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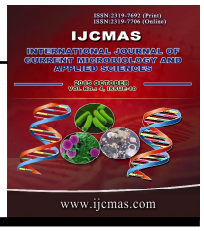


Original Research Article

Extracellular Lignocellulolytic Enzymes by *Phanerochaete chrysosporium* (MTCC 787) Under Solid-State Fermentation of Agro Wastes

Sai Prashanthi Govumoni[1], Jahnavi Gentela[1], Sravanthi Koti[1], V. Haragopal[3], S.Venkateshwar[2] and L.Venkateswar Rao[1]*

[1]Department of Microbiology, Osmania University, Hyderabad, Telangana State, India
[2]Methodist College of Engineering & Technology, Hyderabad, Telangana State, India
[3]Department of Statistics, Osmania University, Hyderabad, Telangana State, India
**Corresponding author*

Keywords

Lignocellulosic substrate, Laccase, Xylanase, Cellulase, Solid state fermentation, *Phanerochaete chrysosporium*

A B S T R A C T

The aim of this work is to study the production of lignocellulolytic enzymes by *Phanerochaete chrysosporeum* (MTCC 787) during solid state fermentation using five lignocellulosic substrates like wheat straw, rice straw, sorghum straw, corn cobs and *Prosopis juliflora.* This is the first report demonstrating the production of laccase, xylanase and cellulase simultaneously using various substrates by *P. chrysosporium*. The enzyme activities were observed in a medium containing 10 g of each substrate, moistened with malt extract broth and incubated for 13 days at 30°C. Among the five agro-residues used, the maximum laccase activity of 10.5 ± 0.4 IU/ml was achieved on 11th day of fermentation using wheat straw when compared with rice straw, *Prosopis*, sorghum and corn cobs. The maximum xylanase activity of 28.5 ± 1.1 IU/ml was obtained on 9th day using corn cobs, with wheat straw it was 20.4 ± 0.9 IU/ml and sorghum showed lowest xylanase activity. Rice straw was found to be most effective for cellulase production resulting in 1.6 ± 0.06 IU/ml on 5th day of fermentation compared to other five substrates. The data when analyzed by three-way ANOVA showed that the enzyme activities were significant for all substrates.

Introduction

There are numerous organisms that rely on biomass degradation for their survival. Current commercial products for biomass treatment are derived from fungi as these organisms produce a complex mixture of enzymes at high productivity and catalytic efficiency, both of which are required for low-cost enzyme supply (Sandra T. Merino, and Joel Cherry, 2007).

Among the microbial populations, fungi are recognized as the prominent source by the researchers, due to their extreme facility in producing a large variety of extracellular enzymes and easy handling; among them wood-rotting Basidiomycete fungi are identified as the best degrader of lignocellulosic materials (Arrivukkarasan Sanjeevirayar *et al.,* 2014-15).

Fungi have two types of extracellular enzymatic systems; the hydrolytic system, which produces hydrolases that are responsible for polysaccharide degradation and a unique oxidative and extracellular ligninolytic system, which degrades lignin and opens phenyl, rings (Carmen Sánchez, 2009). The White rot fungi have the ability to produce laccase, xylanase and cellulase that act together to degrade the components of lignocelluloses. The role of laccase is in lignin degradation (Tien and Kirk, 1984) while xylanase and cellulase are involved in hemicellulose and cellulose degradation. The hyphae of the white rot fungi penetrates inside the lignocellulosic wood and its extracellular enzymatic activity effectively catalyses the degradation. Therefore their application in the bioconversion of agricultural by-products rich in lignocellulose has attracted much attention (Agosin *et al.,* 1985). The white-rot basidiomycete *Phanerochaete chrysosporeum* has been studied widely because of its ability to degrade lignocelluloses as reviewed by Kirk and Farrell (1987). Recent works have shown that the enzyme production has increased using basidiomycetes on lignocellulosic substrates.

Laccases (E.C. 1.10.3.2, *p*-benzenedial: oxygen oxido reductases) are able to catalyze the oxidation of various aromatic compounds with the concomitant reduction of oxygen to water. There have been reports on the production of extracellular laccases by many species of white rot fungi when grown on natural substrates such as cotton stalk (Ardon *et al.,* 1996), molasses waste (Kahraman and Gurdal, 2002), wheat bran and barley bran. Laccase has received much attention because of its relatively low substrate specificity in comparison to most of the enzymes. Xylanases (E.C.3.2.l.8) are inducible enzymes which are responsible for complete hydrolysis of xylan, the major component of the hemicellulosic complex. Different lignocellulosic substrates, such as wheat bran and other agro-wastes, have been used as substrates to produce high yields of xylanolytic enzymes using solid state fermentation (Dobrev *et al.,* 2007). Cellulases, a group of hydrolytic enzymes, hydrolyse the β-glycosidic bonds of native cellulose and related cello-oligosaccharides. It is the key enzyme of potential use for industrial saccharification of cellulosic materials into simple sugars.

The interest towards lignocellulolytic enzymes has been dictated by their wide applications in various fields of industries (Godfrey and West, 1996), particularly in bioremediation, biosensor developments (Nelson and Elisa, 2000; Nelson *et al.,* 2002) and also bioethanol production (Govumoni *et al.,* 2013). However, for effective lignocellulolytic enzyme production it is very important to exploit efficient microorganism with suitable fermentation methods using cheap and abundantly available substrates.

Solid substrate fermentation is a promising technique for the production of industrially-relevant enzymes from large numbers of agro-industrial wastes using different organisms (Rodriguez Couto and Sanroman, 2005). Solid substrate fermentation has gaining importance in enzyme production because of its higher product titers, reduced energy requirements, simpler fermentation media and operating expenses as compared with submerged fermentation (Pandey *et al.,* 2001).

Some species could produce laccase and xylanase while having low activity of cellulase. This property will be beneficial for the removal of xylan from lignin-carbohydrate complexes which will facilitate

the separation of lignin from the fiber cell wall (Maheshwari *et al.,* 2000). Therefore, the present work is focused on the production of lignocellulolytic enzymes (Laccase, xylanase and cellulase) by *Phanerochaete chrysosporeum* (MTCC 787) using low cost lignocellulosic agro-based residues such as wheat straw, rice straw, corn cobs, sorghum straw and *Prosopis juliflora* under solid state fermentation.

Materials and Methods

Organism

The white rot fungi *Phanerochaete chrysosporeum* (MTCC 787) was procured from Institute of Microbial Technology, Chandigarh, India.

Media and growth conditions

P. chrysosporium culture was cultivated on malt extract agar. The medium contained (g/l): glucose, 5.0; malt extract, 2.0; yeast extract, 2.0; NH_4NO_3, 1; KH_2PO_4, 0.8; Na_2HPO_4, 0.2 and $MgSO_4.7H_2O$, 0.5 was autoclaved and the tubes were inoculated and incubated at 37°C for 3 days to allow the spores to germinate.

Substrates used for fermentation process

Wheat straw, rice straw, sorghum straw, corn cobs and *Prosopis juliflora* were screened to identify the suitable substrate for production of lignocellulolytic enzymes in solid-state fermentation. Substrates were oven dried at 50ºC.

Solid state fermentation using different substrates

Fermentation was carried out by mixing 10 g of each substrate with malt extract broth to attain the required moisture. The moisture content was optimized and 80% was chosen for experimentation. The flasks were autoclaved prior to inoculation with five agar plugs (8mm in diameter) of fungal colony grown on malt extract agar. After mixing, all flasks were incubated at 30 °C, maintained in triplicate and harvested on alternate day from 3rd day onwards till 13th day.

Enzyme extraction

A 0.05M acetate buffer (pH- 4.8) was added in each flask (1:20) and kept on a shaker at 150 rpm for 1 h. The content was filtered through filter paper and centrifuged at 10,000 rpm at 4 °C for 30 min and the supernatant was used to perform enzyme assays.

Analytical methods

Laccase activity was determined as described by Vasdev and Kuhad (1994). The reaction mixture contained 1.8 ml of 10 mM guaiacol in 0.05M acetate buffer (pH 5.0), and 0.2 ml crude enzyme solution. The reaction mixture was incubated at 26 °C for 30 min in dark and the absorbance was read at 470 nm. The buffer devoid of guaiacol was treated as blank. One unit of laccase activity was defined as the change in absorbance of 0.01mL^{-1} min^{-1}. Xylanase activity was measured with method described by Bailey *et al.* (1992). The reaction mixture contains 1.8 ml of 1.0% (w/v) birch wood xylan in 0.05M acetate buffer (pH 4.8) and 0.2 ml of appropriately diluted crude enzyme solution, were incubated at 50°C for 10 min and the reducing sugars were assayed by dinitrosalicyclic acid (DNSA) method of Miller (1959). One unit of xylanase was defined as the amount of enzyme to release 1 µmol of xylose per ml per minute. Cellulase activity (FPase) was determined in accordance with the International Union of Pure and Applied Chemistry procedures as

reported by Ghosh (1987). FP cellulase activity was assayed by measuring the release of reducing sugars in a reaction mixture containing Whatman No. 1 filter paper (50.0 mg) as substrate in 50 mM sodium citrate buffer (pH 4.8) at 50°C, after 60 min. Reducing sugars were assayed by dinitrosalicyclic acid (DNSA) method of Miller (1959). One unit of enzyme corresponds to the amount of enzyme to release 1 µmol of glucose per ml per minute.

Statistical analysis

All the experiments were performed in triplicates and the results were represented as mean ± standard deviation. Data was subjected to three-way Analysis of Variance (ANOVA) using the Statistical Package for Social Sciences (SPSS) version 19.

Results and Discussion

Chemical composition of lignocellulosic substrates

The main components of the feed stocks listed in table 1 are lignin, cellulose and hemicellulose. The highest content of lignin (24.6%), cellulose (45.5%) was found in *Prosopis juliflora* and hemicelluloses (37%) in corn cobs when compared with other substrates. In general lignocelluloses can be utilized by fungi as support nutrient and also as an inducer for enzyme production.

The enzyme activities on the lignocellulosic substrates were monitored for a period of 13 days. The results of enzyme activities varied markedly with the substrates, in the range of 10.5–0.04 IU/ml for laccase (Table 2), 28.5–2.55 IU/ml for xylanase (Table 3) and 1.61–0.01 IU/ml for cellulase (Table 4) at different incubation periods.

Laccase enzyme activity was less on 3rd day of incubation and increased up to 11th day, thereafter showing a declining trend. Decrease in laccase activity may be due to the toxicity of lignin degradation products such as phenolic compounds, located in polymer structure of lignin, which probably reduces the growth of the organism and the production of laccase. Similar result was observed by Chmelova *et al.* (2011) using the organism *Ceriporiopsis subvermispora*. Jasmina Cilerdzic *et al.* (2011) reported laccase activity of 2500 IU/L and 910 IU/L with corn stalks and wheat straw using *Tramatus hirusta.* In the current study, maximum laccase activity of 10.5± 0.4 IU/ml was achieved on 11th day using wheat straw as the substrate (Figure 1), it was reduced to 4.41 ± 0.2 IU/ml on 13th day of incubation. It is also evident from the graphical depiction that the organism exhibited greater activities of the enzyme on wheat straw as compared with other substrates at all incubation periods. In the present work, rice straw was found to produce laccase activity of 6.89 ± 0.31 IU/ml on the 11th day of fermentation, whereas according to Abdul Kareem Jasim Hashim (2012) laccase activity of 0.55 U/ml was obtained with sawdust using *Pleurotus ostreatus* on 15th day of fermentation. Sorghum straw, corn cobs and *Prosopis juliflora* showed high laccase activity of 3.88 ± 0.106 IU/ml, 3.47 ± 0.14 IU/ml and 6.23m ± 0.23 IU/ml on 9th day of fermentation respectively. Massimiliano Fenice *et al.* (2003) reported the laccase activity of 1309 ± 20 IU/L on 9th day of fermentation from white-rot fungus *Panustigrinus* using olive-mill wastewater.

In the current study, *P. chrysosporium* produced maximum xylanase activity of 28.58 ± 1.11 IU/ml on 9th day of fermentation using corn cobs as a substrate (Figure 2), the same organism when used by Broda *et al.* (1996), resulted in the xylanase activity of 0.669 IU/ml on 15th day using paddy straw. From the above study it can be

concluded that corn cobs was found to be potential substrate for xylanase production. Christakopoulos *et al.* (1996); Singh *et al.* (2000) also considered corn cobs to be an ideal substrate for xylanase production. In a study conducted by Uma Gupta and Rita Kar (2009) xylanase production was done using corncobs with *Bacillus* sp., however, the xylanase activity of 9.88 IU/ml was obtained after 48 h, it was found to be less when compared with the present study. After corn cobs, wheat straw and rice straw showed enzyme activity of 20.49 ± 0.98 IU/ml and16.11 ± 0.75 IU/ml on 9th day of fermentation. *Penicillium oxalicum* produced xylanase activity of 2.52 IU/ml using rice straw as a substrate under SSF (Muthezhilan *et al.*, 2007). The xylanase activity with sorghum straw and *Prosopis juliflora* were 13.35 ± 0.54 IU/ml and 15.09 ± 0.59 IU/ml on 11th day of fermentation.

Table.1 Chemical composition of lignocellulosic substrates

Lignocellulosic substrate	Lignin (%)	Cellulose (%)	Hemicellulose (%)	Reference
Wheat straw	20.6	32.6	24.7	Govumoni *et al.* (2013)
Rice straw	12	35	22	Srilekha Yadav *et al.* (2011)
Sorghum straw	11	30	31	Theander and Aman (1984)
Corn cobs	16.4	39.1	37	Mingjia Zhang *et al.* (2010)
Prosopis juliflora	24.6	45.5	20.3	Rishi Gupta *et al.* (2009)

Table.2 Laccase production under solid state fermentation using *P. chrysosporium* 787

Lignocellulosic Substrate	Laccase activity (IU/ml)					
	3rd Day	5th Day	7th Day	9th Day	11th Day	13th Day
Wheat straw	0.31±0.015	0.78±0.040	1.28±0.046	3.22±0.104	10.5±0.40	4.41±0.20
Rice straw	0.08±0.004	0.16±0.007	0.27±0.015	0.98±0.041	6.89±0.31	1.39±0.06
Sorghum straw	0.18±0.007	0.32±0.010	1.26±0.061	3.88±0.106	0.22±0.008	0.17±0.007
Corn cobs	0.04±0.002	0.16±0.006	0.92±0.036	3.47±0.14	0.12±0.004	0.11±0.004
Prosopis juliflora	0.06±0.003	0.08±0.003	1.19±0.054	6.23±0.23	2.2±0.055	0.84±0.030

Analyses were carried out in triplicate, Values are mean± standard deviation

Table.3 Xylanase production under solid state fermentation using *P. chrysosporium* 787

Lignocellulosic Substrate	Xylanase activity (IU/ml)					
	3rd Day	5th Day	7th Day	9th Day	11th Day	13th Day
Wheat straw	8.33±0.38	17.91±0.20	18.55±0.90	20.49±0.98	16.55±0.71	2.55±0.11
Rice straw	5.33±0.26	3.47±0.09	11.52±0.47	16.11±0.75	11.99±0.57	6.52±0.20
Sorghum straw	6.15±0.26	8.12±0.33	10.64±0.42	6.66±0.29	13.35±0.54	5.64±0.28
Corn cobs	8.77±0.44	9.41±0.30	12.06±0.48	28.58±1.11	17.87±0.74	10.13±0.3
Prosopis juliflora	8.24±0.27	7.24±0.27	12.67±0.58	10.87±0.43	15.09±0.59	9.65±40. 5

Analyses were carried out in triplicate, Values are mean± standard deviation

Table.4 Cellulase production under solid state fermentation using *P. chrysosporium* 787

Lignocellulosic Substrate	Cellulase activity (IU/ml)					
	3rd Day	5th Day	7th Day	9th Day	11th Day	13th Day
Wheat straw	0.13±0.021	0.45±0.02	0.22±0.009	0.14±0.007	0.02±0.001	0.01±0.002
Rice straw	0.19±0.007	1.61±0.065	0.91±0.025	0.08±0.004	0.05±0.002	0.04±0.002
Sorghum straw	0.02±0.012	0.14±0.005	0.11±0.006	0.02±0.002	0.01±0.002	0.01±0.002
Corn cobs	0.04±0.009	0.12±0.186	0.09±0.005	0.04±0.002	0.02±0.001	0.01±0.001
Prosopis juliflora	0.08±0.008	0.13±0.007	0.09±0.006	0.05±0.002	0.04±0.002	0.03±0.002

Analyses were carried out in triplicate, Values are mean± standard deviation

Table.5 Analysis of variance for lignocellulosic substrates, enzymes and days

	SS	df	MS	F	Sig.
Day	54.610	5	10.922	2279.502	.000
Substrate	32.081	4	8.020	1673.871	.000
Enzyme	146.160	2	73.080	15252.188	.000
Day * Substrate	84.808	20	4.240	884.994	.000
Day * Enzyme	156.607	10	15.661	3268.482	.000
Substrate * Enzyme	49.189	8	6.149	1283.258	.000
Day * Substrate * Enzyme	179.272	40	4.482	935.375	.000
Error	.862	180	.005		
Corrected Total	703.589	269			

*p ≤0.05
SS – sum of squares; df – degrees of freedom; MS – mean square

Figure.1 Laccase production of *P. Chrysosporium* 787 incubated on 11th day of fermentation

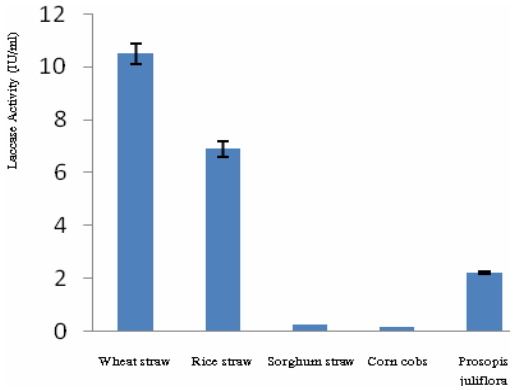


Figure.2 Xylanase production of *P. Chrysosporium* 787 incubated on 9th day of fermentation

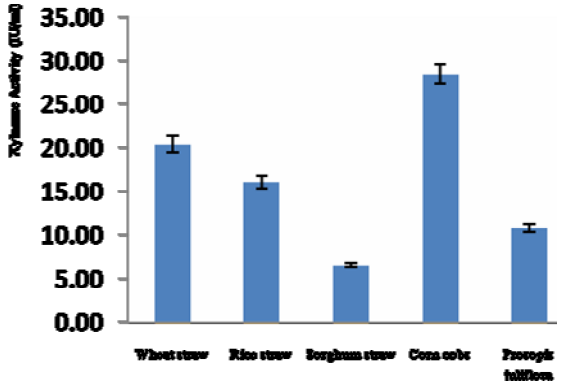
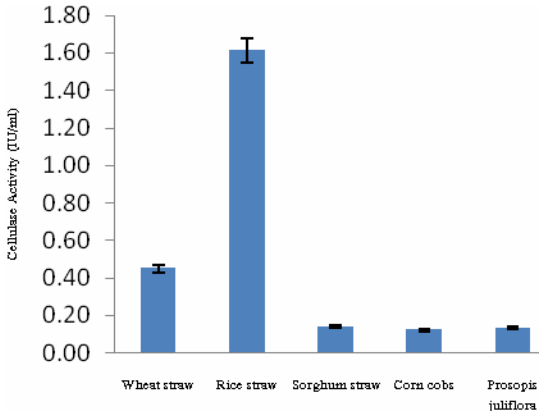


Figure.3 Cellulase production of *P. Chrysosporium* 787 incubated on 5th day of fermentation



The maximum cellulase production was obtained on 5th day of incubation and decreased on further incubation for all the five lignocellulosic substrates. Among the five agro-residues used, rice straw was found to be the most effective for cellulase production resulting in 1.61 ± 0.06 IU/ml (Figure 3), similar result was obtained during solid state fermentation of rice straw using *P. chrysosporium* which produced the highest cellulase enzyme of 1.43 IU/ml on 4th day (Munir *et al.*, 2007). After rice straw, wheat straw showed an activity of 0.45 ± 0.02 IU/ml on 5th day of fermentation, similar result was reported by Ashish vyas and Deepak vyas (2005) with the combination of *Aspergillus terreus* and *Tramatus viride* using ground nut shell as substrate. According to Mohammad Rizwan Pervez (2011), *Rhizopus* sp. gave maximum cellulase activity of 1.58 IU/ml in 120 h of incubation under solid state fermentation using sugarcane bagasse. Cellulase activity of other substrates were found to be 0.14 ± 0.005 IU/ml, 0.12 ± 0.018 IU/ml and 0.13 ± 0.009 IU/ml on 5th day of fermentation using sorghum straw, corn cobs and *Prosopis juliflora* respectively. Though the cellulose content is in low amounts in rice straw, the cellulase activity was high when compared with *Prosopis* and corn cobs. According to Krishna (2005) the type and composition of the carbohydrates present in rice straw are suitable for the induction of cellulases in filamentous fungi under solid-state fermentation. The cellulase production by *P.*

chrysosporeum was found to be low when grown on other lignocellulosic materials like corncobs and *P. juliflora*.

It is suggested that *P. chrysosporeum* utilizes cellulose and hemicellulose as carbon source during the initial growth phase which results in the production of cellulase on 5th day and xylanase on 9th day of fermentation. As the level of carbon source decreases, induction of laccase synthesis initiates, which finally results in the increase of laccase production on 11th day (Machuca and Ferraz 2001). In comparison between laccase, xylanase and cellulase, it is evident that the production of xylanase was approximately two times higher than laccase and eighteen times higher than cellulase. Similar result was achieved by Souza-Cruz *et al.* (2004) and Heidorne *et al.* (2006).

Statistical analysis

This study accentuates the necessity of screening of substrate having different structural constituents to disclose the actual potential of the white rot fungus expressing the lignocellulolytic enzymes. ANOVA was used to analyze the results of the experiment and to determine the variation. The statistical analysis revealed that the substrates were highly significant for the production of three enzymes. Jeffries (1994) stated that the composition and percentages of polymers vary from one plant species to another.

Multiple comparisons was performed between the three enzymes, substrates and days to know their pair wise effect, it was found that all the pairs differ significantly, indicating that they have no similarities among them. From table 5, the F value shows that the model is significant at a 95% confidence level. The p-value being less than 0.05 (<0.0001) indicates that the model is significant. This means that the variables had a significant effect on the response. The outstanding ability of secreting the varieties of hydrolytic and oxidative enzymes by White rot fungi makes them to grow efficiently on the lignocellulosic substrates and they are very indispensable for lignin, cellulose and hemicellulose degradation. Moreover, their secretion pattern depends upon the nature of substrates, organisms employed and nature of the cultivation mode (Arrivukkarasan Sanjeevirayar *et al.*, 2014-15). When laccase production was performed with five different substrates, the activity of laccase in all substrates was significant except in pair of sorghum straw and corncobs. This shows that laccase production is highly affected even by the nature and composition of the lignocellulosic substrate. As far as the production of xylanase is concerned, the pair wise combinations of all five different substrates were found to be significant. In the production of cellulase, no significant difference can be observed in the pair wise combination of sorghum straw, corncobs and *prosopis juliflo*ra. The combinations between other substrates were observed to be significant.

In conclusion, most researchers have investigated the effect of lignocellulosic materials on individual enzymes using solid-state fermentation. As per our literature survey, this is the first report showing the production of laccase, xylanase and cellulase using various lignocellulosic substrates by *P. chrysosporeum*.

The organism can be grown on low cost agricultural wastes and was found to be a promising strain for the production of commercial enzymes. It is evident from experimental and statistical analysis in the present study that, among five different substrates used, wheat straw, corn cobs and

rice straw can serve as the best solid substrates for production of laccase, xylanase and cellulase enzymes respectively by *P. chrysosporeum.*

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