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Comparative studies on glucanases and β -glucosidase activities of *Pleurotus ostreatus* and *P. pulmonarius* in solid state fermentation

Ekundayo FO^{1*}, Ekundayo EA² and Ayodele BB¹

¹Department of Microbiology, Federal University of Technology, Akure, Nigeria

²Department of Biological Sciences, Afe Babalola University, Ado-Ekiti, Nigeria

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Abstract

Agricultural wastes constitute major environmental problem. However, these wastes can be used as substrates for the production of enzymes such as cellulases by filamentous fungi including *Pleurotus* species. This present investigation was therefore aimed at screening *Pleurotus ostreatus* DBUI14 and *P. pulmonarius* DBUI002 for glucanases and β -glucosidase using rice bran, corn cob and sawdust as substrates. Both *P. ostreatus* DBUI14 and *P. pulmonarius* DBUI002 cultivated on pretreated rice bran, corn cob and sawdust were screened for cellulolytic enzymes under solid state fermentation (SSF) for a period of 15 days using standard methods. The protein contents were determined using the method of Lowry. The results showed that lowest activities of these enzymes by *Pleurotus* spp occurred on the third and fifteenth days while the highest activities occurred on the ninth day of fermentation. The highest endoglucanase as well as lowest exoglucanase activities were obtained from *Pleurotus pulmonarius* DBUI002 cultivated on corn cob (PPCC). *Pleurotus ostreatus* DBUI14 cultivated on corn cob (POCC) had the lowest endoglucanase but highest exoglucanase activity when grown on rice bran (PORB). Moreover, lowest β -glucosidase activity was obtained from POCC while PPCC and PORB had the highest β -glucosidase activities. *Pleurotus pulmonarius* cultivated on corn cob and *P. ostreatus* cultivated on rice bran could be harnessed in the commercial production of endoglucanase and exoglucanase. In addition, both *Pleurotus ostreatus* DBUI14 strain and *P. pulmonarius* DBUI002 could be harnessed in synthesis of β -glucosidase.

Key words – agro-industrial wastes bioconversion extracellular cellulolytic enzymes – pretreatment – submerged fermentation

Introduction

Most often than not disposal of agro-industrial wastes constitutes a major environmental problem in developing countries (Khalil 2002, Göğüş et al. 2014). Bioconversion of these wastes to useful substances requires activities of enzymes, namely, β -1,4 glucanases, β -1,4 exoglucanase and β -1,4 glucosidase, pectinases etc (Baker et al. 1995, Mansfield et al. 1998, Birhanlı & Yeşilada 2013, Göğüş et al. 2014). It is advantageous to produce enzymes using microorganisms since they are not influenced by climatic and seasonal factors and can be subjected to genetic and environmental manipulations to increase product yield (Dhillon et al. 2004). The genera *Trichoderma* and

Aspergillus have been extensively used in the production of cellulolytic enzymes (Baig 2005, Narasimha et al. 2006).

Extracellular cellulolytic enzymes can also be produced from macrofungi which are known to be safe. *Pleurotus* spp. have been found to be efficient in utilizing agricultural residues (Zhang et al. 2002, Salmones et al. 2005, Alborés et al. 2006). Goyal & Sony (2011) reported that cellulolytic enzymes were produced by *P. florida*, *P. ostreatus* and *P. sajar-caju* but highest production was by *P. florida*. The major methods used in production of cellulolytic enzymes by fungi are solid state fermentation (SSF) and submerged culture of the microorganisms (Lonsane & Ghildyal 1993, Ishida et al. 2006). However, it is very expensive to produce cellulolytic enzymes by submerged fermentation (Ruth et al. 1999). The importance of solid state fermentation over submerged fermentation cannot be underestimated. The cost of producing cellulolytic enzymes via SSF is low and the yield is high. Also, energy consumed is low, it is simple to carry out and a little waste product is generated (Gupte & Madamwar 1997). Moreover, cellulolytic enzymes produced by SSF are more concentrated in the culture filtrate (Moo-Young et al. 1983). The present work was therefore aimed at determining cellulolytic enzymes by *P. ostreatus* and *P. pulmonarius* using corn cob, saw dust and rice bran as substrates using solid state fermentation.

Materials & Methods

Collection of materials

Pleurotus ostreatus DBUI14 and *P. pulmonarius* DBUI002 were collected from the Department of Botany, University of Ibadan, Oyo State, Nigeria and were used for spawn preparation and mass production of the selected mushrooms. These cultures were cultivated and preserved at 4°C on potato dextrose agar respectively.

The agro-industrial substrates namely rice bran, corn cob and sawdust of *Hannoa klaineana* were obtained from market and timber shed at Akure, Ondo State, Nigeria.

Solid state fermentation of rice bran, corn cob and sawdust

Pretreatment of each substrate was done by soaking in 1.0% (w/v) NaOH solution. Five grammes of pretreated rice bran, corn cob and sawdust were placed in 250 mL flasks and then autoclaved at 121°C for 15 minutes. Both *P. ostreatus* and *P. pulmonarius* (1mm disk) were cultivated separately in 20 mL of 1% carboxyl methyl cellulose (CMC) and the selected agro-industrial wastes as well as czapek dox medium (Na₂HPO₄ 1.0, NaNO₃ 3.0, KCl 0.5, MgSO₄·7H₂O 0.1 and FeSO₄·7H₂O 0.001) at pH 5.0 after autoclaving at 121°C for 15 minutes. The inoculated flasks were incubated at 30 °C in a static position for 15 days (Goyal & Sony 2011).

Extraction of Enzyme

Twenty five millilitres of cooled 0.05 M citrate buffer with pH 4.8 was added to each flask at interval of three days and the filtrates were then subjected to enzyme activity to determine endoglucanase, exoglucanase and β-glucosidase production by the *Pleurotus* species by the dinitrosalicylic acid (DNS) method (Miller 2000) following degradation of appropriate substrate by enzyme extracts supplied from the different flasks. Each assay system contained enzyme extract, substrate and buffer using a standard assay of 0.5 mL. After intervals of 3days incubation periods at 28°C – 30°C, reactions were terminated by the addition of the Dinitrosalicylic acid (DNS) reagent and heating for 5minutes at 100°C. Controls included inclusion of substrate and omission of enzyme extract (Miller 2000).

Endoglucanase activity of *P. ostreatus* and *P. pulmonarius*

Endoglucanase activities of both *Pleurotus ostreatus* DBUI14 and *P. pulmonarius* DBUI002 were done according to the methods described by Mandels et al. (2006). The reaction mixture consists of 1.0 mL of 0.05 M citrate buffer (pH 4.8), 1.0 mL of 1% CMC solution and 0.5 ml of culture filtrate which was then incubated at 50 °C. Determination of reducing sugars released was done by

withdrawing 0.5 mL of sample at zero and 30 minutes interval and the absorbance was measured at 540 nm (Miller 2000).

Exoglucanase activity

Tiny pieces of Whatman no.1 filter paper were used for the determination of exoglucanase activities of *Pleurotus ostreatus* DBUI14 strain and *P. pulmonarius* DBUI002. The cut strips containing 2 mL of 0.05 M citrate buffer (pH 4.8) and 0.5 mL culture filtrate were incubated at 50°C. Determination of reducing sugars released was done at 0 and 60 min intervals by DNS method (Miller 2000). The absorbance was measured at 540 nm.

β -glucosidase activity of *P. ostreatus* and *P. pulmonarius*

The reaction mixture consisted of 1% cellobiose solution (1 mL), 0.05 M citrate buffer (0.5 mL) and enzyme solution (0.5 mL). The mixture was then incubated at 50°C after which the amount of reducing sugars released were measured at 0 and 15 min of incubation period at 540 nm according to DNSA method. One unit of enzyme activity was expressed as the amount of enzyme that corresponded to one micromole of reducing sugars per minute under the assay conditions, using glucose as a sugar standard.

Estimation of soluble protein from *P. ostreatus* and *P. pulmonarius*

Total protein was determined in culture filtrate by the method of Lowry et al. (2009) with bovine serum albumin as standard measured at 540 nm by spectrophotometer (Hannan electronic spectrophotometer) model. Bovine serum albumin (BSA) and distilled water served as the positive and negative controls in protein estimation.

Results

Enzymatic activities of *P. ostreatus* and *P. pulmonarius* on different substrates during solid-state fermentation

Typical enzyme activity occurred in the colonised substrates (rice bran, corn cob and sawdust) by the prepared by the inocula (*Pleurotus ostreatus* DBUI14 strain and *P. pulmonarius* DBUI002) and the enzymes produced were extracted from the whole colonised substrates at three days interval for fifteen days. It took about sixth to twelfth days to colonise the substrates, at which time biomass was visible on the surface of the flask. The three enzymes (β -glucosidase, endoglucanase and exoglucanase) assayed were all present during the degradation of the substrates; rice bran, corn cob and saw dust in solid state fermentation (SSF). The lowest activities of these enzymes were between the third and sixth days. The enzymes reached the peak of their activities around ninth day of growth and then declined to the lowest activities at the fifteenth day of incubation (Figures 1, 2, and 3).

Endoglucanase activities of *P. ostreatus* and *P. pulmonarius* on different substrates during solid state fermentation

Pleurotus ostreatus DBUI14 cultivated on sawdust (POSW) had the highest endoglucanase activity between third and fifteenth days of SSF. On the ninth day of fermentation, maximum endoglucanase activity (15.0 μ mol/min/mL) was recorded for POSW while the minimum (1.0 μ mol/min/mL) for *P. ostreatus* on corn cob (Figure 1).

Exoglucanase activities of *P. ostreatus* and *P. pulmonarius* on different substrates during solid state fermentation

Pleurotus ostreatus DBUI14 cultivated on rice bran (PORB) had the highest exoglucanase activity between third and fifteenth days. On the other hand, *P. pulmonarius* DBUI002 on corn cob (PPCC) had the lowest activity of this enzyme especially on the ninth day of SSF (Figure 2).

Beta-glucosidase activities of *P. ostreatus* and *P. pulmonarius* on different substrates during solid state fermentation

Pleurotus pulmonarius DBUI002 cultivated on corn cob had the highest β -glucosidase activity between the third and fifteenth days. On the ninth day of fermentation, both PPCC and PORB had the same β -glucosidase activity of 33.0 $\mu\text{mol}/\text{min}/\text{mL}$ while POCC had the lowest β -glucosidase activity (Figure 3).

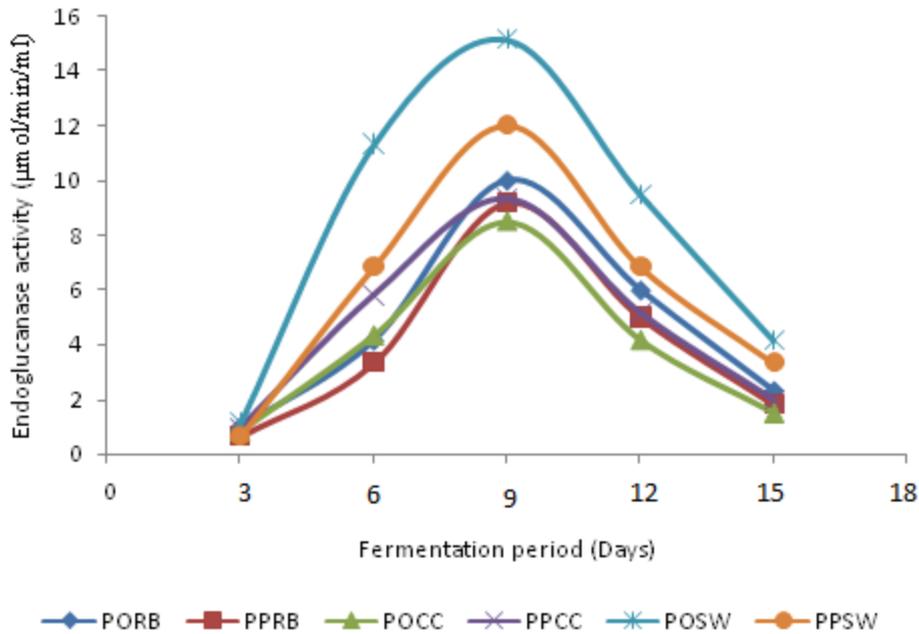


Figure 1 – Endoglucanase activities of *P. ostreatus* and *P. pulmonarius* on different substrates during solid state fermentation. PORB – *P. ostreatus* DBUI14 on rice bran; PPRB – *P. pulmonarius* DBUI002 on rice bran; PPCC – *P. pulmonarius* DBUI002 on corn cob; POSW – *P. ostreatus* DBUI14 on sawdust; PPSW – *P. pulmonarius* DBUI002 on sawdust.

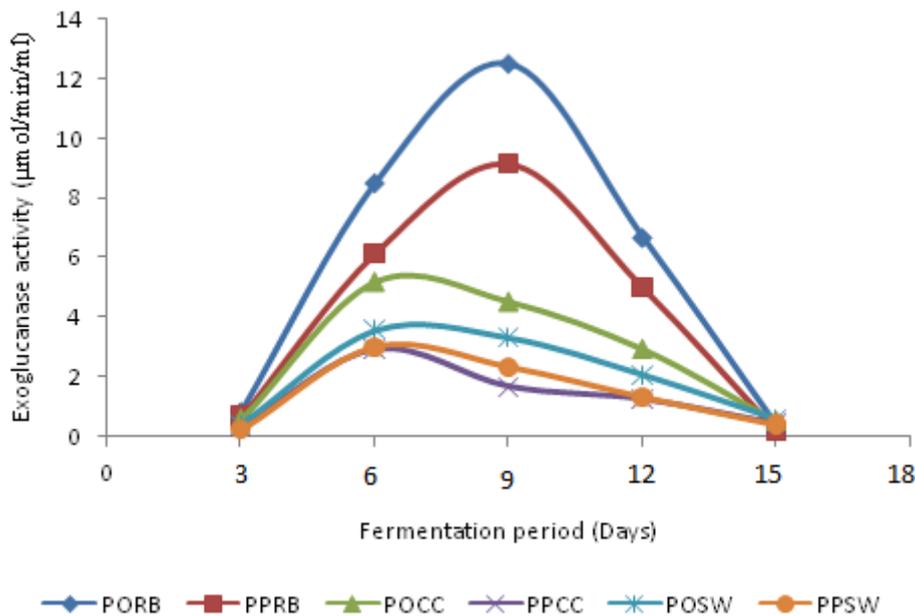


Figure 2 – Exoglucanase activities of *P. ostreatus* and *P. pulmonarius* on different substrates during solid state fermentation. PORB – *P. ostreatus* DBUI14 on rice bran; PPRB – *P. pulmonarius*

DBUI002 on rice bran; PPCC – *P. pulmonarius* DBUI002 on corn cob; POSW – *P. ostreatus* DBUI14 on sawdust; PPSW – *P. pulmonarius* DBUI002 on sawdust.

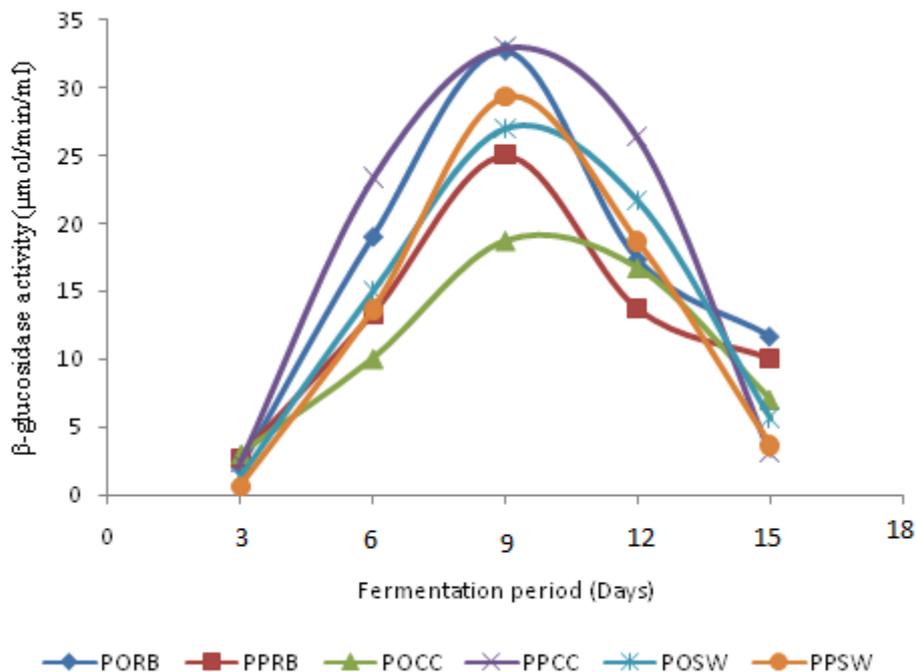


Figure 3 – β-glucosidase activities of *P. ostreatus* and *P. pulmonarius* on different substrates during solid state fermentation. PORB – *P. ostreatus* DBUI14 on rice bran; PPRB – *P. pulmonarius* DBUI002 on rice bran; PPCC – *P. pulmonarius* DBUI002 on corn cob; POSW – *P. ostreatus* DBUI14 on sawdust; PPSW – *P. pulmonarius* DBUI002 on sawdust.

Soluble proteins in fermented products produced by *P. ostreatus* and *P. pulmonarius*

Levels of soluble proteins of endoglucanase were found to reach a peak on the ninth day of fermentation after which the levels diminished gradually. The highest concentration of soluble protein (6.0 mg/mL) was found in *P. ostreatus* DBUI14 cultivated on rice bran while the lowest level (1.0 mg/mL) was observed in *P. pulmonarius* DBUI002 cultivated on sawdust (Figure 4).

Similar trends were observed for both exoglucanase and β-glucosidase. The highest concentration of soluble protein (6.0 mg/mL) was found in *P. ostreatus* DBUI14 cultivated on rice bran while the lowest level (0.0 mg/mL) was observed in *P. Pulmonarius* DBUI002 cultivated on sawdust (Figure 5). Also, the highest concentration (9.0 mg/mL) was found when rice bran was the substrate for *P. ostreatus* DBUI14 while the lowest level (0.5mg/mL) was for *P. pulmonarius* DBUI002 when sawdust was the substrate for exoglucanase (Figure 6).

Discussion

In this study, enzymatic activities reached a peak on the ninth day of fermentation for both *P. ostreatus* and *P. pulmonarius*. Gupte & Madamwar (1997) reported the maximum activity of lignocellulose degrading enzymes at 9–10 days of solid state fermentation (SSF). Khalil et al. (2011) reported that *P. ostreatus* and *P. sajor-caju* reached a peak on the 10th day of fermentation. Zawistowski et al. (1991) reported the highest oxidative enzyme activity at 15 day and carboxyl methyl cellulase activity at 5–9 days on SSF of sawdust and straw in *P. florida*. From this research, exoglucanase, endoglucanase and β-glucosidase activities were much higher in *P. ostreatus* compared to *P. pulmonarius*. Similar results were also reported by Gupte & Madamwar (1997) as well as Khalil et al. (2011).

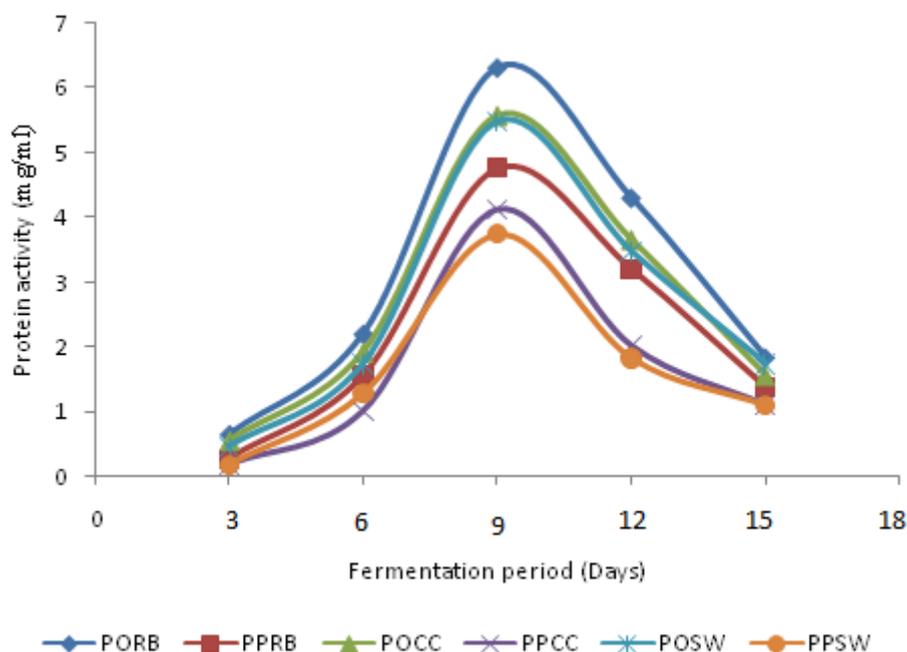


Figure 4 – Time course of protein activity of endoglucanase on selected agro-industrial wastes. PORB – *P. ostreatus* DBUI14 on rice bran; PPRB – *P. pulmonarius* DBUI002 on rice bran; PPCC – *P. pulmonarius* DBUI002 on corn cob; POSW – *P. ostreatus* DBUI14 on sawdust; PPSW – *P. pulmonarius* DBUI002 on sawdust.

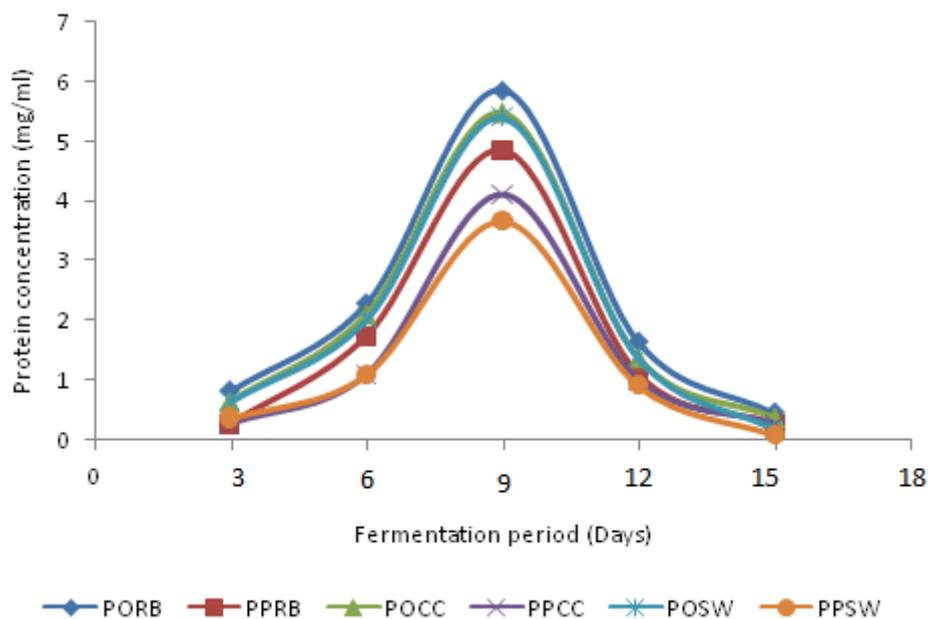


Figure 5 – Time course of protein activity of exoglucanase on selected agro-industrial wastes. PORB – *P. ostreatus* DBUI14 on rice bran; PPRB – *P. pulmonarius* DBUI002 on rice bran; PPCC – *P. pulmonarius* DBUI002 on corn cob; POSW – *P. ostreatus* DBUI14 on sawdust; PPSW – *P. pulmonarius* DBUI002 on sawdust.

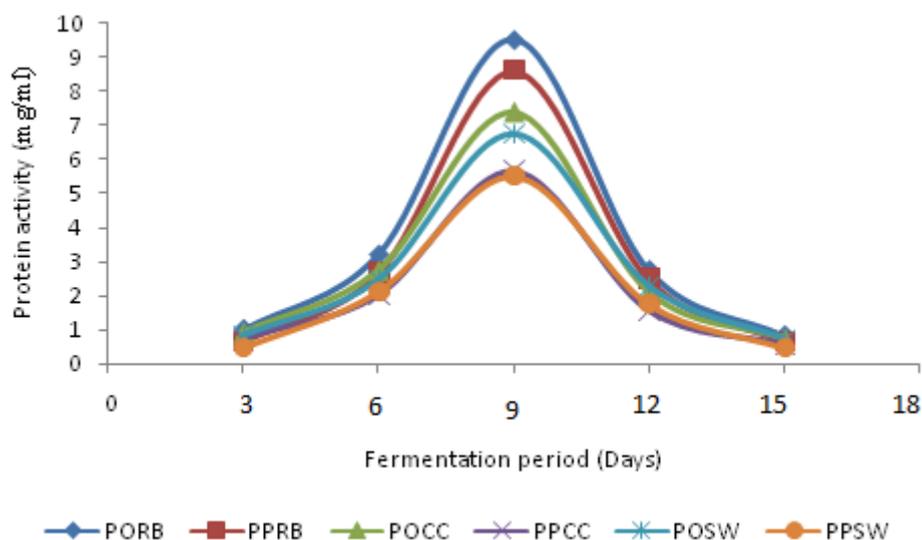


Figure 6 –Time course of protein activity of β -glucosidase on selected agro-industrial wastes. PORB – *P. ostreatus* DBUI14 on rice bran; PPRB – *P. pulmonarius* DBUI002 on rice bran; PPCC – *P. pulmonarius* DBUI002 on corn cob; POSW – *P. ostreatus* DBUI14 on sawdust; PPSW – *P. pulmonarius* DBUI002 on sawdust.

The potency of *P. ostreatus* in lignocellulolytic activity over other mushrooms like *Lentinus* spp. has also been reported (Stamets 2000, Elisashvili et al 2008). It was found that different enzymes peaked at different fermentation period and declined subsequently which could be due to the inactivation and or degradation of these enzymes as also reported by Goyal & Sony (2011). Cellulolytic enzymes produced in this present were low between 12 and 15 days of fermentation period. Hong (2006), Cai et al. (1994) and Azizi et al. (1990), reported maximum production of cellulolytic enzymes by some mushroom on 8th and 12th day of the fermentation period. Both endoglucanase and exoglucanase showed the same activity pattern but with different increasing pattern in which β -glucosidase has highest activity followed by endoglucanase and exoglucanase. Likewise, β -glucosidase showed maximum production at the 9 days of fermentation period and a slightly low production was achieved on 12–15 day of fermentation. Bisaria et al. (2008) detected higher cellulolytic enzymes production from *P. sajocaju* by using treated cotton. In this study, protein reached the maximum peak at 9 days of fermentation.

Conclusion

Based on the results obtained from this study, *Pleurotus pulmonarius* DBUI002 cultivated on corn cob as well as *P. ostreatus* DBUI14 cultivated on rice bran could be harnessed in the commercial production of endoglucanase and exoglucanase. Both *P. ostreatus* and *P. pulmonarius* could be harnessed in synthesis of β -glucosidase. However, further research is necessitated to know the optimum conditions for production of cellulolytic enzymes from these mushrooms.

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