were initially investigated several decades back for the bioconversion of biomass which helps in the industrial application of enzymes in animal feed, food, textiles, detergents and in paper production [1]. It is the most prominent group of hydrolytic enzymes that catalyze the hydrolysis of β-1, 4 linkages present in cellulose to give glucose. This hexose is fed into the central metabolic pathways of various microorganisms to produce various bio-products. Cellulases are primarily produced in nature by plants, fungi, bacteria, and even some protozoa, mollusks, and nematodes [36]. Large numbers of microorganisms are capable of degrading cellulose. However, only a few of these produce significant quantities of cell free enzyme capable of completely hydrolyzing crystalline cellulose [2]. For the production of industrially important enzymes and bioactive secondary metabolites, fungi isolated from tree barks are known to be potential candidates. These enzymes are mostly ligno-cellulolytic in natures which are used to obtain their energy sources [35]. Major constrains in enzymatic hydrolysis of cellulosic materials for the production of fermentation sugar are low productivity and the cost of cellulases [3].

The most abundant renewable carbon source is the cellulosic material. An agricultural waste is a cheap source of cellulose that can be used for the production of different useful products all over the world [2]. Cellulase production from agricultural wastes is economical as compared to production from pure cellulose. The hydrolysis of cellulose can be done by using enzymes to produce glucose, which can be used for the production of ethanol, organic acids and other chemicals [4]. Production of cellulases by fungal isolates requires optimal conditions for their growth which leads to the release of extracellular enzymes. The growth conditions as well as extracellular enzyme production conditions is likely to vary among isolates. The major components of production medium like carbon and nitrogen sources and physical parameters like temperature, pH and incubation time were found to be critically affecting the cellulase production and hence need to be optimized for every isolate [27]. Solid state fermentation (SSF) for the production of cellulase is rapidly gaining interest as a cost effective technology for the production of enzyme and higher yield of cellulase is produced under SSF compared to submerged fermentation (SmF) [29].

SSF has many returns over other fermentation processes in a sense that the culture media are simpler and nutrients present in the substrate support growth due to the natural metabolism of the microorganism can secrete enzymes while growing in the solid substrate. It can be of special interest in those processes where the crude fermented product may be used directly as the enzyme source [33]. Cellulase produced by fungus under SSF has advantages for different industrial purposes, namely, in food and fermentation industries, microbial enzymes have attained significant role in biotransformation involving organic solvent media, mainly for bioactive compounds. Cellulolytic enzymes are produced by a number of microorganisms. Fungi and bacteria are the main agents of natural cellulose degradation in the environment. Fungi however are known to secrete cellulases in large amounts [16].

The mushrooms of the genus *Pleurotus* rank second in the world mushroom market and are the most popular mushroom. The *Pleurotus* of the class basidiomycetes belongs to a group known as white rot fungi [34] as they produce a white mycelium and are generally cultivated on non-composted lignocellulosic substrates in which various kinds of *Pleurotus* are commercially cultivated and have considerable economic values. The white rot fungi, *Pleurotus* species are commonly known as oyster mushrooms. Most of the known species of the genus *Pleurotus* are edible. One of the most important aspects of *Pleurotus* species is related to the use of their lignocellulolytic system for a variety of applications.

These includes, bioconversion of lignocellulosic wastes into valuable products, for animal feed and other food products and the use of their ligninolytic enzymes for the biodegradation of organopollutants, xenobiotic and industrial contaminants [30]. *P. ostreatus* which is well-known among cultivated oyster mushroom species are characterized by its white to lilac-gray spore print and in their natural growing environment, easily recognized by way it grows on the wood in aggregate. Cellulase production by fungi is influenced by culture conditions such as nature and concentration of carbon and nitrogen sources, media composition, pH, temperature, and presence of inducers. This study is initiated in order to produce, characterize, and evaluate for effective cellulase produced by *P. ostreatus* from the environment and thus contribute to different industries utilizing in their processes. More novel cellulases are needed in the bio-based and bioenergy industry, this study therefore is intended to characterize and evaluate cellulases from *Pleurotus ostreatus*. This will provide possibility for locally developed combinations of cellulases that can be used in the production of bioethanol.

2. Material and methods

2.1. Description of the study area

The study was conducted at Holetta Agricultural Research Center, National Biotechnological Research Laboratory which is located at 28 km west of Addis Ababa in the Oromia Special Zone Surrounding Finfinne, Oromia Region. Geographically, HARC is located at latitude, longitude, and an altitude of 9°3' N, 38° 30' E and 2391 meters above sea level, respectively. Typically it has a bimodal rainfall pattern with a mean annual precipitation of about 1100 mm. The main rainy season extends from June to September while the short rainy season from February to April. Mean annual maximum and minimum temperatures are 21°C (ranging from 20°C and 27°C) and 6°C (ranging from 2°C and 9°C), respectively [32].

2.2. Oyster Mushroom Collection and Identification

Fruiting bodies of *Pleurotus ostreatus* were collected from the bark of live old *Eucalyptus* tree around Holetta Agriculture Research Center (HARC) by using sterile forceps and handled in sterile polythene bags. The fungi were collected based on their morphology following the methods of *Pleurotus* species identification guideline given by the Consensus Document on the Biology of Pleurotus species [24]. The major morphological traits considered during fruiting body collection were; occurrence, stem or stipe, odor, shape, and color of the fruiting body.

The fungi grow in shelf-like clusters, nearly absent stem, smooth and thick flesh the whitish kidney-shaped cap having anise odor were considered. The fungi having the above mentioned features were collected from 10 (ten) different trees in early September 2017 and were immediately kept in a freezer at -20°C until cultured on potato dextrose agar (PDA) media. One week later PDA media were prepared and the mushrooms were inoculated on it under aseptic condition following standard microbiological procedures: first the fruiting bodies (gills of the fungi) were cut into pieces of nearly 3 mm size and subjected to surface sterilization by dipping successively in sterile distilled water, 70% ethanol, and 5% NaOCl, each for 4 minutes and finally repeatedly washed with sterile distilled water.

Then after the pieces were kept on sterile filter paper for a few minutes for drying and then placed on prepared PDA plates. All plates were incubated at 28°C and daily observation was made. After a week, sub-culturing was done by taking fungal mycelia from incubated plates and transferred to new PDA plates to get purer cultures. The major microscopic features considered during sub-culturing were spore print and colony morphology. The fungi having smooth white to lilac-grey spore with cylindrical to long elliptical shaped morphology were considered as *Pleurotus ostreatus* [24]. After pure *P. ostreatus* cultures were obtained, they were maintained on agar slants by sub-culturing every month and preserved at 4°C in a refrigerator according to [21].

2.3. Screening of Cellulase Producing P. ostreatus

Carboxymethylcellulose containing cellulose as the sole carbon source was inoculated with *P. ostreatus.* The plates were incubated for 4 days at 30°C and positive cellulolytic activity was detected by the formation of clear zones of hydrolysis on a plate after flood the plates with 0.1% aqueous Congo red followed by repeated washing with 1 M NaCl. The composition of the CMC plate agar medium was (g/l): urea 0.3; ammonium sulphate 1.4 K2HPO4 1, Fe2SO4 0.3, CaCl 0.3, Yeast Extract agar 0.25, Peptone 0.75, and CMC 10, The final pH of the medium was adjusted to 5.5 prior to sterilization [31].

2.4. Source and Preparation of Growth Substrates

The growth substrates like Teff straw, wheat straw, bean straw, *Eucalyptus* tree bark and wood fiber were obtained from HARC. The selection of substrate for enzyme production in SSF processes depends upon several factors related with cost and availability of the substrates, and thus may involve screening of several agro-industrial residues. The lignocelluloses substrates were oven dried and ground using a manual grinder. All the resulting powder could pass through a 1 mm size mesh, and then the powder passed through 1 mm size mesh used for submerged fermentation and substrate above 1mm used for solid-state fermentation.

2.5. Preparation of Media for Enzyme Production

2.5.1. Media for submerged fermentation

Media used for submerged fermentation were arranged in four separate Erlenmeyer flasks of 250ml capacity containing each 10g of a single type of lignocellulosic substrate (*Eucalyptus* tree, wood fiber, Teff straw, wheat straw and bean straw smaller than 1mm). Experiments were performed in triplicate at room temperature (25±2 °C) with shaking at

120rpm. The respective substrates were submerged in 200ml basal medium consisting of (g/l): $(NH4)_2SO4$, 1.4; KH2PO4, 2; CaCl2, 0.3; MgSO4, 0.3; FeSO4.7H2O, 0.5; MnSO4.7H2O, 0.16; ZnSO4.7H2O, 0.14; CoCl2, 0.2; and Tween 80, 1ml; with a final pH of 5.5. The initial pH of the medium was adjusted to 4.0 prior to sterilization by adding 1N HCl. The media was autoclaved at 121°C for 20 min and cooled at room temperature [26].

2.5.2. Media for solid state fermentation

Media used for solid-state fermentation were arranged in four separate flasks of 250ml capacity containing each 10g of a single type of lignocellulosic substrate Bean straw, *Eucalyptus* tree bark, Teff straw, wood fiber and Wheat straw. Then 30 ml of distilled water was added to all bottles following [7]. Experiments were performed in triplicate using Erlenmeyer flasks at 30°C. Then, the glass bottles were wrapped and autoclaved at 121˚C for 20 minutes.

2.6. Preparation of Inoculum

PDA inoculum was prepared and the fungal stock was stored at 4°C.then transferred on to PDA plates with a sterilized needle and allowed to grow at 30°C for 7 days. The growing edges of the spore were cut with a sterilized 4mm core borer and used as inoculum. The inoculum piece was placed inverted down so that they had good contact with the agar surface [11].

2.7. Growth of P. ostreatus and Extraction of cellulase

2.7.1. Submerged fermentation

Three mm^2 of spore-containing PDA was used to inoculate the flasks containing submerged media. After 7 days of mushroom cultivation, biomass was filtered through cotton gauze and the solids separated by centrifugation 10000rpm for 10 min at 4°C. The supernatant was filtered through Whatman no. 1 filter paper and the clear filtrate was used as crude enzyme extract.

2.7.2. Solid state fermentation

The solid-state media were inoculated with three mm square of spore-containing PDA. After mixing, the flasks were incubated at 28 ± 2°C temperatures for 7 days under static conditions. After incubation, 100ml citrate buffer (0.05M at pH 5.3) was added and rotated on the rotary shaker at 120rpm for at least 2hr at room temperature for maximum enzyme extraction. The liquid homogenate was then filtered through cotton gauze and centrifuged at 10000rpm for 10 minutes at 4°C to remove solid particulate matter and the supernatant was filtered through Whatman no. 1 filter paper and the clear filtrate was used as crude cellulase.

2.8. Enzyme assay

2.8.1. Filter paper activity (FPase) assay

Filter paper degrading activity of cellulase (FPase) is a measure of total cellulolytic activity resulting from different enzyme components present in the culture filtrate. Whatman filter paper strip with 50 mg weight was suspended in 1 ml of 0.05M sodium citrate buffer pH 4.8 at 50°C in a water bath. Suitable aliquots of enzyme source were added to the above mixture and incubated for 60 minutes at 50°C. Enzyme blank or without enzyme were run simultaneously in the same manner as specified above. After incubation, 3, 5-dinitrosalicyclic acid (DNS) was mixed. All samples, enzyme blanks, and glucose standards were vigorously boiled for exactly 5 minutes in a water bath after cooled measured at 540nm Spectrophotometer. The activity of cellulase was expressed in filter paper units. One unit of filter paper unit (FPU) was defined as the amount of enzyme releasing one µ mole of reducing sugar from filter paper per minute [8].

2.8.2. Endoglucanase activity (CMCase) assay

The reaction mixture contains 1.0ml of 1% carboxymethylcellulose (CMC) in 0.2M acetate buffer pH 5.0. These reaction mixtures were pre-incubated at 50°C in a water bath for 20 minutes. Crude cellulase was added to the reaction mixture and incubated at 50°C in a water bath for 60 minutes. Appropriate controls devoid of substrate or enzyme were simultaneously run. The reducing sugar produces in the reaction mixture was determined by dinitrosalicyclic acid method. The reagent 3, 5-dinitrosalicyclic acid reagent was added to aliquots of the reaction mixture and the color developed was read at wavelength 540nm in a spectrophotometer. One unit of endoglucanase activity was defined as the amount of enzyme releasing one µ mole of reducing sugar per minute.

2.8.3. β-D-glucosidase activity

β-D-glucosidase activity in the culture filtrate was determined according to the method of β-D-glucosidase activity measured in assay mixture containing 0.2ml of 5mM *p*-nitro phenyl β-D-glucopyranoside dissolved in 0.05M citrate buffer pH 4.8 and 0.2ml of dilute enzyme solution with appropriate controls. After incubation for 30min at 50°C, the reaction was stopped by adding 4ml of 0.05M NaOH-glycine buffer pH 10.6. The yellow color *p*-nitrophenol liberated was determined using Spectrophotometer at 405nm. β- D-glucosidase activity was defined as the amount of enzyme liberating one μ mole of *p*-nitrophenol per minute under standard assay conditions.

2.9. Fungal Cellulase Production

2.9.1. Cellulase production

Cellulase production was carried out in 250ml Erlenmeyer flasks each containing Mandels medium pH 4.2 that comprises of Na_2HPO_4 15g, $CaCl_2$ 0.8g, $MgSO_4.7H_2O$ 1.2g, $MnSO_4.7H_2O$ 0.0016g, $FeSO_4$ 0.00271g, $ZnSO_4$ 0.0014g, Urea 3g, Peptone 0.75g, Yeast extract 0.3g and CMC 10g in 200ml media. To each flask 5 disc spores were added and incubated at 30°C, with shaking at 130rpm and samples were collected every 24hrs until maximum cellulase activity was reached. The culture was centrifuged at 3500rpm for 10 minutes using a centrifuge and the supernatant was stored at -20°C until required for cellulase assay.

2.10. Assay for Cellulase Activity

2.10.1. Dinitrosalicylic acid assay method for cellulase activity

Cellulase activity was measured using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1960), through the determination of an amount of reducing sugars liberated from 1% carboxymethylcellulose (CMC) solubilized in 50 mM citrate buffer pH 5.0. A combination of 100μl enzymes and 900μl of 1% CMC substrate solution were incubated at 50°C for 5 min. The reaction was stopped by addition of DNS solution and reaction mixture was boiled for 15 min, cooled in water for color stabilization, and the optical density was measured at 540 nm. Cellulase activity was determined by using a calibration curve obtained from standard solutions of glucose. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of glucose per minute.

2.11. Characterization of cellulase produced by P. ostreatus

2.11.1. Effect of pH on cellulase activity

The optimum pH, of cultures was determined by cultivating in a 250ml flask containing 50ml medium with different pH ranges from 4 to 8. The pH of the medium was adjusted by using 1 N HCl or 1 N NaOH. The flasks were incubated at 28°C for 5 days and centrifuged at 10,000rpm for 10min. Pellet was discarded and supernatant for assay of cellulase.

2.11.2. Effect of temperature on enzyme activity

The optimum temperature of the enzyme for hydrolysis of Carboxymethylcellulose (CMC) in 50 mM Citrate buffer pH 5.0 by *P. ostreatus* was determined by incubating the mixture of the enzyme 100μl and 1% CMC 900μl for 15 min at different temperatures ranging from 40 to 80°C. The reaction was stopped by the addition of DNS solution and cellulase activity was determined.

2.11.3. Effect of pH on enzyme activity and stability

The optimum pH for cellulase activity was determined by incubating the mixture of the enzyme 100μl and 1% CMC 900μl in the presence of appropriate buffers; 50 mM citrate buffer pH 3, 4, 5 and 6, 50mM sodium phosphate pH 6, 7 and 8, 50mM Tris-HCl pH 8 and 9 and 50mM glycine-NaOH pH 9, 10 and 11. The reaction mixtures in various pH buffers were incubated for 5min at 50°C after which the cellulase activity was assayed by the DNS method.

2.12. Optimization of culture conditions for cellulase production

2.12.1. Effect of pH on cellulase production

After preparation of the basal liquid medium containing cellulose, suitable aliquots were adjusted at pH 3, 4, 5, and 6 using 0.1M NaOH and 0.1N HCl buffer system. After 4 days of incubation at 30°C, the amount of cellulase was assayed by DNS method.

2.12.2. Effects of temperature on cellulase production:

The effect of temperature was evaluated by inoculating the flasks containing 50ml of sterile production medium, with 100µl culture. The inoculated flasks were incubated at a different temperature varying from 20°C, 25°C, 30°C, 35°C for 5 days and centrifuged at 10,000rpm for 10min. Pellet was discarded and supernatant for assay of cellulase.

2.12.3. Effect of carbon sources on cellulase enzyme production:

The effect of carbon sources on enzyme production was studied at different concentration 0.5%, 1.0%, 2.0% and 2.5% (w/v). Fifty ml of production medium was distributed into the flasks and supplement with different concentration of carbon compounds Glucose, Sucrose, and Lactose. The mixture incubated without carbon source served as a control. The flask was then inoculated with 100µl culture and incubated in an incubator at 28°C for 7 days and centrifuged at 10,000rpm for 10min. The optimum carbon sources were found by analyzing the result of cellulase production.

2.12.4. Effect of nitrogen sources on Cellulase enzyme production

The effect of nitrogen sources on enzyme production was studied at different concentration 0.5%, 1.0%, 2.0% and 2.5% (w/v). Fifty ml of production medium was distributed into flasks and supplement with different concentration of nitrogen compounds Ammonium sulfate, beef extract and yeast extract. The flasks were inoculated with 100µl culture and incubated in incubator shake at 28°C for 7 days at 130rpm and centrifuged at 10,000rpm for 10min. The mixture incubated without nitrogen source served as a control. The optimum nitrogen sources were found by analyzing the result of cellulase production.

2.12.5. Effect of substrate concentration on Cellulase activity

Rate of CMC hydrolysis was determined by incubating 900µl substrate (CMC) at various concentrations (0.1%, 0.5%, 1%, 1.5%, 2%, 2.5% and 3%) with 100µl of crude cellulase. The enzymes incubated without CMC served as a control for 10 minutes. The effects of substrate concentration were assayed by DNS method.

2.13. Saccharification of substrates by cellulase

Agro-waste was ground into powder using a grinder. 10g/l of the substrate was suspended in 50mM acetate buffer (pH 5) and autoclaved at 121°C for 15min and 15ml of substrate suspension taken into 100ml of Erlenmeyer flasks and the substrate was added into 5ml of culture filtrate obtain from cellulolytic fungi. Saccharification was performed in a water bath at 27±2°C for 24hrs and the supernatant was centrifuged at 2500rpm for 15min and total reducing sugar was assayed using the DNS method. The percentage saccharification was calculated as:

$$Saccharification(\%) = \frac{\text{Glucose (mg/ml)}}{\text{substrate(mg/ml}}\ x\ 100$$

2.14. Data Analysis

The study was carried out experimentally with three replications and the data was fed into Microsoft Excel and presented on the average of three replicates obtained from there independent experiments. The results were expressed as the mean of data obtained from triplicate experiments ±SD.

3. Results and discussion

3.1. Identification of P. ostreatus

Identification of *P. ostreatus* was done at their natural habitat living *Eucalyptus* tree based on the color difference of their fruiting bodies. This well-known mushroom is easily recognized by the way it grows on the wood in shelf-like clusters, its relatively large size, its whitish gills that run down a stubby, nearly-absent short, stipe, and its whitish to lilac-gray spore print. Only white colored fruiting bodies forming overlapping shelves or clusters on logs were collected as those were the features of *P. ostreatus*. In the laboratory, the fungus was identified based on its colony morphology as well as microscopic visualizations spore print. The organisms producing smooth and white to lilac-gray colored spore print with cylindrical to long-elliptical shape were considered. The microscopic observations showed smooth, cylindrical to narrowly kidney shaped features. These colony characteristics and microscopic visualizations were similar to the genus *P. ostreatus* (OECD, 2005); hence the isolated fungus was concluded to be *P. ostreatus*. The fungus was further sub-cultured for cellulase production detection.

3.2. Screening of *P. ostreatus* for cellulase production

Screening of cellulase producing fungi was performed on CMC agar plates flooded with 0.1% Congo red and washed with 1M NaCl. Congo red has the ability to bind with cellulose and produce bright red color, if the enzyme acts on the substrate, the cellulose may be used up, so it ends with a zone of decolorization.

Depending on the diameter of the clear zone around the colony, potential fungal isolates were identified as cellulase producing fungi and its initial identification was done by fungal staining and colony morphology. The *Pleurotus ostreatus* show maximum clearance around the colony (Table 1) [22]. Out of these strains, some isolates showed hydrolyzing zones on agar plates containing CMC as core carbon source, after Congo-red staining the hydrolyzing zone diameter and colony diameter were listed & given below in Table 1.

Table 1 Clear zone of CMC hydrolysis of different isolates

Serial No	Isolate No	Colony Diameter(mm)	Zone Diameter(mm)	Zoneof hydrolysis (mm)
1	OM1	8.7	10	1.3
2	OM2	7.5	9	1.5
3	OM3	9.2	10	0.8
4	OM4	8.6	12	3.4
5	OM5	8.6	9.3	0.7
6	OM6	9	9	-
7	OM7	8.8	9.5	0.7
8	OM8	8	8	-
9	OM9	9	9.8	0.8
10	OM10	9.4	10.4	1

3.3. Cellulase Production on Different Lignocellulosic Substrates

Production of cellulase enzyme by *P. ostreatus* has grown on different lignocellulosic substrates; bean straw (BS), *Eucalyptus* bark (EB), Teff straw (TS), wheat straw (WS) and wood fiber (WF) under solid state and submerged fermentation condition was evaluated and the results are summarized and presented in Table 2. Each substrate was served as a sole nutrient source in both media form.

Table 2 Cellulase production (U/ml) by *P. ostreatus* grown on different lignocellulosic substrates under SSF and SmF

		SSF			SMF	
Substrate	**FPase**	**CMCase**	**β-glucosidase**	**FPase**	**CMCase**	**β-glucosidase**
BS	0.191	0.867	0.398	0.122	0.101	0.098
EB	0.104	0.258	0.208	0.124	0.217	0.158
TS	0.098	0.228	0.215	0.081	0.112	0.116
WF	0.106	0.114	0.158	0.083	0.094	0.088
WS	0.216	0.444	0.245	0.117	0.14	0.115

When different substrates were used in both solid-state and submerged fermentation medium, the highest enzyme production was obtained on bean straw and wheat straw which is, 0.191 U/ml, 0.867 U/ml and 0.389 U/ml, and 0.216 U/ml, 0.444 U/ml, and 0.245 U/ml of FPase, CMCase and β-glucosidase in solid-state fermentation and the lowest was obtained in media containing wood fiber, 0.106 U/ml, 0.114 U/ml and 0.158 U/ml, and 0.083 U/ml, 0.094 U/ml, and 0.088 U/ml of FPase, CMCase and β-glucosidase in solid-state and submerged fermentation, respectively (Table 2). This

indicated that bean straw and wheat straw are the most suitable substrate for cellulase production by *P. ostreatus* when compared with other study substrates.

Wheat straw was the second highest in SSF and SmF. The production of cellulase by *P.ostreatus* was higher in SSF, using different substrates as a carbon source when compared to the results obtained in SmF with the same substrates.

Based on the substrate selection criteria bean straw was not selected for further study in this experiment. Study substrates were analyzed by an economical point of view, its availability around study area and management to use it as substrate. Bean straw is more scare and needs high management because of easily perishable. Wheat straw is an inexpensive, agricultural by-product, which contains a lot of cellulose.

Therefore; further studies were conducted using wheat straw as a carbon source. *Eucalyptus* tree bark is a natural substrate for the study organism, but the study showed that it results in the lowest production of cellulase. This is due to nutrient content found in it, which the organism highly need protein nutrient to grow (why it is highest in bean straw). The production of cellulase by *P. ostreatus* was higher in SSF, using different substrates as a carbon source when compared to the results obtained in SmF with the same substrates (Table 2). A higher efficiency on enzymatic production SSF is described by several authors for various enzymes and microorganisms [12].

While in submerged fermentation (SmF), the fungus is exposed to hydrodynamic forces; in SSF growth is restricted to the surface of the solid matrix. Another factor is that the use of solid systems (SSF) provides the fungus with an environment closer to its natural habitat wood and decayed organic matter, which stimulates the fungi to produce more hemicellulolytic enzymes [6]. Since this enzyme has an ability to hydrolyze hemicellulosic substrates and function at a wide range of temperature and pH, it can be a great attraction to be utilized in industry after pretreatment of hemicellulose from agricultural wastes to fermentable sugars.

3.4. Optimization of culture conditions for cellulase production

3.4.1. Effect of incubation period on cellulase production

The rate of production of cellulase by *P. ostreatus* in shake flasks (Figure 2) shows the production of cellulase increased with the increase in the incubation period and reached a maximum after the 5th day of incubation. Further increased the incubation period, however, resulted in the gradual decrease in the production of cellulase. Therefore, the incubation period of the 5th day was found to be optimal for cellulase production by *P. ostreatus*. The optimization of the time course is of prime importance for cellulase biosynthesis by fungi [14]. The decrease in the production of cellulase by *P. ostreatus* after the 5th day of the incubation period is due to the depletion of nutrients in the medium which stressed the fungal physiology resulting in the inactivation of secretary machinery of the enzymes [23].

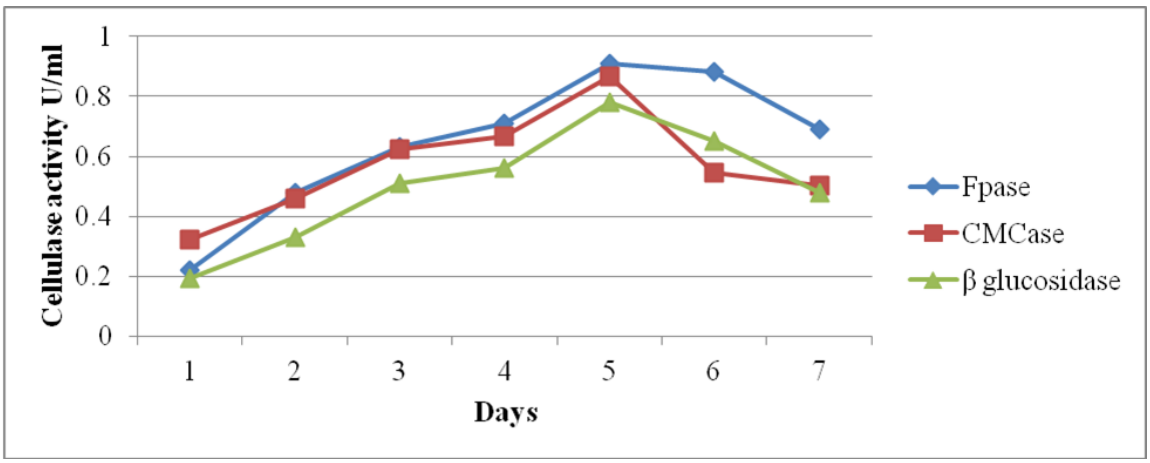

Figure 1 Effect of incubation period on cellulase production

3.4.2. Effect of pH on enzyme production

Effect of pH on enzyme production was evaluated at pH values 3, 4, 5 and 6. Cellulase yield by *P. ostreatus* appear to depend on pH value. Result illustrated by (Figure 3) clearly show that cellulase production, expressed as enzyme activity, gradually increased as the pH value increased from 3 to 4 reached its maximum at pH of 4 which is (0.844 U/ml), (0.72 U/ml) and (0.621 U/ml) of FPase, CMCase, and β-glucosidase. The enzyme production was favored in acidic

range of pH 4 to 6 and result of the present study were considerably similar to the results reported by where *Trichoderma reesei* was shown to produce maximum cellulase at a pH of 4. The effect of pH on cellulase production by these fungi was found to be in agreement with the findings of [18] who reported that CMCase, Avicelase and FPase activities exhibited an optimum pH approximately 4, while the pH optimum for β glucosidase production was between pH 5 and 6.

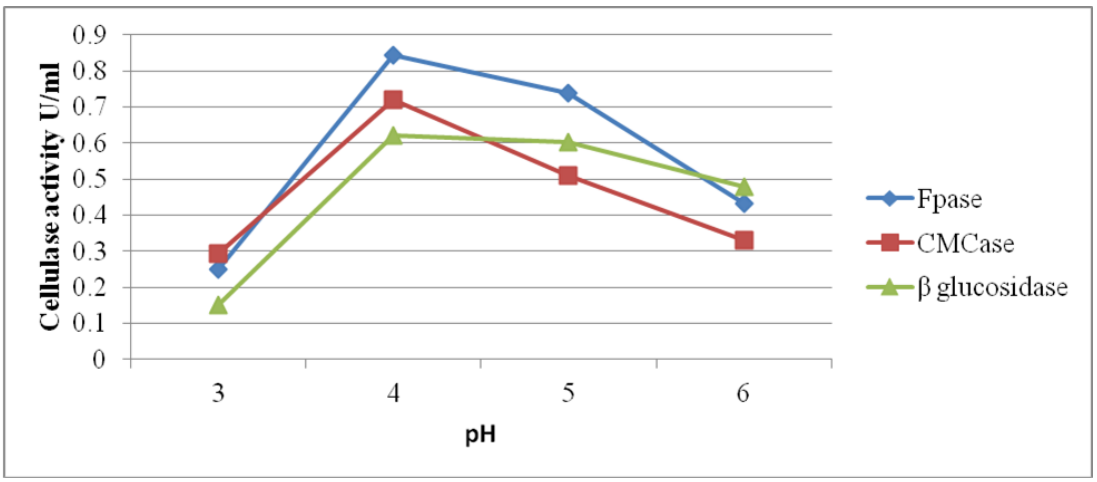

Figure 2 Effect of pH on cellulase production under SSF

3.4.3. Effect of temperature on cellulase production

Incubation temperature plays an important role in the metabolic activities of a microorganism. Even slight changes in temperature can affect enzyme production. Since enzyme is a primary metabolite produced during exponential growth phase, the incubation at high temperature could lead to poor growth and thus a reduction in enzyme yield. The effect of temperature on cellulase activity was determined by incubating the culture at different temperature, i.e. 20°C 25°C, 30°C and 35°C. The results showed that the optimum temperature for the production of cellulase, i.e. FPase (0.542 U/ml), CMCase (0.46 U/ml) and β glucosidase (0.38 U/ml) by *P. ostreatus* at 30°C**.** This result was, however, different from the findings of [10] who reported that the optimum temperature for cellulase production by *A. niger & A. fumigatus* was 40°C. The optimum temperature for cellulase production depends on the strain variation of the microorganism. The present result is probably different from that of [10] because of differences in the studied fungal species.

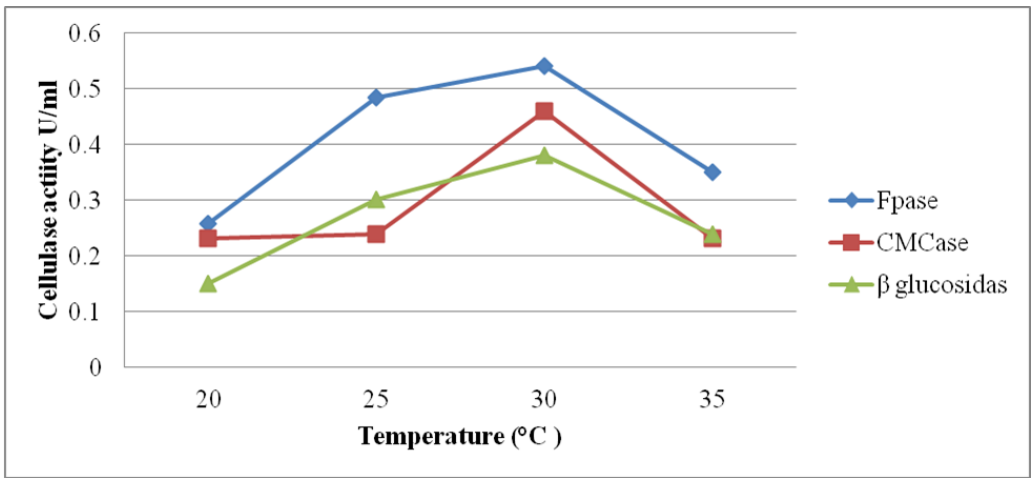

Figure 3 Effect of temperature on cellulase production under SSF

3.4.4. Effect of carbon source on cellulase production

The production of cellulase is a key factor in the hydrolysis of cellulosic material and is essential to make the process economically viable. Cellulase production by *P. ostreatus* increased with increase in initial sugar concentration from 0.5% to 1.0%. Regardless of the type of carbon source utilized, highest cellulase production was obtained at 1% concentration of sugars tested. With further increase in sugar concentration, reduction in enzyme activity was observed

when compared with control [20] also reported the maximum yield of cellulase at 1% concentration with different carbon sources in *Trichoderma viride*. Sucrose was the most effective carbon source for cellulase production, i.e. for FPase (2.67 U/ml), CMCase (2.16 U/ml) and β-glucosidase (2.05 U/ml) production followed by glucose and lactose. Same carbon sources have been optimized by different workers for cellulase production [28].

Table 3 Effect of carbon source on cellulase production under SSF (±SE)

Carbon source	Concentration (%)	FPase	CMCase	β- glucosidase
	0	0.32±0.03	0.38±0.04	0.41±0.04
Glucose	0.5	0.68±0.04	0.48±0.03	0.42±0.02
	1	2.32±0.08	1.92±0.07	1.90±0.09
	2	0.96±0.05	0.97±0.04	0.92±0.05
	2.5	0.63±0.04	0.73±0.04	0.54±0.04
	0	0.41±0.03	0.31±0.04	0.41±0.05
Lactose	0.5	0.43±0.05	0.34±0.04	0.47±0.05
	1	1.86±0.08	1.92±0.07	1.89±0.08
	2	1.27±0.06	0.86±0.05	1.62±0.05
	2.5	0.82±0.05	0.52±0.04	1.11±0.04
	0	0.26±0.02	0.34±0.03	0.37±0.03
Sucrose	0.5	0.68±0.05	0.51±0.06	0.41±0.04
	1	2.67±0.07	2.16±0.07	2.05±0.08
	2	1.56±0.05	0.87±0.05	0.91±0.06
	2.5	0.84±0.04	0.64±0.04	0.73±0.05

3.4.5. Effect of nitrogen source on enzyme production

Results indicated that the source of nitrogen greatly affected the production of cellulase. The microorganisms which have an important industrial application can utilize inorganic and organic nitrogen sources.

Table 4 Effect of nitrogen source on cellulase production under SSF (±SE)

Nitrogen sources	Concentration (%)	FPase	CMCase	β- glucosidase
	0	0.24±0.04	0.19±0.03	0.15±0.03
Yeast Extract	0.5	0.32±0.03	0.31±0.04	0.31±0.02
	1	2.21±0.07	1.93±0.07	1.94±0.05
	2	1.52±0.07	0.83±0.05	0.89±0.04
	2.5	0.92±0.07	0.63±0.05	0.44±0.03
	0	0.39±0.05	0.22±0.03	0.31±0.03
Beef Extract	0.5	0.72±0.05	0.31±0.04	0.37±0.03
	1	2.11±0.07	1.92±0.06	1.94±0.06
	2	1.52±0.07	0.83±0.05	0.92±0.06
	2.5	0.92±0.07	0.63±0.05	0.46±0.05
	0	0.21±0.03	0.16±0.06	0.11±0.03
Ammonium sulfate	0.5	0.23±0.03	0.18±0.03	0.14±0.02
	1	1.72±0.05	1.62±0.06	1.68±0.05
	2	0.88±0.04	0.83±0.04	0.87±0.05
	2.5	0.62±0.03	0.68±0.05	0.28±0.02

Data revealed that the supplementation of the production medium by organic and inorganic nitrogen sources (i.e. beef extract, yeast extract and ammonium sulfate) ranging in concentrations from 0.5 to 2.5 % stimulated the cellulase yield and activity. Evidently, the data (Table 4) indicated that *P. ostreatus* exhibited maximum cellulase activity at 1% concentration of different nitrogen sources. Yeast extract proved to be the best nitrogen source for cellulase production [FPase (2.21 U/ml), CMCase (1.93 U/ml) and β-glucosidase (1.91 U/ml)] followed by Beef extract and ammonium sulfate. Amongst different nitrogen sources tested, growth was boosted in the presence of organic nitrogen source when compared to control [37].

3.5. Characterization of Cellulase

3.5.1. Effects of pH on activity of cellulose

In order to determine the optimum working pH for cellulase enzyme, the crude extract was first harvested and incubated at 50°C within different pH range from 4-8 for 15 minutes. In this study as the pH value increased, cellulase activity also increased and its highest activity was observed at pH 4 to 5 (Figure 5). The maximum activity for CMCase and β-glucosidase was observed at pH 4, while for FPase the activity was highest at a pH of 5. This supports the findings of [18] who reported that CMCase, Avicelase and FPase activities exhibit a pH optimum of 5 and 6. The instability of these enzymes at very low or very high pH values is due to the fact that they are proteins that are generally denatured at extreme pH values.

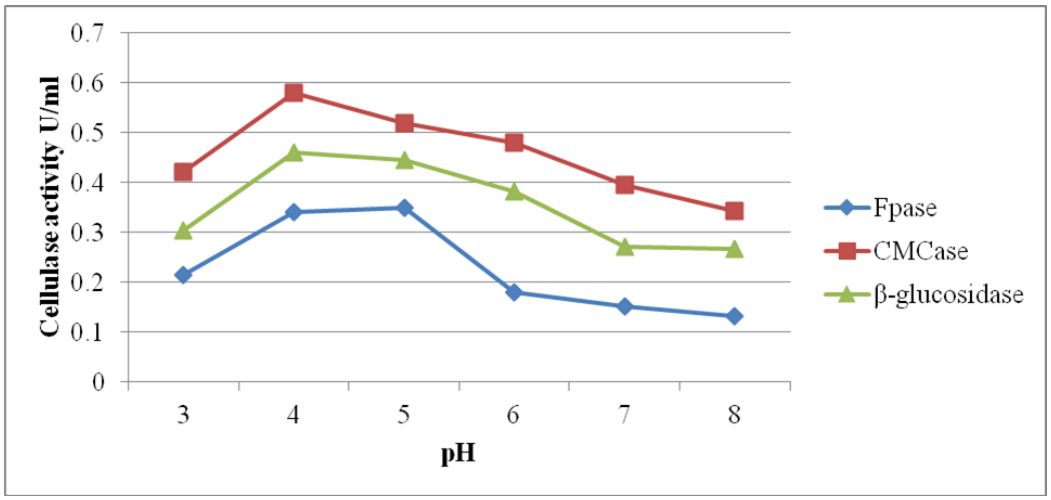

Figure 4 Effect of pH on cellulase activity

The result is in agreement with that of [1] who indicated that enzyme activity has a bell shaped profile with an optimum pH. The report also showed that the optimum pH for cellulase activity was 4.0. The dependence of cellulase activity on pH usually renders a bell-shaped profile.

3.5.2. Effect of temperature on activity of cellulose

The optimum temperature for activity of the cellulases was determined at temperatures ranging between 40 and 80°C. The results showed that as temperature increases cellulase activity also increases up to a certain level of temperature. The highest activity of cellulase (FPase, CMCase, and β-glucosidase) was observed in the range of 40-60°C and the maximum activity was observed at 50°C. However, a further increase in temperature resulted in a decrease in cellulase activity. The least activity was observed at 80°C (Figure 6). Temperatures above 60°C generally result in the loss of the moisture content of the substrate which affects metabolic activities, reduces growth and enzyme production by microorganisms. Due to high temperature, the growth of fungal isolates was greatly inhibited and hence, enzyme formation was seriously hampered.

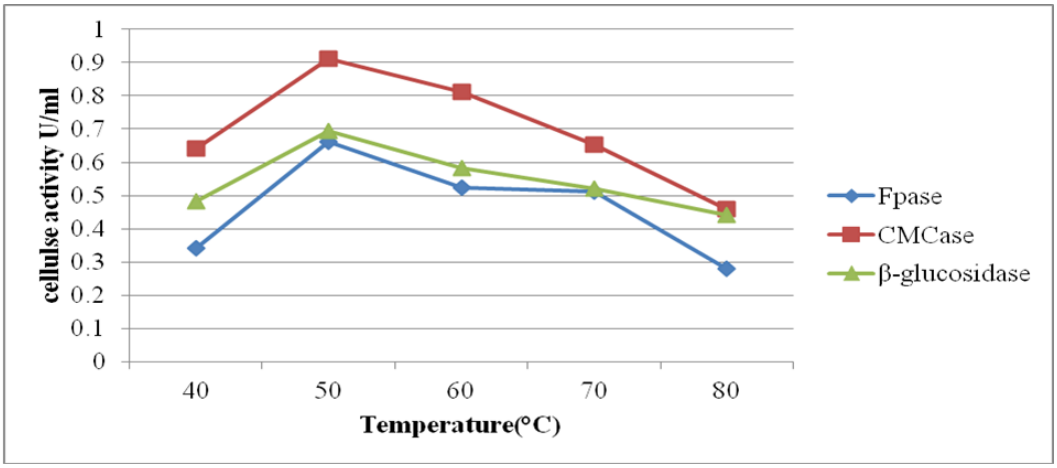

Figure 5 Effect of Temperature (°C) on cellulase activity

3.5.3. Effect of substrate concentration on cellulase activity

The increased in the enzyme activity at substrate concentration ranging from 0.1% to 3% of the CMC suggests the ability to produce more of the cellulase at an optimal substrate concentration of 2.5% as shown in (Figure 7). Since the substrates contain different minerals apart from carbon, which may serve as nutrient supplements, an increase in substrate concentration leads to an increase in these nutrients that may adversely affect the cell concentration. The increased in enzyme production until the optimum was obtained due to the availability of cellulose in the medium and decreased in production beyond optimum concentration is as a result of an inhibiting effect of accumulated cellobiose and cellodextrin of low degree of the polymerization of the growth medium [9] further stated that it might also be due to specific binding of the enzyme with substrates.

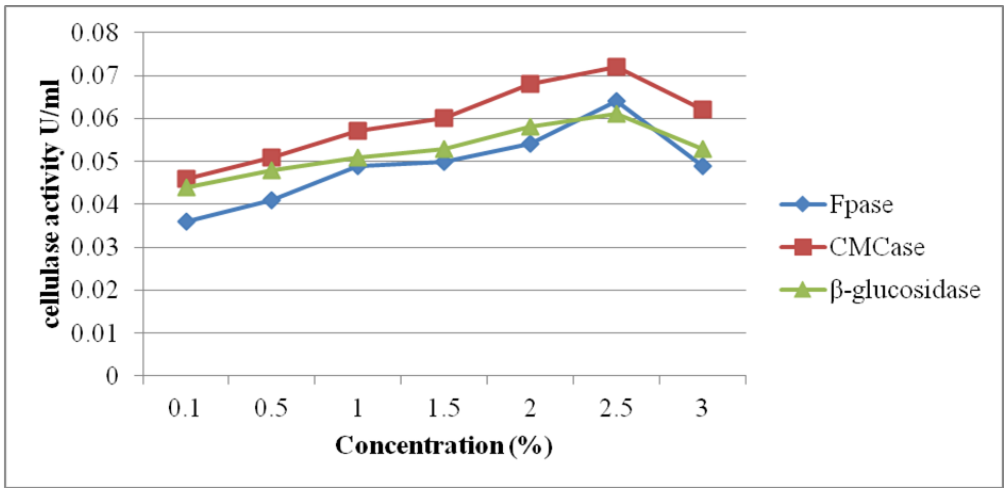

Figure 6 Effect of substrate concentration on cellulase production

3.6. The extent of saccharification of lignocellulosics by *P. ostreatus* cellulose

Saccharification of wheat straw, *Eucalyptus* bark and Teff Straw by the cellulase of *P. ostreatus* was evaluated. The bioconversion efficiency was assessed via quantification of released monosaccharides in 24hrs. The maximum saccharification by crude cellulase was recorded from wheat straw, followed by *Eucalyptus* bark and Teff straw when compared with control.

Table 5 Saccharification of lignocellulosic substrates by *P. ostreatus* cellulose

Saccharification (%)			
Substrate	**FPase**	**CMCase**	**β- glucosidases**
Wheat Straw	17.7	20.2	18.1
Teff Straw	13.5	14.6	13.9
E.Bark	14.6	15.5	15.1
Control	1	1.02	0.993

The culture supernatant crude enzyme contained the complete cellulose degrading system for hydrolysis of cellulose to glucose produced by the fungus. β- glucosidases hydrolyze cellobiose to glucose in order to eliminate cellobiose inhibition and it is not only required for more complete hydrolysis of cellulose but also stimulates the overall cellulolytic reaction in a synergistic manner. Cellulase enzyme systems are not merely an agglomeration of enzymes representing the three enzymes, but rather act in a coordinated manner to efficiently hydrolyze cellulose into glucose [19].

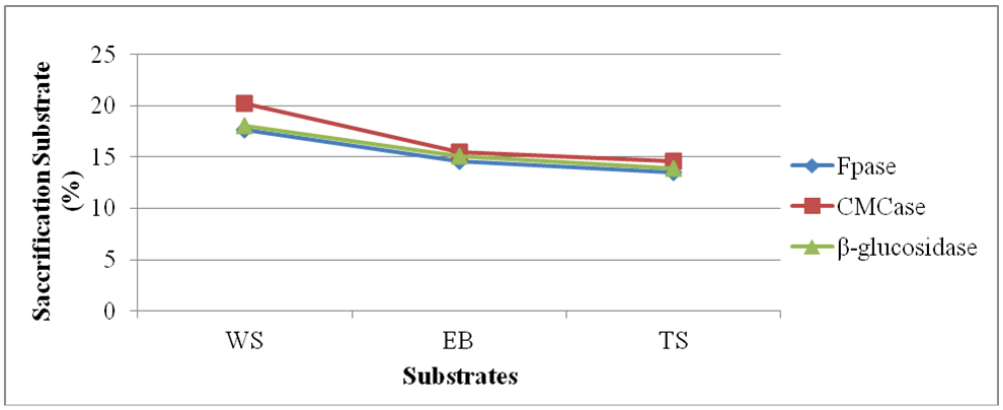

Figure 7 Saccharification of lignocellulosic substrates by cellulase

Enzymatic conversion of cellulose to food, fuel and chemical feedstock is a well-established process. The enzymatic conversion of the carbohydrate part of the lignocellulosic material has received considerable interest during recent years. This source of raw material is available in abundance and generally free of cost. This could be converted into fermentable sugars. To reduce the production cost and enhance the formation of cellulases, which are both essential for the utilization of the carbohydrate components of lignocellulosic, different strategies can be applied. Lignocellulosic waste of wheat straw, teff straw and *Eucalyptus* bark left over otherwise for natural degradation in the field were effectively used as a component in the medium for the production of enzymes [3].

Subsequently, these enzymes produced on the medium containing wheat agro-waste can be further implicated in the saccharification of the same agro-waste. *P. ostreatus* synthesized cellulases were used for saccharification of wheat straw, *Eucalyptus* bark, and teff straw. The cellulolytic enzyme complex when incubated with the agro-waste released sugars. The degree of saccharification was assayed on the basis of the release of the reducing group. The saccharification of wheat straw by an enzyme produced by *P. ostreatus* indicates the specificity of the enzyme towards the substrates. Enzymes synthesized on wheat straw medium released more glucose. This can be attributed to the other enzymes like ligninase and hemicellulases in the agro-waste medium, synthesized along with cellulases.

4. Conclusion

This study was conducted to screen cellulase producing oyster mushroom species collected from *Eucalyptus* tree bark in Holetta agricultural Research center, to evaluate the *in vitro* production of cellulase by *P. ostreatus* using different lignocellulosic substrates, and to produce characterize and evaluate the effective degradation of cellulose by cellulase produced from *P. ostreatus* with respect to changes in pH, temperature, and concentration of substrates. A total of 10 mushroom specimens were randomly collected from *Eucalyptus* tree bark in the premise of Holetta Agricultural

Research Center campus. The collected mushroom specimens were identified morphologically and biochemically as *P. ostreatus* and also screened for cellulase production depend on the clear zone formation on carboxymethylcellulose containing cellulose as a sole carbon source.

Screened mushroom specimen were cultivated using both solid state and submerged fermentation systems supplemented with different substrates (wheat straw, teff straw, bean straw, wood fiber and *Eucalyptus* tree bark) to identify the most suitable medium for the production of cellulase. The highest enzyme production was obtained on bean straw and wheat straw in solid-state fermentation and the lowest was obtained in media containing wood fiber in solid-state fermentation and submerged fermentation, respectively. Production of cellulase by study organism was better in SSF system than SMF system and among tested lignocellulosic substrates; Wheat Straw was selected for further growth parameter optimization.

Crude cellulase extract was harvested by sodium acetate buffer (pH 4.8) and centrifuged at 10000 rpm at 4°C for 10 minutes and later on, the clear supernatant was collected through decantation which was used for cellulase activity determination. The production of cellulase was higher at the 5th day of the incubation period, and the maximal pH and incubation temperature were 4 and 30°C, respectively. Sucrose and Yeast extract at 1% concentration was found to be the most preferred carbon and nitrogen source for cellulase production by *P. ostreatus*. The optimum working pH and temperature of cellulase were found to be 4 and 30°C, respectively. Generally, cellulases are stable and active in a temperature range of 20-50°C. These characteristics hopefully would make this enzyme potentially attractive in a variety of industrial applications like animal feed treatments. There was a linear relation between cellulase and its substrate concentration; there is an increase in activity with increasing substrate concentration. The relationship between the rate of reaction and substrate concentration depends on the affinity of the enzyme for its substrate. *P. ostreatus* synthesized cellulases were used for saccharification of wheat straw, *Eucalyptus* bark, and Teff straw. The cellulolytic enzyme complex when incubated with the agro-waste released sugars.

Recommendations

- Further study is recommended at a molecular level to enhance the production of cellulase from P. ostreatus for animal feed processing and other possible industrial application.
- This study is also strongly recommended that researches have to be done on large scale production, further purification and characterization of cellulase from P. ostreatus.
- Optimization of P. ostreatus cellulase production would be done, to be used in industrial level.
- Further study should be made to optimize the potential of P. ostreatus for production of other ligninolytic enzymes.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare that they have no competing interests

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