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Production of cellulase by *Pleurotus ostreatus* and *Pleurotus sajor-caju* in solid state fermentation of lignocellulosic biomass

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Abstract: Bioconversion of cellulosic components into fermentable sugars has currently been accomplished with the help of the microbial enzyme, cellulase. In this study, *Pleurotus ostreatus* (Jacquin ex Fr.) Kummer and *Pleurotus sajor-caju* (Fr.) Singer were tested for cellulase production by solid state fermentation (SSF) using saw dust, sugarcane bagasse, and paddy straw as substrate. To enhance production, the substrates were pretreated with 1% and 4% NaOH solutions. Supernatants of extracted enzyme solutions were used to estimate reducing sugar and soluble protein, as well as enzyme activities. Maximum production of reducing sugar and soluble protein were recorded in 12 and 10 day fermented products, respectively. In the production of reducing sugars, *P. ostreatus* (42.8 mg g^{-1}) was found to be more efficient than *P. sajor-caju* (10.9 mg g^{-1}), while *P. sajor-caju* was more efficient in soluble protein production (40.2 mg g^{-1}) compared to *P. ostreatus* (3.1 mg g^{-1}). *P. ostreatus* was found to be more efficient in the individual components of cellulase enzyme production than *P. sajor-caju*. All the enzyme activities were found to attain a peak at 10 days of fermentation. The highest activities of endoglucanase, exoglucanase, and β-glucosidase were recorded as 7.08, 7.36, and 3.60 units (μmole of glucose released/min/g substrate), respectively, for *P. ostreatus*, while that of *P. sajor-caju* were 1.9, 2.03, and 2.63 units, respectively. Total cellulase activity was found to reach a maximum at 10 days of fermentation for both strains. For *P. ostreatus* it was recorded as 3.51 units, while for *P. sajor-caju* it was 0.82 units. This study suggests *P. ostreatus* as an important source of cellulase enzymes.

Key words: Cellulase, *Pleurotus ostreatus*, *Pleurotus sajor-caju*, solid state fermentation

Introduction

All over the world, there is a growing concern about the over dependence on fossil fuels as well as their possible roles in global warming. Because of this, there is a tremendous search for a biofuel to use as an alternative source of energy, utilizing the existing lignocellulosic biomolecules (Sharma et al. 2004). In this endeavor, ethanol produced from foodstuffs such as wheat, maize, soybean, etc., is under scrutinized experimentation, while victimizing the food security of the world (Somerville 2006). Though it is proclaimed that ethanol produced in this way is eco-friendly, its production consumes more energy than it releases (Antoni et al. 2007). Therefore, it can give

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rise to further environmental damage, rather than repairing it (Farrell et al. 2006). The use of cellulase is the most *promising* technology in the conversion of *lignocellulosic biomass.* (Demain et al. 2005). *Cellulosic ethanol* is a biofuel produced from *wood, grasses,* and the *nonedible parts of plants.* (Ingram et al. 1999). Lignocellulosic biomass is an important resource for the production of biofuels because of its abundance nature, is inexpensive, and production of such resources is ecofriendly. Agricultural residues are the major source of lignocellulosic biomass, which is renewable and inexpensive. These resources include corn fiber, sugarcane bagasse, rice husks, woody crops, and forest residues. Furthermore, there are plenty of sources of lignocellulosic waste from industrial and agricultural processes, such as citrus peel waste, sawdust, paper pulp, industrial waste, municipal solid waste, and paper mill sludge (Maki et al. 2009). Most of these agricultural residues are nonedible lignocellulose which is used for ethanol production (Brusstar and Bakenhus 2008). Hemicellulose must be degraded, at least in part, before the cellulose in plant cell walls can be effectively degraded by cellulolytic organisms (Blackwell 1992).

Cellulose and hemicellulose occur in plants, algae, many fungi, and in the cysts of some protozoa. Lignin is found not only in higher plants, but also in certain fungi and algae (Das et al. 1995). The enzymatic degradation of cellulose is a complex process that requires the participation of at least 3 types of cellulolytic activity: exo-β-l, 4-glucanase, endo- β-1, 4-glucanase, and β-glucosidase. In the hydrolysis of native cellulose, exo- and endoglucanase act synergistically to produce cellobiose, which is then degraded into glucose by β-glucosidase (Enari and Markkanen 1977). Some important bacteria and fungi are the most prominent sources of cellulase enzymes, which participate in the conversion of cellulose to glucose (Bisaria and Ghose 1981). Among these organisms, fungi have been studied extensively because their elongated hyphae create mechanical pressure on the cellulose structure, causing them to produce large amounts of cellulase (Schwarz 2001). Most fungal cellulases have the capability to digest cellulose and they also produce the enzymes necessary to hydrolyze lignin and hemicelluloses (Singh et al. 1989). Sodium hydroxide (NaOH) pretreatment of cellulose is one of the best-known methods of increasing the digestibility of cellulosic materials (Lynd et al. 2002). The prime requirement for production of any enzyme is a high yielding organism for which the selection of a cultivation technique and composition of media and culture conditions of supporting media should be properly formulated (Dequin et al. 1999; Doran et al. 2000). Hydrolytic enzymes used in industries can be produced in 2 ways: a) solid state fermentation (SSF) and b) submerged culture of the microorganisms (Ishida et al. 2006). *SSF* has numerous *advantages over* submerged liquid fermentation, including *superior enzyme productivity*, minimum *capital investment*, *low energy* consumption, process simplicity and less wastewater output, better *product recovery*, and negligible liquid waste product (Gunju et al. 1990; Gupte and Madamware 1997; Mukhopadhyey and Nandi 1999). The present study aimed to initiate and develop a biotechnological process, especially by SSF, for the production of cellulase from easily available substrates using *P. ostreatus* and *P. sajor-caju*.

Materials and methods

Biological materials of the study and screening of cellulolytic activity

Subcultures of *Pleurotus ostreatus* (Jacquin ex Fr.) Kummer and *Pleurotus sajor-caju* (Fr.) Singer were collected from the National Mushroom Cultivation and Extension Center, Dhaka, Bangladesh. Saw dust (SD), paddy straw (PS), and sugarcane bagasse (SB) were used as lignocellulosic substrates. Potato dextrose agar (PDA) medium with 0.1 μg L^{-1} L-arginine added, was prepared in petri plates and formed into slants in test tubes, which were stored at 4 °C. Both *P. ostreatus* and *P. sajor-caju* were grown on PDA at temperatures of 25, 28, 30, 35, and 40 °C. Both mushrooms were grown on PDA media at pH levels of 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0. All PDA plates were incubated at same temperatures and under the same conditions. Every 24 h, growth of colonies was recorded. The maximum colony diameter was 7.5 cm.

As alkali treatment on lignocellulosic substrates results in a disruption of the lignin seal, hydration and swelling of the cellulose, and a decrease in crystallinity (Kato et al. 2004; Patel et al. 2006),

pretreated lignocellulosic substrates were used as the sole source of carbon to detect the cellulolytic activity of the selected fungal strains. The substrates (saw dust, paddy straw, and sugarcane bagasse) were sun-dried, shredded into 4-6 cm segments, and powdered by an electric grinder. These substrate powders were then soaked individually in 1% and 4% NaOH solutions at a ratio of 1:15 (substrate:solution), heated in a boiling water bath for 1 h, and then cooled. The alkali treated substrates were separated from the solution by filtering with muslin. Excess alkali was removed by repeated washing with tap water until the pH was neutral. The substrates were then squeezed to remove excess water, spread over newspaper and allowed to dry overnight in an oven at 90 °C (Kubo et al. 2000; Kaur et al. 2004).

Enzyme extraction by solid state fermentation

Five grams of lignocellulosic substrates were taken individually in 250 mL conical flasks. To give a final moisture content of 80%, 20 mL of distilled water was added individually to each flask. The pH of the medium was adjusted to 5.5. The flasks were then cotton plugged and autoclaved at 121 °C for 20 min. Then, a 6-mm disc of PDA agar block was used to inoculate 1 flask. A sterilized cork borer was used to cut the blocks from the growing edge of the culture, thus inoculating the flasks individually. The inoculations were done under aseptic conditions in a laminar airflow cabinet. The flasks were incubated at 2, 4, 6, 8, 10, 12 and 14 day intervals. As the strains were shown to grow optimally at room temperature, the flasks were incubated at room temperature. After incubation, 50 mL of 0.05 M citrate buffer (pH 5.0) was added to each flask and left for shaking on a rotary shaker at 150 rpm for 1 h. The fermented samples were then filtered through muslin. Because the cellulase was strongly absorbed by the cellulose (Goel and Ramachandran 1983; Kim et al. 1997; Van-Wyk 1997), 50 mL of 0.05 M citrate buffer was added in 2 successive washings to recover the enzyme successfully. The filtrates were centrifuged at 1000 × *g* for 30 min. The supernatants were taken as crude enzyme extracts and stored at 4 °C.

Estimation of reducing sugar

The estimation of reducing sugar was done using the DNS (3, 5-dinitrosalicylic acid) method (Sadasivam and Manickam 2005) with a slight modification; 1 mL of appropriately diluted fermented extract, 2 mL of distilled water, and 3 mL of DNS reagent were added. The tubes were heated in a boiling water bath for 15 min and 1 mL of 40% sodium potassium tartarate was added while the tubes were still warm. The tubes were allowed to cool and the absorbance was measured at 540 nm. Standard glucose was used as the positive control and distilled water was used as the negative control.

Estimation of soluble protein

Total protein was extracted from fermented substrates by NaOH treatment, and then the concentrations of soluble protein at various fermentation stages were determined using the method of Lowry et al. (1951). In this method, protein reacted with Cu+ in the alkali and formed the protein-Cu complex and the tyrosine and the tryptophan residue of the protein reduced the phosphotungstic and molybdic acids of the folin reagent to tungsten and molybdenum blue. The colored complex was measured at 660 nm by spectrophotometer. Bovine serum albumin (BSA) was used as the positive control and distilled water was used as the negative control in protein estimation.

Enzyme assay

There are 3 main types of enzymes present in the cellulase system: endo-β-1; 4-glucanase, exo-β-1; 4-glucanase, and β-glucosidase. 'μmole of glucose released/min/g substrate' was taken as an enzyme unit. The enzymes were analyzed as follows: (1) Combined assay for endo- and exo-glucanase and β-glucosidase: For the endoglucanase assay, 1% carboxymethylcellulose (CMC) and for the exoglucanase assay, 1% cellulose solution was prepared. For the combined assay of endo- and exo- glucanase, Strips (1 × 6 cm) of Whatman No. 1 filter paper were used as substrate. Standard cellulase enzyme was used as the positive control and distilled water was used as the negative control. For the β-glucosidase assay, p-nitrophenyl-β-D-glucopyranoside (PNPG) solution (0.3 g PNPG dissolved in 40 mL distilled water) was used as substrate (pH was maintained neutral: 7.0). Endoglucanase hydrolyzed carboxymethylcelulose was used to produce free carboxymethyl glucose units. The free carboxymethyl glucose units reacted with 3, 5-dinitrosalicylic acid (DNS) reagent to form a colored complex, which was detected

spectrophotometrically at 540 nm (Martins et al. 2008). Exoglucanase removed glucose from non-reducing ends of the major chain or shorter chains of cellulose. The carbonyl group of this glucose and other reducing sugars reacted with 3, 5-dinitrosalicylic acid to form a colored complex which was detected spectrophotometrically at 540 nm (Martins et al., 2008). β-glucosidase hydrolyzed p-nitrophenyl-β-D-glucopyranoside and released p-nitrophenol, which was determined by spectrophotometer at 410 nm (Martins et al. 2008). (2) Filter paper assay (FPase) method: 1 mL of appropriately diluted enzyme solution was added in test tubes and 1 mL of 0.05 M citrate buffer (pH 4.8) and 50 mg (1 × 6 cm strip) of Whatman No. 1 filter paper that had been curled around a glass rod was added. The test tubes were incubated for 60 min at 50 °C. After incubation, 3 mL of DNS reagent was added. Then the tubes were boiled for 15 min in a boiling water bath and 1 mL of 40% sodium potassium tartarate was added, while the tubes were still warm. After cooling to room temperature, absorbance was measured at 540 nm (Martins et al. 2008). Standard cellulase enzyme was used as the positive control and distilled water was used as the negative control.

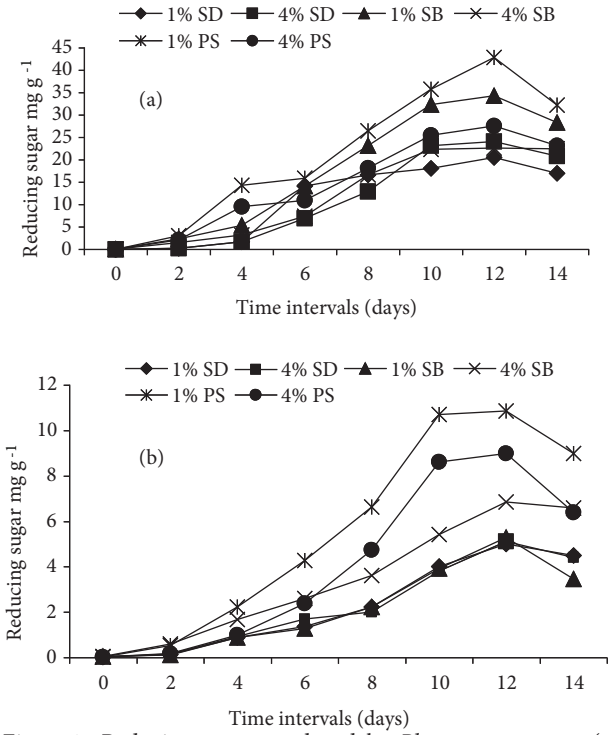

Figure 1. Reducing sugar produced by *Pleurotus ostreatus* (a) and *Pleurotus sajor-caju* (b) (Mean value of n = 4). SD = saw dust, SB = sugarcane bagasse, PS = paddy straw; 1% and 4% stand for 1% and 4% NaOH pretreatment, respectively.

Results

Effect of temperature and pH on the growth of *P. ostratus* and *P. sajor-caju*

Maximum colony diameter was obtained at room temperature (25-28 °C) for both strains. Colonial growth was increased gradually during the incubation period. No growth was observed at 40 °C for either strain and very poor growth was observed at 35 °C for both strains. Growth pattern and colony diameter (in mm) of the strains were studied regularly. Poor growth was observed at a pH of 4.0, 4.5, and ≥7.0 and thin mycelia mat was found for both strains. Maximum colony diameter was recorded at a pH of 5.5 for both *P. ostratus* and *P. sajor-caju*.

Reducing sugar in fermented products produced by *P. ostreactus* and *P. sajor-caju*

Levels of reducing sugar were found to reach a peak at 12 days of fermentation in *P. ostreatus* (Figure 1a), after which the levels diminished gradually. The highest concentration (42.8 mg g^{-1}) was found when 1% NaOH treated paddy straw (1% PS) was the substrate, while the lowest level (20.5 mg g^{-1}) was for 1% NaOH treated saw dust (1% SD). In the case of *P. sajor-caju*, levels of reducing sugar were found to reach a peak at 12 days of fermentation (Figure 1b), after which the levels diminished gradually. The highest concentration (10.9 mg g^{-1}) was found when 1% NaOH treated paddy straw (1% PS) was the substrate, while the lowest level (5 mg g^{-1}) was for 1% NaOH treated saw dust (1% SD).

Soluble proteins in fermented products produced by *P. ostreatus* and *P. sajor-caju*

Levels of soluble proteins were found to reach a peak at 10 days of fermentation in *P. ostreatus* (Figure 2a), after which the levels diminished gradually. The highest concentration (3.1 mg g^{-1}) was found when 1% SB was the substrate, while the lowest level (1.8 mg g^{-1}) was for 1% SD. Levels of soluble proteins were found to reach a peak at 10 days of fermentation in *P. sajor-caju* (Figure 2b), after which the levels

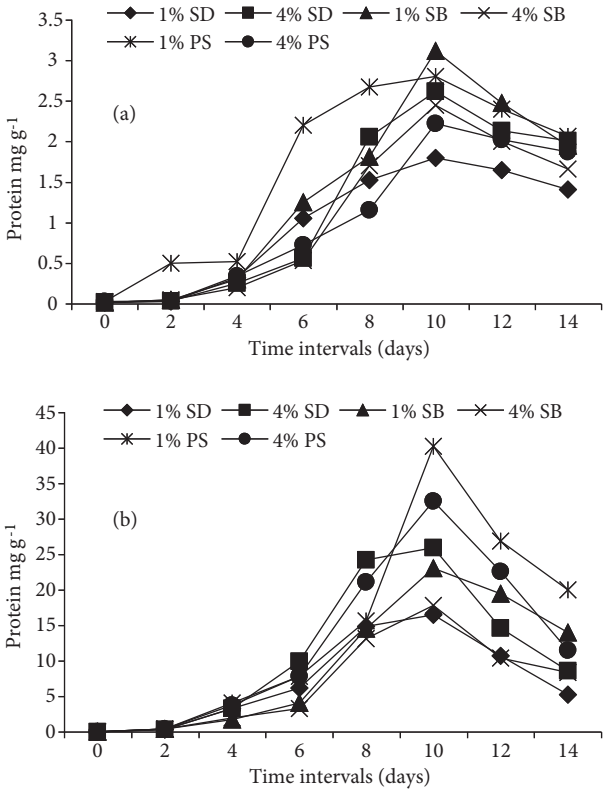

Figure 2. Soluble protein produced by *Pleurotus ostreatus* (a) and *Pleurotus sajor-caju* (b) (Mean value of n = 4). SD = saw dust, SB = sugarcane bagasse, PS = paddy straw; 1% and 4% stand for 1% and 4% NaOH pretreatment, respectively.

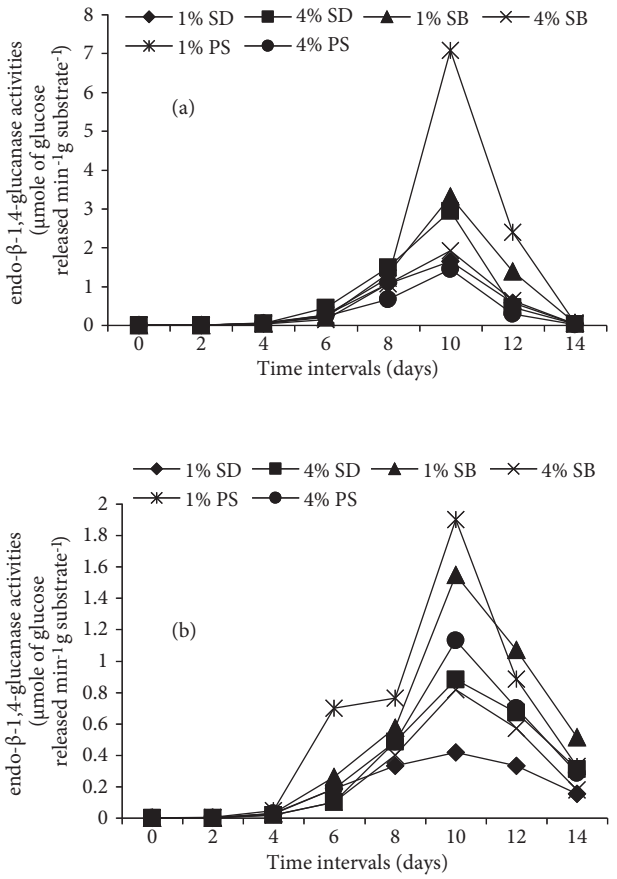

Figure 3. Endo-β-1, 4-glucanase activities of *Pleurotus ostreatus* (a) and *Pleurotus sajor-caju* (b) (Mean value of n = 4). SD = saw dust, SB = sugarcane bagasse, PS = paddy straw; 1% and 4% stand for 1% and 4% NaOH pretreatment, respectively.

diminished gradually. The highest concentration (40.2 mg g^{-1}) was found when 1% PS was the substrate, while the lowest level (16.5 mg g^{-1}) was for 1% SD.

Enzyme activity

Endo-β-1, 4-glucanase activity was found to reach a peak at 10 days of fermentation in *P. Ostreatus* (Figure 3a), after which the levels diminished gradually. The maximum activity (7.08 units) was found when 1% PS was the substrate, while the minimum activity (1.45 units) was for 4% PS. Endo-β-1, 4-glucanase activity was found to reach a peak at 10 days of fermentation in *P. sajor-caju* (Figure 3b), after which the levels diminished gradually. The maximum (1.9 units) was found when 1% PS was the substrate, while the minimum level (0.41 units) was for 1% SD. Exo-β-1, 4-glucanase activity was found to reach a peak at 10 days of fermentation in *P. Ostreatus* (Figure 4a), after which the levels diminished gradually. The highest activity (7.36 units) was found when 1% PS was the substrate, while the lowest level (1.88 units) was for 1% SD. Again the exo-β-1, 4-glucanase activity was found to reach a peak at 10 days of fermentation in *P. sajor-caju* (Figure 4b), after which the levels diminished gradually. The maximum activity (2.03 units) was found when 4% SB was the substrate, while the minimum level (0.38 units) was for 1% SD. β-glucosidase activity was found to reach a peak at 10 days of fermentation in *P. osreatus* (Figure 5a), after which the levels diminished gradually. The maximum activity (3.60 units) was found when 1% PS was the substrate, while the minimum level (1.29 units) was for 1% SD. β-glucosidase activity was found to reach a peak at 10 days of fermentation in *P. sajor-caju* (Figure 5b), after which the levels diminished gradually. The maximum activity (2.63 units) was found when 1%

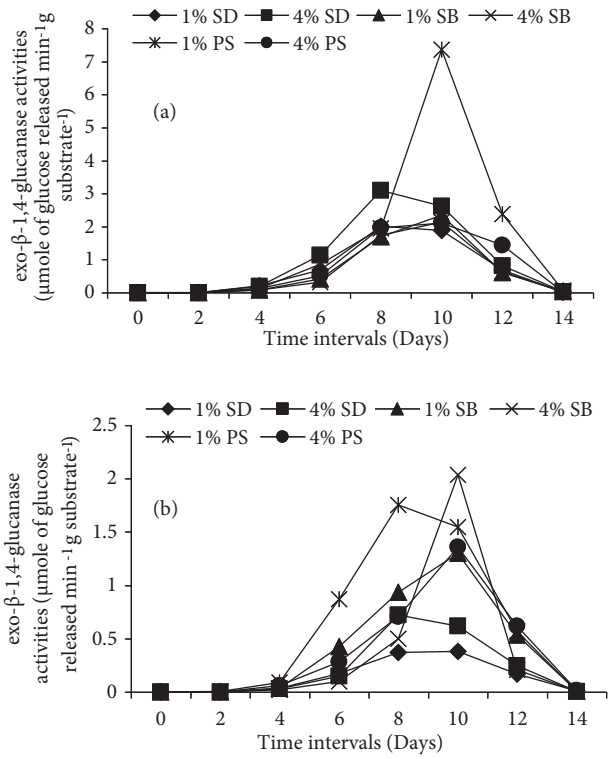

Figure 4. Exo-β-1, 4-glucanase activities of *Pleurotus ostreatus* (a) and *Pleurotus sajor-caju* (b) (Mean value of n = 4). SD = saw dust, SB = sugarcane bagasse, PS = paddy straw; 1% and 4% stand for 1% and 4% NaOH pretreatment, respectively.

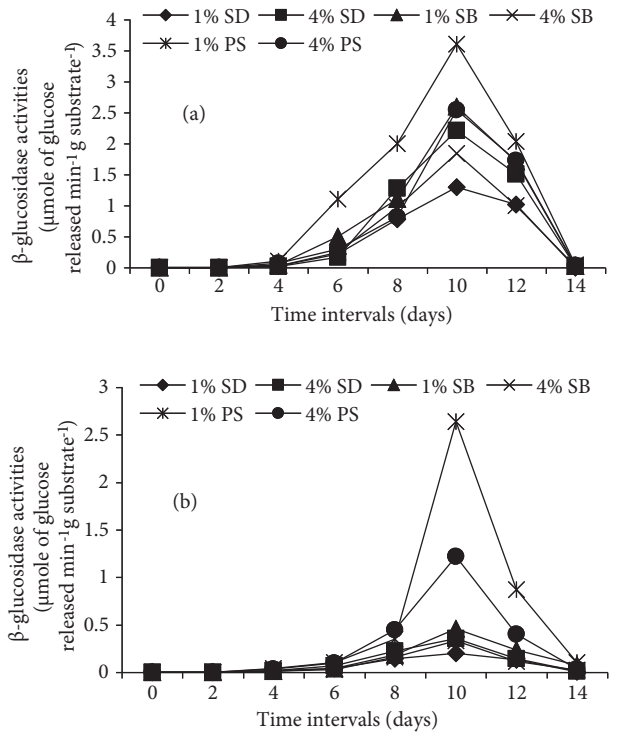

Figure 5. β-glucosidase activities of *Pleurotus ostreatus* (a) and *Pleurotus sajor-caju* (b) (Mean value of n = 4). SD = saw dust, SB = sugarcane bagasse, PS = paddy straw; 1% and 4% stand for 1% and 4% NaOH pretreatment, respectively.

PS was the substrate, while the minimum level (0.20 units) was for 1% SD. For the FPase assay, maximum enzyme activity was found at 10 days of fermentation in *P. osreatus* (Figure 6a), after which the levels diminished gradually. The maximum activity (3.51 units) was found when 1% PS was the substrate, while the minimum level (0.86 units) was for 1% SD. The maximum enzyme activity was found at 10 days of fermentation in *P. sajor-caju* (Figure 6b), after which the levels diminished gradually. The highest activity (0.83 units) was found when 1% SB was the substrate, while the lowest level (0.19 units) was for 1% SD.

Discussion

Alternative fuels made from renewable resources such as fuel ethanol provide numerous benefits in terms of environmental protection, economic development, and national energy security (Antoni et al. 2007). Cellulosic biomass like trees, grasses, and crops are renewable energy, such as biofuels/bioenergy, important for human development (Farrell et al. 2006). Cellulose is converted into fermentable sugars by the enzyme cellulase, and cellulase based biorefinery technologies are versatile and flexible because they utilize cheaper substrates for enzyme synthesis (Mane et al. 2007). Lignin prevents lignocelluloses from swelling and is responsible for the fiber integrity and rigidity of lignocelluloses (Smith and Wood 1991). Thus, the presence of lignin constitute the factors which are responsible for the most recalcitrant component of lignocellulosic material to enzymatic hydrolysis by restricting the enzyme accessibility (Martinez et al. 2005); therefore, the pretreatment of lignocellulosic mass increases the reaction rate of enzymatic hydrolysis. The most renowned method for the pretreatment of cellulosic materials to increase digestibility of cellulosic materials is probably NaOH treatment (Gharpury et al. 1983). Optimum growth of fungus was found

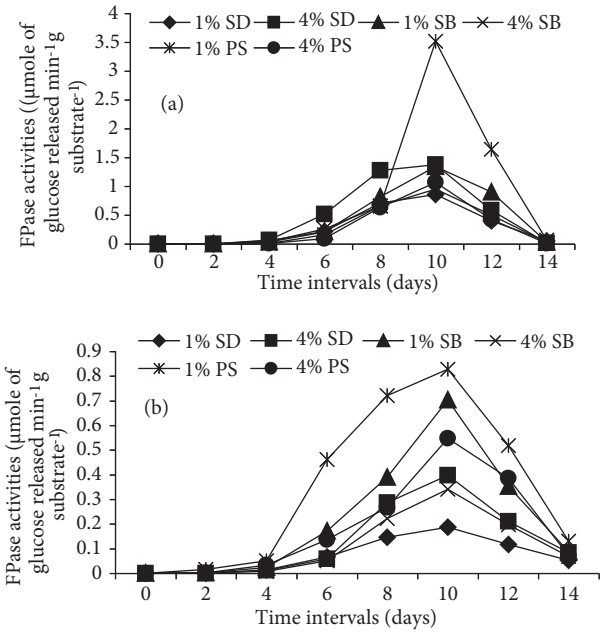

Figure 6. FPase activities of *Pleurotus ostreatus* (a) and *Pleurotus sajor-caju* (b) (Mean value of n = 4). SD = saw dust, SB = sugarcane bagasse, PS = paddy straw; 1% and 4% stand for 1% and 4% NaOH pretreatment, respectively.

by using PDA media at room temperature and at pH 5.5. Cellulase is produced either by solid-state or submerged culture. Fungi grow on relatively dry substrates on which bacteria cannot easily grow. Thus, for large scale production of Cullulase, fungi are used by solid state fermentation (Hartree 1987). Moreover, SSF is easy and simple, and also requires less energy compared to the liquid fermentation process. Considering all of these reasons, we chose the SSF method for this study. Since the selected strains grew optimally at room temperature and a pH of 5.5, these conditions were maintained in the solid state cultivation. The other factors important for SSF are moisture content, carbon, and nitrogen sources in the fermentation media. Cellulosic wastes themselves acted as the carbon source, so no additional carbon source was required in the fermentation medium. L-asparagine was added as a source of organic nitrogen. The whole fermentation medium worked as a complex medium for growth of fermentating fungus.

In this study, we have found enzymatic activities to reach a peak at 10 days of fermentation (for both *P. ostreatus* and *P. sajor-caju*). Ghosh et al. (1998) reported the maximum activity of lignocellulose degrading enzymes at 16-24 days of SSF. They used banana pseudostem as lignocellulosic biomass. However, Reddy et al. (2003) found the maximum enzyme activity at 10 days in pseudostem and at 20 days on leaf biomass fermentation in both *P. ostreatus* and *P. sajor-caju*. In this study, we have found higher protein and reducing sugar content after 10-12 days of fermentation. Reddy et al. (2003) also reported that protein production increases up until the 12th day and then decreases, only to increase again, and reach another peak at 20-25 days. Kurt and Buyukalaca (2010) reported the highest laccase enzyme activity at 10 days and carboxymethyl cellulase activity at 5-8 days on SSF of saw dust and straw in *P. ostreatus* and *P. sajor-caju.* Therefore, fewer days required for maximum enzyme activities, and the increased sugar and protein production on saw dust, straw, and baggase, compared to banana leaves or pseudostems may be due to the effect of NaOH pretreatment, or lignocellulolytic enzyme activities being higher on these substrates.

In most cases, it was found that 1%-NaOH treated substrates were much more efficient compared to 4% NaOH treated substrates. This occurred because strong alkali treatments dissolved the lignin seal instead of removing it. The dissolved lignin has been regarded as an effective inhibitor of cellulase (Berlin et al. 2006), since when dissolved, lignin becomes available to microorganisms, which then switch to the lignin degrading metabolism to gain energy. This is due to the fact that the *Pleurotus* species are more prominent in ligninase production compared to cellulase. However, 1% NaOH treated paddy straw showed greater efficiency than 1% NaOH treated sugarcane bagasse. This may be due to the fact that paddy straw contains less lignin compared to bagasse.

From these results, it is clear that exoglucanase activity is much higher in *P. ostreatus* compared to *P. sajor-caju*. This is also true for generating endoglucanase and β-glucosidase. Similar results were also reported by Ghosh et al. (1998). The potency of *P. ostreatus* in lignocellulolytic activity over other mushrooms like *Lentinus* spp. has also been reported (Elisashvili et al. 2008).

Conclusions

From this study, it can be concluded that *P. ostreatus* is much more efficient in cellulase enzyme production compared to *P. sajor-caju*. Therefore, *P. ostreatus* can be considered as an efficient source of cellulase enzyme and further research may help us to reach the ultimate goal of producing eco-friendly bioethanol. In the current study, even though *P. sajor-caju* has produced cellulase enzymes, cellulase production using *P. sajor-caju* may not be a practical proposition, since the activity was very low. This strain may continue to be used as an edible protein rich food, since it produces a higher level of soluble protein in the medium.

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