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Qualitative Assays and Quantitative Determinations of Cellulolytic Enzymes of Wood Rot Fungi

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Abstract: Cellulolytic enzymes are a group of hydrolytic enzymes capable of hydrolyzing cellulose to sugars. The enzymes have enormous potential in food, beverages, textile, paper and pulp industries. In this study, wood rot fungi were screened for cellulolytic activities by inoculating on carboxymethylcellulose (CMC) as solid media. The degradation of the CMC substrate was expressed through the formation of clear zone around the colony of the test fungus. Thus the clear zones were measured and enzymatic indexes (EI) were calculated. Cellulolytic enzymes were further quantified both in submerged fermentation (SmF) and solid state fermentation (SSF) experimental procedures for the efficient fungal isolates. Isolates *Phellinus tremulae* 030-1D, *Pholiota adipose* 026-2D and *Armilleria mellea* 033-1G showed significantly wider clear zones than other tested fungal isolates. The fungal isolates also differently responded to the incubation period both under SmF and SSF conditions and SSF yielded more enzyme amounts. The highest CMCase activity (15.81±0.98 U/ml) was obtained from isolate 033-1G on 12th day and followed by the CMCase activities from isolate 033-1G on the 15th day and from isolate 026-2D on the 12th day. From the obtained results showed that 45°C and pH 5 were optimum for the activities of CMCases. It was observed that optimization of the culture conditions at different growth conditions and media supplementations improved enzyme yields indicating that the isolates could be used for hydrolysis of lignocellulosic substrates to sugars which could be used for different industrial applications.

Key words: Cellulolytic Enzymes · CMCase · Wood Rot Fungi · Lignocellulose

INTRODUCTION

Lignocellulosic materials include agricultural wastes, forestry residues, grasses and woody materials. Most lignocellulosic biomass comprised of about 10-25% lignin, 20-30% hemicellulose and 40-50% cellulose [1, 2]. Conversion of the recalcitrant lignocelluloses to fermentable sugars requires three sequential steps: size reduction, pretreatment and enzymatic hydrolysis [3].

Wood rot basidiomycete fungi can decompose all major components of lignocellulosic materials due to their oxidative and hydrolytic enzymes they produce [4]. Cellulose is commonly degraded by cellulolytic enzymes of wood rot fungi. Its hydrolysis is completed by the synergistic action of endo- and exo-glucanases [5]. Recent studies have shown that hydrolytic enzymes can be produced using lignocellulosic materials both in SmF (submerged fermentation) and SSF (solid state fermentation) [6].

Environmental and physical factors rule the microbial extracellular enzyme secretions [7]. Culture conditions such as incubation period, temperature, pH and type and concentrations of culture media supplementations highly influence the growth and the enzyme productions by wood rot fungi. Variation in the enzyme activity has also been accounted for microbial strain variation [8]. The objective of the current study was therefore to assess the cellulolytic activities of wood rotting basidiomycte fungi collected from Dagaga and Gambo forests of the Arsi forest and wildlife enterprise and optimize their production.

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MATERIALS AND METHODS

Qualitative Assays of Cellulolytic Wood Rot Fungi: In order to assess the cellulolytic activities of wood rotting fungal isolates, agar plates containing 2.5 g carboxy methylcellulose (CMC), 5.0 g yeast extract, 0.2 g K_2HPO_4 and 20.0 g agar in a liter of distilled water was prepared [9]. For the purpose of the study 56 fungal species were used. A 5 mm disc from 5 days old culture was inoculated at the center of the sterile plates. The plates were incubated at 28±1°C for 7 days and flooded with Congo red stain (0.1% w/v) for 15 minutes undisturbed. Then, the stain was poured off, washed with distilled water and de-stained by flooding the plates with 1M NaCl solution for another 15 minutes. The formation of a clear zone around the fungal colonies was used as an indication of the hydrolysis of CMC. Thus, for each of the plates, to determine the enzyme index (EI), the diameter of the clear zone and the diameter of the colony were measured and EI was calculated using the equation below [10, 11].

$$EI = \frac{Diameter\ of\ hydrolysis\ zone}{Diameter\ of\ colony}$$

The cellulolytic fungi with EI greater than 1.5 were considered efficient and selected for quantitative determination of cellulases.

Quantitative Determination of Cellulolytic Enzymes: noculum preparation: Inocula of the cellulolytic fungi (Table 1) were prepared following the procedures described in Altaf *et al*. [12]. The medium contained 10.0 g glucose, 3.0 g yeast extracts, 3.0 g peptone, 1.0 KH_2PO_4 and 0.5 g $MgSO_4.7H_2O$ in a liter of distilled water and its pH was adjusted to 6.0 with 2M NaOH and HCl. 100 ml of the medium was added to each 250 ml flasks and autoclaved at 121°C for 15 minutes. After cooling, four disks (5 mm diameter each) from each isolate were inoculated and grown on a rotary shaker at 150 rpm at room temperature. After six days of fungal growth, mycelial pellets were harvested, homogenized and used as inocula for the SmF and SSF experiments.

Submerged Fermentation (SmF): SmF of the test fungi was carried out using a modified czapek dox liquid medium containing 10.0 g/l CMC, 3.0 g $NaNO_3$, 0.5 g KCl, 1.0 g KH_2PO_4, 0.5 g $MnSO_4.7H_2O$, 0.01 g $FeSO_4.7H_2O$ per liter of distilled water [13]. Each 250 ml flask containing 100 ml of sterile media was inoculated with 3.0 ml fungal inoculum and incubated at room temperature and 150 rpm. At exactly five, eight and twelve days of growth, the culture contents were filtered using nylon cloth and then centrifuged at 4000 rpm for 15 minutes. The cell free culture supernatant from each isolates was used as crude cellulolytic enzyme.

Solid State Fermentation (SSF): 10.0 gram CMC was moistened with 12 ml of the czapek dox liquid medium [13] in 250 ml flasks. Each sterile flask was inoculated with 3 ml of mycelial homogenate and incubated at room temperature. Exactly after seven, twelve and fifteen days of incubation, the extracellular enzymes were extracted from the whole biomass twice with 25 ml of distilled water (total volume 50 ml). The solids were separated by filtration through nylon cloth and centrifuged at 4000 rpm for 15 minutes. The cell free culture supernatant from each isolate was used as crude cellulolytic enzyme.

Determination of FPase Activity: FPase (total cellulase) activity was determined as described by Gadgil *et al*. [14]. One ml of the crude enzyme supernatant was added to 2 ml of 0.5 M sodium acetate buffer (pH 5.0) in a test tube. A size of 1 cm by 6 cm Whatman No. 1 filter paper (approximately weighing 50 mg) was cut and inserted into the test tube and then incubated. Enzyme blanks (without enzyme) were run simultaneously in the same manner and incubated for one hour at 50°C.

Determination of Carboxy Methylcellulase Activity: Carboxy methylcellulase (CMCase) (endoglucanase) activity was determined according to Wang *et al.* [15]. Test tube containing one ml of the crude enzyme extract and 2 ml of 1% CMC (prepared in 0.5 M sodium acetate buffer solution of pH 5.0) were incubated for 10 minutes at 50°C. Control tubes were made by adding one ml of 1% CMC solution and 2 ml of distilled water and incubated under the same condition.

Resulting sugars were quantified according to the Miller's modified method of DNS [5]. Absorbance of the mixtures was measured at 540 nm by using spectrophotometer (Jenway Model 6305, UK). A standard curve was constructed using known solution of glucose to determine the released reducing sugars. Enzyme activities were calculated according to Firmani *et al*. [16] using the following equation.

$$Enzyme\ activity\ = \frac{[G]}{Mol.Wt.G} x \frac{v}{pxq} df$$

where [G] = Glucose concentration (g/l), Mol. Wt.G = Molecular weight of glucose (g/mol), v = total volume of sample in each tube experiments (ml), p = volume of enzyme (ml), q = incubation time (minutes), df = dilution factor. One CMCase unit (U) was defined as the amount of enzyme that released one ìmol of reducing sugar in one minute under the assay conditions.

Characterization of Crude CMCase: To determine the optimum temperature for CMCase activity, one ml CMCase extract obtained from 12th day SmF growth in CMC supplemented medium was incubated with 2 ml of 1% CMC in 5.0 pH of Na-acetate solution for 10 min at 35-60°C (at 5°C intervals). Heat stability studies of the crude CMCase were performed by pre-incubating the crude enzymes in 0.05 M Na-acetate buffer (pH 5.0) at different temperatures (35-60°C) for 120 minutes. Finally, the released reducing sugar was measured and CMCase activity was determined.

The pH of 1% CMC reaction mixtures were adjusted using Na-acetate buffer solution (pH 3.0-5.0) and sodium phosphate buffer solution (pH 6.0-8.0). pH optima experiments were conducted by incubating CMC-enzyme mixture at different pH for 10 minutes. For the pH stability experiments, the crude extracts in buffers were pre-incubated under different pH initial conditions (3.0-8.0) for 120 minutes at 30°C. After 120 minutes of crude enzyme incubation. Finally, the released reducing sugar was determined and CMCase activity was determined.

Optimization of Culture Conditions for CMCase Productions: Effect of incubation temperatures on CMCase production was determined by SmF using modified czapek dox liquid medium at 5°C intervals in the range of 20-40°C. Effect of incubation pH on CMCase production was determined by SmF on czapek dox liquid medium at different pH levels ranging from 3.0-8.0. After 12 days of SmF incubation, the cell free extracts from each flask were analyzed for CMCase activity.

Effect of different carbon sources on CMCase production was determined by SmF on czapek dox liquid medium supplemented with 1% glucose, xylose, sucrose, CMC or xylan. Effect of nitrogen sources on CMCase productions was determined by supplementing 1% malt extract, yeast extract, peptone, $NaNO_3$ or NH_4NO_3 in to the medium. 0.05% metallic ions ($CaCl_2$, $CuSO_4$, $MgSO_4$, $FeSO_4$, $MnSO_4$ or $ZnSO_4$) were supplemented in the medium for determination of effects of ions on CMcase production. The test fungi in all cases were incubated for 12 days at room temperature and then the cell free extracts from each flask were analyzed for CMCase activity.

Statistical Analysis: All experiments were performed in triplicates. The means of three replicate values for all quantitative data in the experiments obtained were tested in a one way ANOVA at P = 0.05 using SPSS software and Tukey's test was used to evaluate mean differences between treatments.

RESULTS AND DISCUSSION

Qualitative Assays of the Cellulolytic Fungal Isolates: Of the 56 wood rotting fungal isolates qualitatively screened on CMC supplemented agar media, many showed positive cellulolytic activities with different enzymatic indexes and results of ten isolates are presented (Table 1). Isolates 033-1G, 026-2D and 030-1D exhibited the highest EI range of 3.5-4.5. Furthermore, isolates 027-1D, 029-2D and 004-2G expressed enzyme activity of EI ranging between 2.5-3.5 while the remaining fungal isolates displayed less than 2.5 EI activities. The three aforementioned isolates displayed higher enzyme indexes compared to the 40 isolates screened by Duncan *et al*. [17] indicating that probably the fungal isolates secreted higher amount of cellulolytic enzymes. Similarly, different authors indicated that qualitative screening of cellulolytic fungi on solid media is a good method to estimate the secreting potential of [18, 19].

Quantitative Determination of Cellulolytic Activities

Determination of Cellulolytic Activities in SmF: All tested isolates, except 027-1D and 033-1G, displayed their respective highest CMCase activities on 12th day of incubation (Fig. 1). But isolate 033-1G displayed its highest CMCase activity under the three incubation periods of SmF compared to other isolates but its own CMCase activity was peaked (15.68±0.16 U/ml) on 8th day of incubation. The next two highest CMCase activities were displayed on 12th day of incubation by isolates 026-2D (14.86±0.16 U/ml) and 030-1D (12.75±0.11 U/ml). On the other hand, isolate 030-1D displayed the highest FPase activity (7.83±0.09 U/ml) on the 12th day of

Table 1: Enzymatic indexes of some of the qualitatively screened cellulolytic fungi

No	Isolate code	Fungal species	CMCase activity scale
1	011-1D	*Trametes gibbosa*	++
2	022(b)-1D	*Gymnopus eucalyptorum*	++
3	027-1D	*Bjerkandera adusta*	+++
4	030-1D	*Phellinus tremulae*	++++
5	026-2D	*Pholiota adipose*	++++
6	029-2D	*Lenzites betulina*	+++
7	033-1G	*Armilleria mellea*	++++
8	042-1G	*Stereum rugosum*	++
9	092-1G	*Polyporus cinnabarinus*	++
10	004-2G	*Lentinellus cochleatus*	+++

[a] EI calculated on the 7th day of growth: + = EI<1.5, ++ = 1.5<EI<2.5, +++ = 2.5<EI<3.5, ++++ = 3.5<EI<4.5

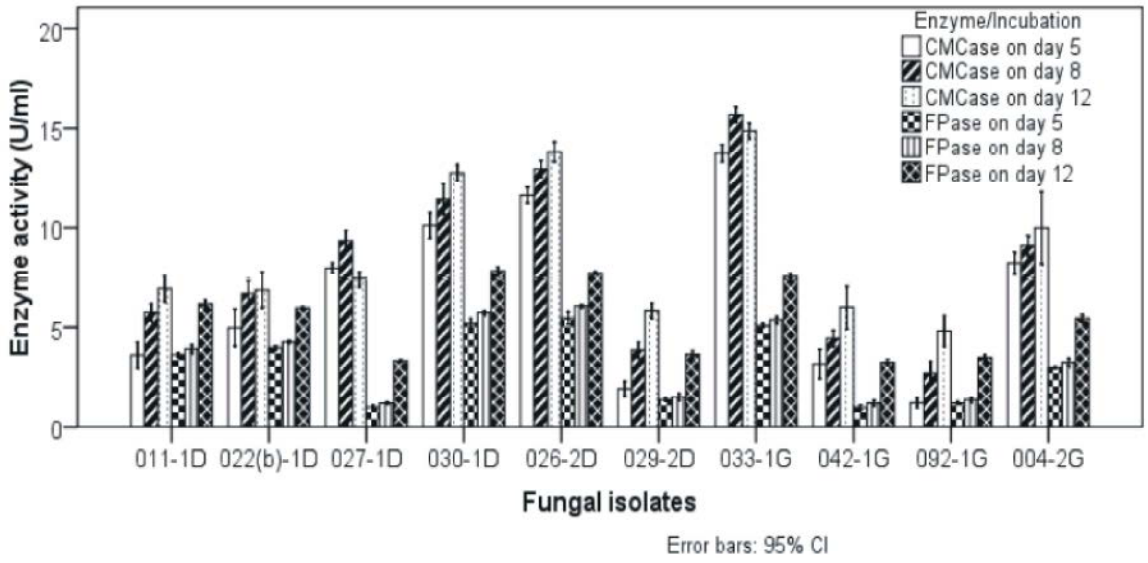

Fig. 1: CMCase and FPase activities of the fungal isolates incubated for 5-12 days in SmF

incubation which was followed by the FPase activity of isolate 026-2D (7.73±0.06 U/ml) on the same day of incubation. Elisashvili *et al.* [20] cultivated *P. ostreatus* and *Trametes hirsute* fungi on mandarin peelings in SmF and reported CMCase activities of 12.0 U/ml and 11.0 U/ml, respectively, after 10 days. Some isolates of this research took relatively longer days but displayed higher CMCase activities. Elisashvili *et al*. [21] also reported lower CMCase (9.8 U/ml) and FPase (3.0 U/ml) activities by cultivating *Pleurotus tuberregium* on tree leaves after 8 days of incubation in SmF. On the other hand, Jadhav *et al*. [5] incubated lower fungus, *Aspergillus niger*, on wheat bran in SmF and reported CMCase and FPase activities of 8.9 U/ml and 12.4 U/ml, respectively on 5th day.

Determination of Cellulolytic Activities in SSF: The results revealed that, like in the SmF, the fungal isolates responded differently to the different incubation periods under the SSF too. Isolates 033-1G, 030-1D, 026-2D, 022(b)-1D and 004-2G displayed their respective highest CMCase activities on the 12th day of SSF and the remaining five fungal isolates displayed their highest CMCase productions on the 15th day (Fig. 2). The highest CMCase activity (15.81±0.98 U/ml) was obtained from isolate 033-1G. The next two highest CMCase activities were recorded for isolate 033-1G on the 15th day and isolate 026-2D on the 12th day of incubation. All isolates except isolates 022(b)-1D and 026-2D displayed their highest FPase activities on the 15th day (Fig. 3). The highest FPase activity was exhibited by isolate 026-2D (7.98±0.04 U/ml) on the 12th day and followed by those of isolates 030-1D (7.83±0.09 U/ml) and 026-2D on the 15th day of incubations. In general, three isolates (033-1G, 026-2D and 030-1D) exhibited significantly higher CMCase and FPase activities than the remaining tested fungal isolates. Similar CMCase activities of 16.0 U/ml and 10.0 U/ml were reported by Elisashvili *et al.* [20] by cultivating

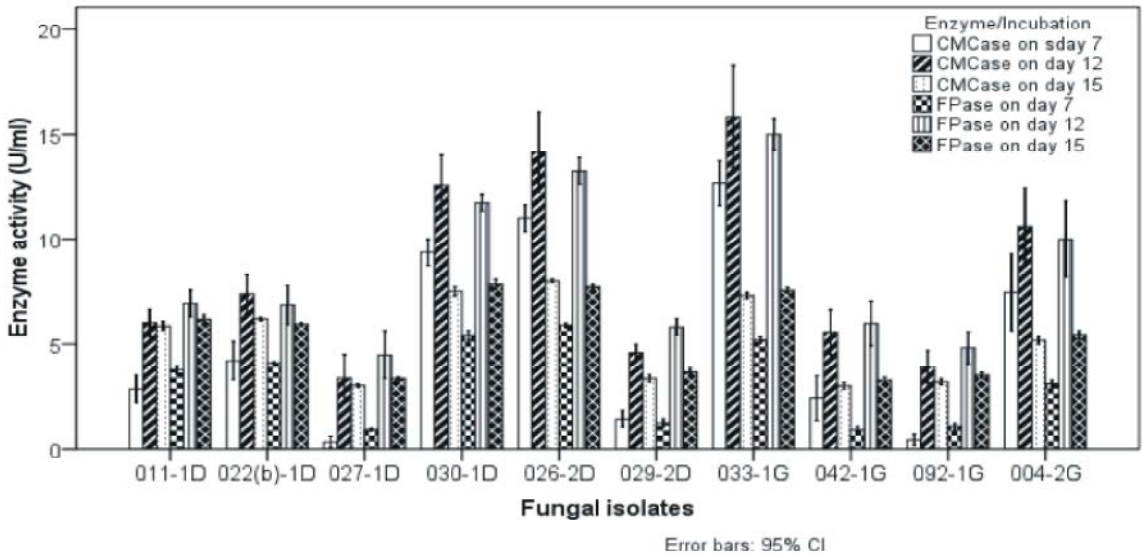

Fig. 2: CMCase and FPase activities of the fungal isolates incubated for 7-15 days in SSF

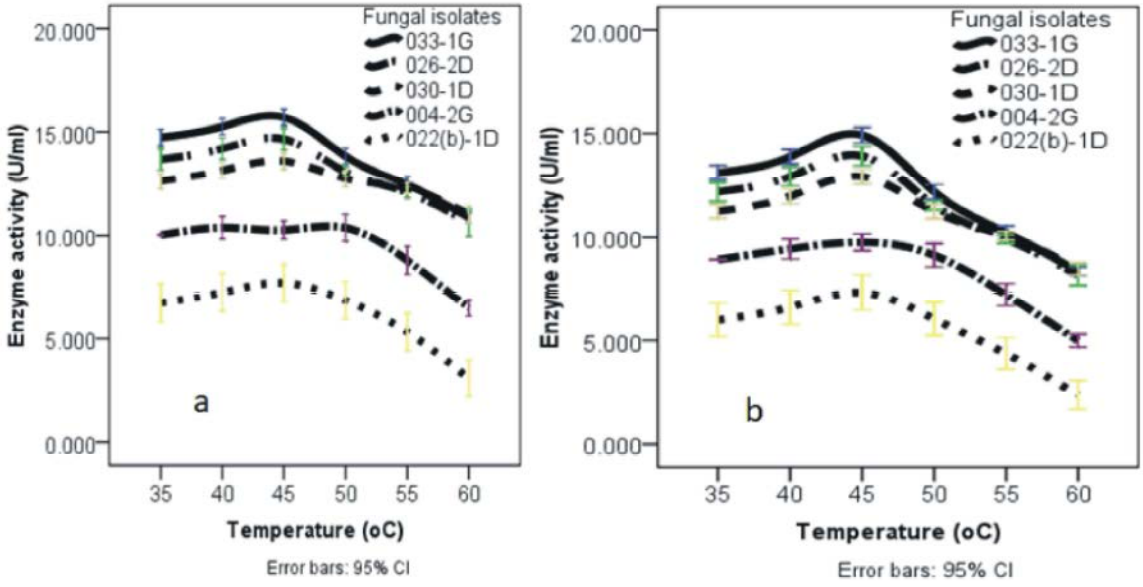

Fig. 3: Temperature optima (a) and stabilities (b) of the CMCase of the wood rot fungal isolates

T. hirsute and *T. ochracea*, respectively, on mandarin peelings in SSF on 14th day. On other hand, Elisashvili *et al.* [21] reported lower CMCase (3.0 U/ml) and FPase (1.7 U/ml) activities by cultivating *P. tuberregium* on tree leaves on 10th day of incubation in SSF. It could be safely concluded that the amount of cellulolytic enzyme secretion depends on the inherent characteristic of a given wood rot fungus and each fungus needs its own incubation day for displaying its highest enzyme activity.

Characterization of Crude CMCase Extracts: Temperature optima and stabilities of CMCase: The highest CMCase activity was obtained from isolate 033-1G (15.71±0.16 U/ml) at 45°C and followed by CMCase activity of 14.65±0.21 U/ml from isolate 026-2D at 45°C and of 13.84±0.16 U/ml from isolate 033-1G at 50°C (Fig. 3a). The temperature range of 40-50°C was found optimum for CMCase activities of all fungal isolates and the activities before and after this range declined. It was also observed that isolate 033-1G was the most stable isolate at 45°C

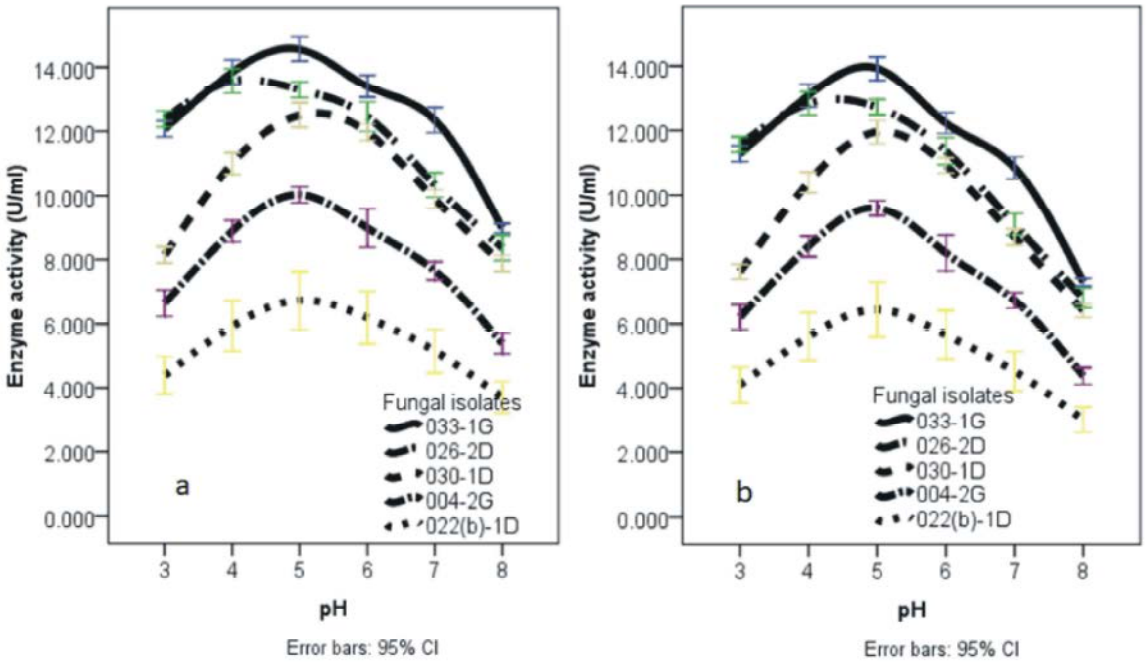

Fig. 4: pH optima (a) and stabilities (b) of the CMCase of the wood rot fungal isolates

exhibiting CMCase activity of 14.92±0.15. This was followed by the CMCase activities of 13.92±0.19 and 12.90±0.14 both at 45°C from isolates 026-2D and 030-1D, respectively. The enzymes were found sensitive to temperature stability tests and declining activities were observed in all tested fungal isolates (Fig. 3b). An enzyme activity beyond its optimum and stable temperature decreases because the temperature denatures the enzyme [22]. Similarly, Talekar *et al*. [23] reported 40ºC as optimum temperature for Cellulase from *Tribolium castaneum*. The authors also reported a decrease of thermostability beyond 40ºC. Wood rot fungal isolates tested in this research showed more tolerance to the increasing incubation temperatures. On the contrary, Bilal *et al.* [24] reported lower optimum temperature (28°C) for CMCase activity (4.5 U/ml) from *Trichoderma viride* after which the activity began to drop.

pH Optima and Stability of CMCase: Isolate 033-1G exhibited the highest CMCase activity (14.56±0.19 U/ml) at pH 5.0 which was followed by isolate 026-2D (13.57±0.16 U/ml) at pH 4.0 (Fig. 4a). Beyond pH 5.0, a similar pattern of decreasing CMCase activities were observed. CMCase activity of isolate 026-2D was found stable at pH 4.0 (12.82±0.15 U/ml) (Fig. 6). On the other hand, isolate 033-1G had the most stable CMCase activity at pH 5.0 (13.92±0.18 U/ml). Bilal *et al.* [24] reported the highest CMCase activity of 4.69 U/ml at pH 5.5 by growing *Trichoderma reesei* on cellulose powder in SmF for 7 days. The authors also reported CMCase activity of 5.89 U/ml on wheat bran and further increase in pH declined the enzyme activity. Similarly, Rehman *et al*. [25] reported optimum CMCase activity from *Volvariella displosia* at pH 5.4 and *Tribolium castaneum* at pH 4.8. Das *et al*. [26] also reported cellulase stability at pH 6.0 by cultivating the edible mushroom *Pleurotus pulmonarius* in SmF. Data of this research and the reported pH effects on CMCase activity showed that pH 5.0 was most suitable for many fungi [27].

Optimization of Culture Conditions for CMCase Production

Effect of Temperature on Production of CMCase: Of all the isolates the highest CMCase productions of 15.17±0.16 U/ml and 14.76±0.14 U/ml were obtained from isolate 033-1G at 30°C and 35°C, respectively (Fig. 5). For the tested isolates, as the temperature increased from 20°C to 30°C, the CMCase activity increased progressively and then decreased on further temperature increases. This may be due to the fact that higher temperature reduces the metabolic activity of the fungi or denatures the enzymes produced. Similarly, Ahmed *et al.* [28] optimized CMCase production from *Trichoderma harzianum* and reported maximum CMCase activity at

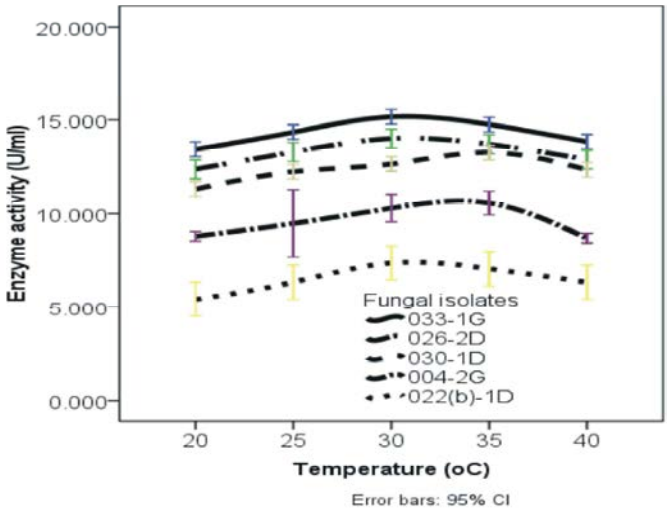

Fig. 5: Effect of temperature on CMCase productions of the wood rot fungal isolates

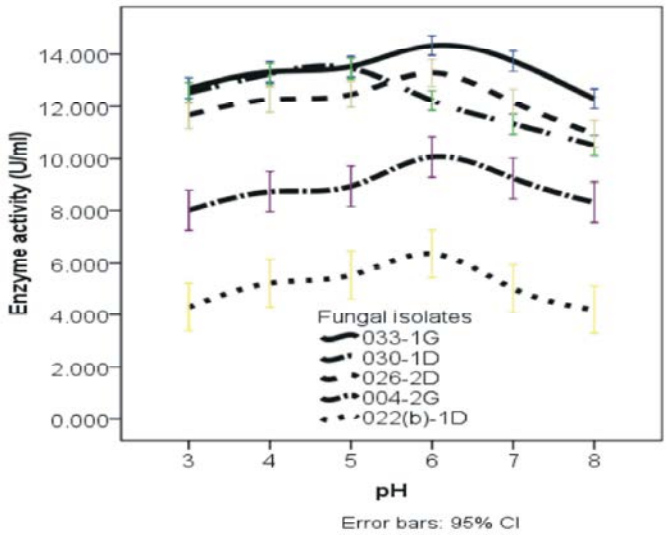

Fig. 6: Effect of initial pH on CMCase productions of the wood rot fungal isolates

28°C and a decreased enzyme production with further increased incubation temperatures. Maurya *et al.* [29] incubated *Trichoderma reesei* in SSF at temperature between 25-45°C and reported the maximum cellulase yield of 2.63 U/ml at 30°C, further increase in temperature reduced cellulase yield. On the other hand, Devi and Kumar [30] grew *A. niger* on sawdust and reported 45°C, higher than temperature optima reported by this research, as optimum temperature for cellulase production (3.75 U/ml).

Effect of Initial pH on Production of CMCase: The fungal isolates showed a wide range of pH tolerance for CMCase productions (Fig. 6). The highest CMCase was produced from isolate 033-1G (14.35±0.16) at pH 6.0 with decreasing productions at lower and higher pH values. The next two highest CMCases were produced by isolates 030-1D (13.74±0.16 U/ml) at pH 7.0 and 026-2D (13.53±0.21) at pH 5.0. These fungal isolates required slightly higher pH to dictate their highest CMCase secretions compared to reports made by different authors. For most of the fungal species, maximum CMCase release reached between pH of 5.0 and 6.0 and further increase in pH resulted in decreased enzyme productions. pH 5.5 was reported as most optimum for CMCase production by *T. harzianum* [28]. According to the authors, *P. sanguineus* preferred pH 5.0 for maximum CMCase production and at higher pH values the enzyme productions dramatically reduced.

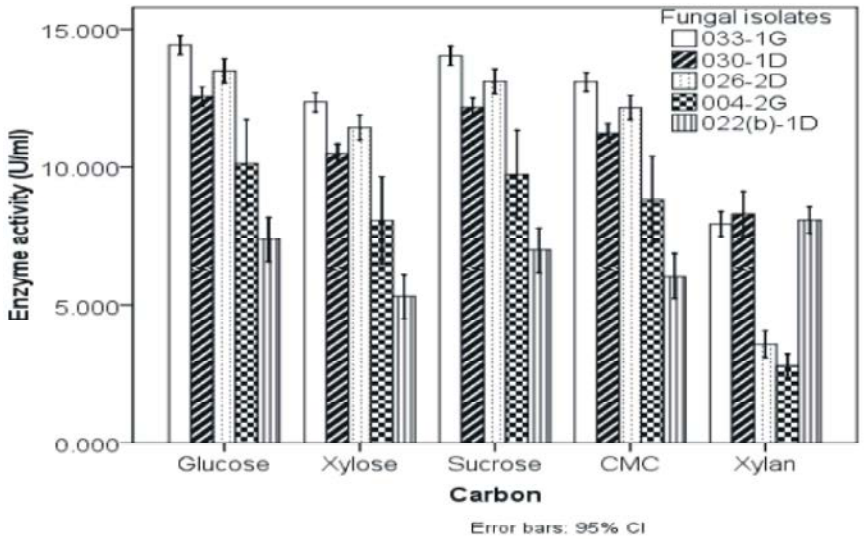

Fig. 7: Effect of carbon source on CMCase productions from the wood rot fungal isolates

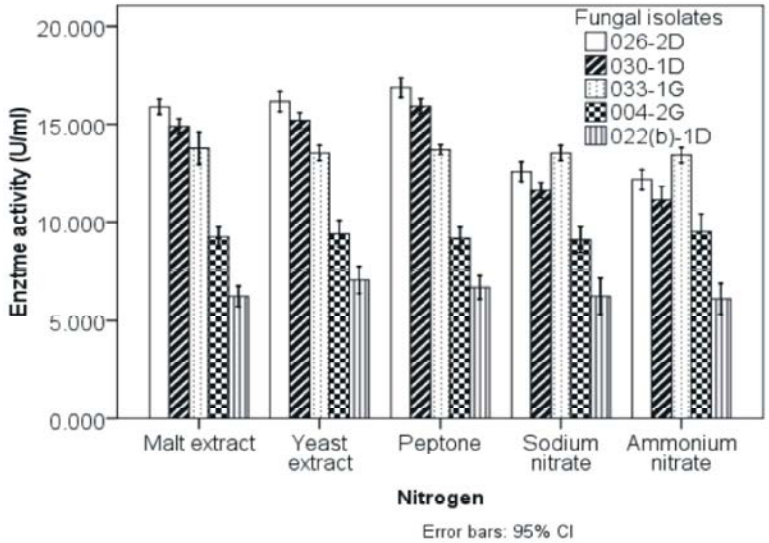

Fig. 8: Effect of nitrogen source on CMCase productions from the wood rot fungal isolates

Maurya *et al*. [29] determined the effect of pH on cellulase production by *T. reesei* in SSF and their finding indicated that the enzyme production was favored in acidic range of pH 4.0-6.0 with optimum enzyme yield of 2.32 U/ml at pH 5.2.

Effect of Media Supplementations on CMCase Production: All isolates secreted their highest CMCases when glucose was supplemented as carbon source (Fig. 7). The highest CMCase amount was exhibited by isolate 033-1G (14.43±0.15 U/ml) and followed by the amount produced by isolate 026-2D (13.50±0.18 U/ml) and isolate 030-1D (12.57±0.14 U/ml). Results also showed that supplementation of sucrose and CMC were resulted in good CMCase productions. Similarly, the highest CMCase production of 7.8 U/ml from *T. harzianum* was reported by Ahmed *et al*. [28] when glucose was supplemented as carbon source in SmF. On the other hand, Ibrahim *et al*. [31] used oil palm empty fruit bunches as carbon source and reported productions of 24.7 U/ml CMCase from *Trichoderma asperellum* UPM1. Gomathi *et al.* [32] also reported wheat bran to be the best carbon source for CMCase production of 1.23 U/ml by *Aspergillus flavus*. Preferring different carbon sources by different fungal isolates may be accounted to their physiological requirements.

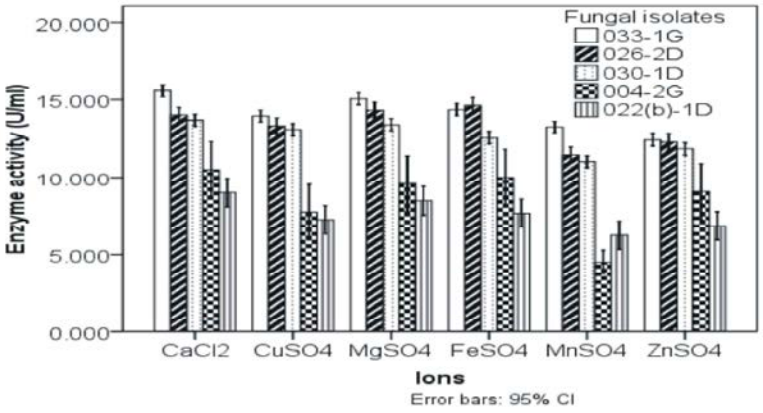

Fig. 9: Effect of divalent metallic ion on CMCase productions from the wood rot fungal isolates

Isolate 026-2D produced the highest amounts of CMCase with supplementation of peptone (16.87±0.20 U/ml) and followed by the yield when yeast extracts supplemented (16.15±0.20 U/ml) (Fig. 8). Isolate 030-1D secreted CMCase amount of 15.92±0.12 U/ml when peptone was supplemented. On the other hand, isolate 033-1G exhibited high CMCase production of 13.53±0.15 U/ml when sodium nitrate was supplemented. Similarly Kachlishvili *et al*. [33] reported peptone to be the best nitrogen source for CMCase productions by *L. edodes* and *P. dryinus*. The authors also recommended supplementation of $(NH_4)_2SO_4$ as nitrogen source for CMCase production in SSF.

Different metallic ions supplementations into the growth medium affected CMCase productions by the fungal isolates (Fig. 9). The maximum CMCase production (15.57±0.19 U/ml) was achieved from isolate 033-1G in the presence of $CaCl_2$. This was followed by CMCase amount of 15.06±0.11 U/ml produced by isolate 033-1G in the presence of $MgSO_4$ and 14.62±0.27 U/ml produced by 026-2D in the presence of $FeSO_4$. Study by Bhavsar *et al.* [34] revealed that metal ions Ca^{2+} and Mn^{2+} activated cellulases of *Aspergillus niger*, which was moderately inhibited by Co^{2+} and Fe^{2+} and strongly inhibited by Cu^{2+}. On the other hand, Saha [35] recommended lower concentration of Zn^{2+} for CMCase productions by reporting 26.7 U/ml and 8.7 U/ml CMcase productions at 10 mM and 20 mM of Zn^{+2} supplementations, respectively, for *Chalara paradoxa*.

CONCLUSION

Wood rot fungal isolates collected from Arsi branch of Oromia forest and wildlife enterprise were screened for cellulolytic activities on agar media supplemented with CMC. The FPase and CMCase were also quantified in SmF and SSF growth conditions. Results indicated that the production of cellulolytic enzymes by hydrolytic fungi in SmF and SSF significantly depends on fungal isolate type and fermentation type. SSF was superior to SmF in cellulase productions. Growth temperature and pH had significant effects on the activities and productions of the enzymes. Supplementations of different media ingredients and adjusting optimum conditions yielded higher cellulolytic enzymes. Thus higher amount of cellulolytic enzymes could be produced using the wood rot fungi which could be used in hydrolysis of lignocellulosic substrates into fermentable sugars. It is expected that the fungi with highest cellulase production and activity will release more sugar components from the lignocellulosic material for bioethanol conversion.

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REFERENCES

1. Anwar, Z., M. Gulfraz and I. Muhammad, 2014. Agro-industrial lignocellulosic biomass a key to unlock the future bio-energy: A brief review. J. Rad. Res. and Appl. Sci., 7: 163-173.
2. Iqbal, H.M.N., I. Ahmed, M.A. Zia and M. Irfan, 2011. Purification and characterization of the kinetic parameters of cellulase produced from wheat straw by *Trichoderma viride* under SSF and its detergent compatibility. Advances in Biosci. Biotechnol., 2(3): 149-156.

3. Menon, V. and M. Rao, 2012. Trends in bioconversion of lignocellulose: biofuels, platform chemicals and biorefinery concept. Prog. Energy Combust. Sci., 38: 522-50.
4. Baldrian, P. and V. Valaskova, 2008. Degradation of cellulose by basidiomycetous fungi. FEMS Microbiol. Rev., 32: 501-521.
5. Jadhav, A.R., A.V. Girde, S.M. More, S.B. More and S. Khan, 2013. Cellulase production by utilizing agricultural wastes. Res. J. Agri. Fores. Sci., 1(7): 6-9.
6. Montoya, S., O.J. Sanchez and L. Levin, 2015. Production of lignocellulolytic enzymes from three white-rot fungi by solid-state fermentation mathematical modeling. Afr. J. Biotechnol., 14(15): 1304-1317.
7. Mishra, S., P. Kumar and A. Malik, 2013. Effect of process parameters on the enzyme activity of a novel *Beauveria bassiana* isolate. Int. J.Curr. Microbiol. App. Sci., 2(9): 49-56.
8. Mustafa, U. and G. Kaur. 2010. Studies on extracellular enzyme production in *Beauveria bassiana* isolates. Int. J. Biotechnol. Biochem., 6: 701-713.
9. Singh, P., O. Sulaiman, R. Hashim, L.C. Peng and R.P. Singh, 2012. Biodegradation study of *Pycnoporus sanguineus* and its effects on structural and chemical features on oil palm biomass chips. Lignocellulose, 1(3): 210-227.
10. Damaso, M.C.T., S.D. Terzi, X.A. Farias, C.P. De Oliveria, M.E. Fraga and S. Couri, 2012. Selection of cellulolytic fungi isolated from diverse substrates. Braz. Arch. Biol. Technol., 55(4): 513-520.
11. Florencio, C., S. Couri and C.S. Farinas, 2012. Correlation between agar plate screening and solid-State fermentation for the prediction of cellulase production by *Trichoderma* Strains. Enzyme Research, doi:10.1155/2012/793708.
12. Altaf, S.A., D.M. Umar and M.S. Muhammad, 2010. Production of xylanase enzyme by *Pleurotus eryngii* and *Flamulina velutipes* grown on different carbon sources under submerged fermentation. W. Appl. Sci. J., 8: 47-49.
13. Hussain, A., A. Shrivastav, S.K. Jain, R.K. Baghel, S. Rani and M.K. Agrawale, 2012. Cellulolytic enzymatic activity of soft rot filamentous fungi *Paecilomyces variotii*. Advances in Bioresearch, 3(3): 10-17.
14. Gadgil, N.J., H.F. Daginawala, T. Chakarabarti and P. Khanna, 1995. Enhanced cellulase production by a mutant of *Trichoderma reesei*. Enzyme Microb Technol., 17: 942-946.
15. Wang, C.T., C. Hseu and C. Huang. 1988. Introduction of cellulase by cello-oligosaccharides in *Trichoderma konigii* G-39. J. Biotechnol., 9: 47-60.
16. Firmani, U., N. Matuzahroh and S. Sumarsih, 2016. Activity of crude extract cellulase produced by *Cellvibrio mixtus* UV4 that isolated from Daduk. Int. J. Sci. Technol. Engin., 2(7): 245-248.
17. Duncan, S.M., R. Minasaki, R.L. Farrell, J.M. Thwaites, B.W. Held, B.E. Arenz, J.A. Jurgens and R.A. Blanchette, 2008. Screening fungi isolated from historic Discovery Hut on Ross Island, Antarctica for cellulose degradation. Antarctic Sci., 20(5): 463-470.
18. Sunitha, V.H., D. Nirmala Devi and C. Srinivas, 2013. Extracellular Enzymatic Activity of Endophytic Fungal Strains Isolated from Medicinal Plants. World Journal of Agricultural Sciences, 9(1): 01-09. DOI: 10.5829/idosi.wjas.2013.9.1.72148.
19. Atri, N.S. and S.K. Sharma, 2012. Qualitative Estimation of Cellulase and Lignin Modifying Enzymes in Five Wild Fungal Species Collected From Northern West India. Academic Journal of Plant Sci., 5(1): 23-27. DOI: 10.5829/idosi.ajps. 2012.5.1.235.
20. Elisashvili, V., E. Kachlishvili, N. Tsiklauri, E. Metreveli, T. Khardziani and S.N. Agathos, 2009. Lignocellulose-degrading enzyme production by white-rot basidiomycetes isolated from the forests of Georgia. W. J. Microbiol. Biotechnol., 25: 331-339.
21. Elisashvili, A., M. Penninckx, E. Kachlishvili, N. Tsiklauri, E. Metreveli, T. Kharziani and G. Kvesitadze, 2008. *Lentinus edodes* and *Pleurotus* species lignocellulolytic enzymes activity in submerged and solid-state fermentation of lignocellulosic wastes of different composition. Bioresour. Technol., 99: 457-462.
22. Kang, S.W., Y.S. Park, J.S. Lee, S.I. Hong and S.W. Kim, 2004. Production of cellulases and hemicellulases by *Aspergillus niger* KK2 from lignocellulosic biomass. Bioresour. Technol., 91: 153-156.
23. Talekar, S., V. Ghodake, S. Chavare, R. Ingrole, A. Kate, S. Magdum and M. Pillai, 2011. Production and characterization of cellulase by local fungal isolate of India using water hyacinth as carbon source and reuse of fungal biomass for dye degradation. IJEST, 3(4): 3236-3241.
24. Bilal, T., B. Malik, R. Rehman and M. Kumar, 2015. Influence of various parameters on cellulase and xylanase production by different strains of *Trichoderma* Species. Austin J. Anal. Pharm. Chem., 2(1): 1-5.

25. Rehman, F.U., M. Aslam, M.I. Tariq, A. Shaheen, A.J. Sami, N.H. Naveed and A.I. Batool, 2009. Isolation of cellulolytic activities from *Tribolium castaneum* (red flour beetle). Afr. J. Biotechnol., 8(23): 6710-6715.
26. Das, N., D. Dey and S. Mishra, 2015. Isolation and physico-chemical characterization of extracellular lingo-cellulolytic enzymes of *Pleurotus pulmonarius* in submerged fermentation. Int. J. Appl. Biol. Pharmaceutical Technol., 6(3): 15-23.
27. Ahmed, S., A. Bashir, H. Saleem, M. Saadia and A. Jamil, 2009. Production and purification of cellulose degrading enzymes from filamentous fungus *Trichoderma harzianum*. Pak. J. Bot., 41(3): 1411-1419.
28. M.A.M. Abo-S tate, M. Swelim, A.I. Hammad and R.B. Gannam, 2010. Some Critical Factors Affecting Cellulase(S) Production by Aspergillus terreus Mam-F23 and *Aspergillus flavus* Mam-F35 under Solid-State Fermentation of Wheat Straw. World Applied Sciences Journal, 9(10): 1171-1179.
29. Maurya, D.P., D. Singh, D. Pratap and J.P. Maurya, 2012. Optimization of solid state fermentation conditions for the production of cellulase by Trichoderma reesei. J. Environ. Biol., 33: 5-8.
30. Devi, M.C. and M.S. Kumar, 2012. Production, optimization and partial purification of cellulase by *Aspergillus niger* fermented with paper and timber sawmill industrial wastes. J. Microbiol. Biotech. Res., 2(1): 120-128.
31. Ibrahim, M.F., M.N.A. Razak, L.Y. Phang, M.A. Hassan and S. Abd-Aziz, 2013. Crude cellulase from oil palm empty fruit bunch by *Trichoderma asperellum* UPM1 and *Aspergillus fumigatus* UPM2 for fermentable sugars production. Appl. Biochem. Biotechnol., 170: 13-20.
32. Gomathi, D., C. Muthulakshmi, D.G. Kumar, G. Ravikumar, M. Kalaiselvi and C. Uma, 2012. Submerged fermentation of wheat bran by *Aspergillus flavus* for production and characterization of carboxy methylcellulase. Asian Pacific J. Trop. Biomed., pp: S67-S73.
33. Kachlishvili, E., M.J. Penninckx, N. Tsiklauri and V. Elisashvili, 2005. Effect of nitrogen source on lignocellulolytic enzyme production by white rot basidiomycetes under solid state cultivation. World J. Biotechnol., 22(4): 391-397.
34. Bhavsar, N.H., B.V. Raol, S.S. Amin and G.G. Raol, 2015. Optimization and characterization of fungal cellulase for enzymatic saccharification of lignocellosic agro-waste. Int. J. Curr. Microbiol. App., Sci., 4(3): 30-46.
35. Saha, B.C., 2004. Production purification and properties of endoglucanase from a newly isolated strain of *Mucor circinelloides*. Proc. Biochem., 39: 1871-1876.