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Full Length Research Paper

Studies on lignocellulose biodegradation of coir waste in solid state fermentation using *Phanerocheate chrysosporium* and *Rhizopus stolonifer*

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The solid state fermentation is one of the most economically viable processes for the bioconversion of lignocellulosic coir waste is represented by *Phanerochaete chrysosporium* and *Rhizopus stolonifer.* Coir pith is a waste lignocellusic material; it consists of lignin, cellulose, hemicellulose and protein. The two fungal cultures are able to synthesis varying quantities of lignocellulytic enzymes (cellulase, xylanase, ligninase, and protease, laccase and lignin peroxidase) that are required for substrate bioconversion. For example, *P. chrysosporium* produces two extracelluler enzymes (laccase and lignin peroxidase). They have been associated with lignin depolymerisation in other fungi. Fermentation was carried out over 35 days and the bioconverted sample was analyzed at 7 days intervals, the highest and most significant lignocellulytic enzyme activity ($P < 0.05$) as well as lignocellulosic compound ($P < 0.05$) conversion was observed on day 35 in *P. chrysosporium* and coculture mediated fermentation. *P. chrysosporium* and coculture was more efficient than *R. stolonifer*. The maximum amount of laccase and lignin peroxidase produced by *P. chrysosporium* and coculture was approximately (5 and 8.1 IU/ml, respectively) after 28 days of fermentation.

Key words: Laccase, lignin peroxidase, cellulase, xylanase, protease, *Phanerochaete chrysosporium, Rhizopus stolonifer*, coculture, solid state fermentation, coir pith.

INTRODUCTION

White-rot fungi, which have lignocelluloses degrading enzymes, play important roles in carbon recycling in nature, because lignin, next to cellulose, is the second most abundant organic carbon compound on earth. The white-rot fungi degrade lignins not only to use them as carbon sources but also to remove a physical barrier against cellulose utilization. Due to their powerful degrading capabilities towards various recalcitrant chemicals, white-rot fungi and their lignin degrading enzymes have long been studied for biotechnical applications such as biobleaching (Takano et al., 2001), biodecolorization (Dias et al., 2003) and bioremediation (Beltz et al., 2001; Cheong et al., 2006). The lignin degrading enzymes consist of laccase, lignin peroxidase, manganese peroxidase and H_2O_2-supplying glucose oxidase for the peroxidase reactions. *Phanerochaete chrysosporium* is one of the most widely studied white-rot fungi with regards to lignin degrading enzymes (Tien and Tu, 1987). It has drawn considerable attention as an appropriate host for the production of lignin-degrading enzymes or direct application in lignocellulose bioconversion processes (Ruggeri and Sassi, 2003; Bosco et al., 1999).

Coir pith is a lignocellulosic waste material consists of lignin 20 - 40%, cellulose 40 - 50%, hemicellulose 15 - 35% and protein 2.04% (Sjostrom, 1993). Coir is produced from the fibrous of coconut (*Cocos nucifera* L). More than 1423 million coconuts were produced in Tamilnadu with an average of 10,000 nuts/ha from which one ton of coir fiber and another one ton of coir pith became available. The estimated annual production of coir pith in coir industries of India is about 7.5 million tons (Kamaraj, 1994) and accumulates every year which leads

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to pollution of the environment. In recent years these agro waste materials were converted to biofertilizers using several microbes and their enzymes synthesized during solid state fermentation (SSF). Coir-wastes are a very suitable raw material for the production of lignolytic enzyme by microorganisms in solid state fermentation.

Solid state fermentation is an attractive process to produce fungal microbial enzymes (Chahal et al., 1996; Haltrich et al., 1996; Jech, 2000). SSF is characterized by the complete or almost complete absence of free liquid or water, which is essential for microbial activities. The water is present in an absorbed or in complexes form with the solid matrix and the substrate (Cannel and Moo-Young, 1980). These cultivation conditions are especially suitable for the growth of fungi, known to grow at relatively low water activities. As the microorganisms in SSF grow under conditions closer to their natural habitats and they are capable of producing enzymes and metabolites that will not be produced or will be produced only in low yield in submerged conditions (Jech, 2000). SSF are considered practical for complex substrate fermentation including agricultural, forestry and food-processing residues and wastes which are used as the carbon source (Haltrich et al., 1996).

The present study focuses on cellulytic and lignolytic enzymes has produced by indigenous fungal micro flora such as *P. chrysosporium* and *Rhizopus stolonifer* on coir waste substrate. These fungi have been successfully used for the enhancement of digestibility of lignocelluloses (Weiland, 1988) and improvement of feed value (Dias-da-silva and Sundstol, 1986).

MATERIALS AND METHODS

Agro waste material

Coir waste material was collected from Sri Ram Mills, Chozhavanthan, Madurai, Tamilnadu and India. It was sun dried and sieved (5 mm mesh) to remove long fibers, stored in gunny bags and used within one month after procurement.

Procurement and selection of microorganisms

P. chrysosporium (NCIM 1197) was procured from the national chemical laboratory Pune, India. The cultures were maintained on PDA slants and stored at 4°C. *R. stolonifer* was previously isolated by primary selection from a sample of naturally contaminated coir waste by serial dilution and pour plate technique. The isolated culture was identified by their morphology, colony characteristics and electron microscopy.

Solid state fermentation

Solid state bioconversion or fermentation (SSF) of coir waste was carried out in 250 ml Erlenmeyer flasks. 20 g of coir waste was placed in individual flasks and 60 ml of distilled water was added to give a moisture content of 70%.The flasks were plugged with cotton and autoclaved at 121°C for 15 min. Agar blocks (8 mm disc) were removed from the plates containing 7 days old cultures of *P. chrysosporiom* and *R. stolonifer* and used as inoculums for SSF experiments. A single block was removed for each organism and aseptically inoculated into the individual flask containing the substrate. The fermenting organisms, *P. chrysosporium* and *R. stolonifer* were simultaneously inoculated into the individual or same flasks containing the substrate. Three replicates were maintained for each organism. All the flasks were incubated at a room temperature for 35 days. Bioconverted coir waste samples were withdrawn at intervals of 7 days, oven-dried at 60°C and analyzed their cellulose, hemi cellulose, lignin, protein and reducing sugars content. Also estimated, the amount enzymes (CMC ase, filter paper activity, β- glucosidase, protease, laccase, lignin peroxidase and xylanase) were produced during the course of fermentation (Jech, 2000)

Analytical procedure

The concentration of cellulose was estimated by using the method described by Updegraff (1969). Hemicellulose concentration was determined using the method described by Deschatelets and Yu (1986). Lignin concentration was determined using the gravimetric method of Chesson (1978).

Reducing sugar concentration was determined using the DNS (dinitro salicylic acid) method (Miller, 1959). Protein content was determined by using the method described by Lowry et al. (1951).

Enzyme assays

The activity of the enzyme cellulase was measured by using the method described by Ray et al. (1993). Xylanase enzyme assays were carried out with some modification described by Bailey et al. (1992). Commercial xylose (Sigma) was used as a standard. One unit of xylanase activity was defined as the amount of enzyme that catalysed the release of 1 µm of xylose per min.

Protease enzyme activity was assayed by the method of Dawson et al. (1959). One unit of enzyme activity was defined as the amount of enzyme required to solubilize 1 M of TCA soluble material calculated as tyrosine per 120 min at 30°C.

Among the lignolytic enzymes the activity of laccase enzyme was measured by using the method described by Dhaliwal et al. (1991). The enzyme lignin peroxidase (LiP) activity was assayed by calculating the difference in absorbance as adapted by (Perumal and Kalaichelvan, 1996). One unit of enzyme activity was defined as the amount of enzyme causing the change of one absorbance unit per min.

Statistical analysis

The results were expressed as means ± standard error and the data were analyzed using Turkey new multiple range test for significant differences among compound and enzymes with an SPSS package. A level of $P < 0.05$ was accepted as statistically significant.

RESULTS AND DISCUSSION

In the preliminary experiment, out of 6 fungal strains, only two fungal strains (*P. chrysosporium* and *R. stolonifer*) were able to degrade the coirwaste supplemented with urea. Among these two, the white rot fungus *P. chrysosporium* showed faster linear growth and mycelial proliferation than filamentous fungi *R. stolonifer* (Table 1).

Table 1. Linear growth and mycelial density of different fungi on coir waste+urea substrate.

Fungi	Linear growth (days)	Mycelial density
Aspergillus niger	19	+
Aspergillus terreus	22	+
Aspergillus flavus	24	+
Penicellium sps	17	++
Rhizopus stolonifer	14	+++
Phanerochaete chrysosporium	12	++++

Results are mean ± S.E of 3 replicates.
+ Poor; ++ moderate; +++ good; ++++ dense.

Table 2. Measurement of lignin degradation and coir waste utilization by *R. stolonifer* and *P. chrysosporium*.

Fungi	Tannic acid medium		Radial growth (mm)
	Diffusion zone (mm)	Class	
R. stolonifer	78 ± 2.0	V	38 ± 1.0
P. chrysosporium	90 ± 2.5	III	44 ± 1.5

Results are mean ± S.E of 3 replicates.

These results closely agree with the reports of Onions et al. (1981). Based on the linear growth and mycelial density, the white rot fungi *P. chrysosporium* and filamentous fungi *R. stolonifer* were selected for lignin degradation. Although their lignin degradation ability was analyzed in both solid tannic acid medium and coir waste medium prepared by coir waste replacing lignin in Day et al. (1949) medium, *P. chrysosporium* showed more brown color diffusion zone in both tannic acid (90 ± 2.5) and coir waste medium (44 ± 1.5). Whereas *R. stolonifer* showed less zone than white rot fungi, *P. chrysosporium* (Table 2). Based on their degradation ability and diffusion zone, they were classified as class III and IV. These results have positively correlated with results of Subba Rao (1993). Akin et al. (1995) reported that the wood rot fungi decompose and utilize the various agricultural wastes and suggested their cultivation for recycling of agricultural waste into food and feed.

Many authors reported that substrate lignin content was negatively correlated with saccharification of cellulose and hemicellulose (Ray et al., 1993; Ramamoorthy et al., 1999). But we observed that the higher lignin content of coir waste did not affect the cellulose and hemi cellulose degradation. Maximum 67 % of cellulose and 71% of hemi cellulose degradation was observed in the coir waste substrate during the growth of co culture; this might be due to the smaller particle size of coir waste, which was one of the important factors for biodegradation of lignin (Chahal, 1991). The highest rate (25 and 26%) of lignin degradation was monitored when *P. chrysosporium* and coculture *(P. chrysosporium + R. stolonifer*) was grown in the coir waste substrate (Figure 1). These results were similar with reports of Geetha and Sivaprakasam (1998).

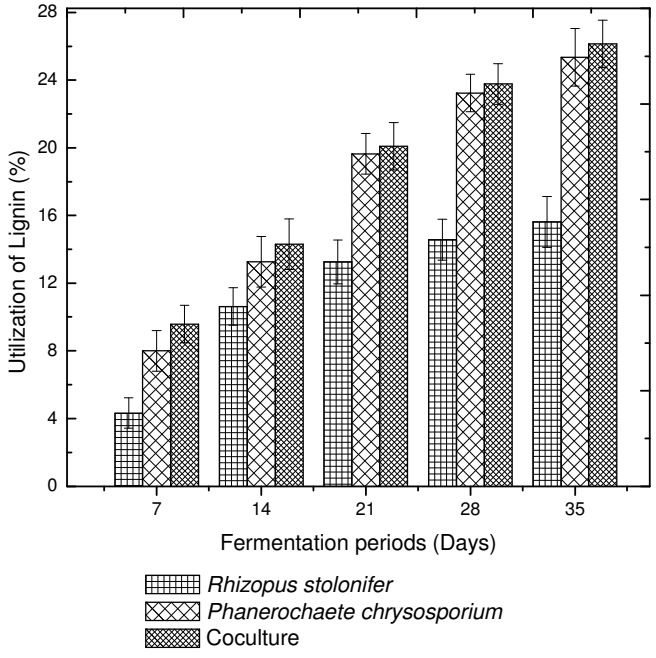

Figure 1. Utilization of lignin (%) in coirwaste substrate during solid state fermentation with *R. stolonifer, P. chrysosporium* and coculture. Mean ± S.E indicated. The lignin conversion was significantly at probability level ($P < 0.05$).

The rate of cellulose degradation was higher (67.1 ± 0.03) in day 21 of fermentation period, while lignin degradation was higher between 21 and 28 days of fermentation. These results were supported by the work of Kannan and Obilisamy (1990) who studied the degra-

Table 3. Production of reducing sugar (mg/g) in coir waste supplemented with urea substrate during solid state fermentation with selected fungi.

Fungi	Fermentation periods (Days)					
	0	7	14	21	28	35
Rhizopus stolonifer	10.2 ± 0.7	11.5 ± 0.9	13.8±0.8	1.9 ± 0.9	22.5 ± 1.2	1.3 ± 1.1
P. chrysosporium	-	12.5 ± 0.8	14.5 ± 0.8	24.3 ± 1.2	28.4 ± 1.3	28.5 ± 1.4
Coculture	-	14.4 ± 1.1	16.3 ± 1.2	26.2 ± 1.8	30.5 ± 1.5	29.2 ± 1.4

Results are mean ± S.E of 3 replicates.

dation of paper mill sludge by *Pleurotus sajor – caju.* Loss of hemi cellulose was higher than cellulose and lignin in coir waste supplemented with urea (Figure 3). Similar results have been reported by Ghose and Nandi (1995) during water hyacinth degradation by *Pleurotus sps*. Most of the white rot fungi including *P. chrysosporium* have higher lignin degrading ability and it's coupled with higher amount of enzyme xylanases production.

Table 3 depicted that higher amounts (30.5 ± 1.5 mg/g) of reducing sugars were monitored when the growth of coculture in coir waste medium. However the fungus *R. stolonifer* and *P. chrysosporium* produced near to the value of coculture at 28 days of fermentation. Also the coculture showed maximum cellulase enzyme activities on the 28 days of incubation while the activities of *R. stolonifer* and *P. chrysosporium* cellulase enzymes were observed maximally on the same day of fermentation (Figure 4). Ray et al. (1993) reported that the higher amount of cellulase and xylanase enzyme production by *Aspergillus terreus* during the course of solid state fermentation.

The tested organisms grown in coir waste supplemented with urea could increase the mycelial protein content more than 0.3 fold over the control values even after 28th days by *R. stolonifer* (Table 4). The coculture and monoculture of *P. chrysosporium* produced 0.4 fold mycelial protein on the same period. The crude protein content of mold infused coir waste ranged from 18.9 to 27.5 mg/g under urea supplemented coir waste substrate. The protein content was increased by the loss of inorganic matter (Singh et al., 1994; Puniya et al., 1996). Rangasami et al. (1975) have reported that the rate of lignocellulosic material degradation was increased by supplementation of various nitrogen sources. From the above results, our study indicated that all parameters related to growth in the composition of coir waste were influenced more by addition of urea.

The higher amount of cellulose was utilized by coculture than that of *R. stolonifer* and *P. chrysosporium* (Figure 2). *R. stolonifer* utilized 52.42% of cellulose in 35 days of fermentation. But cellulose utilization was performed more potentially by *P. chrysosporium* than *R. stolonifer*. Ray et al. (1993) indicated that the cellulase production was essential for the efficient degradation of cellulose.

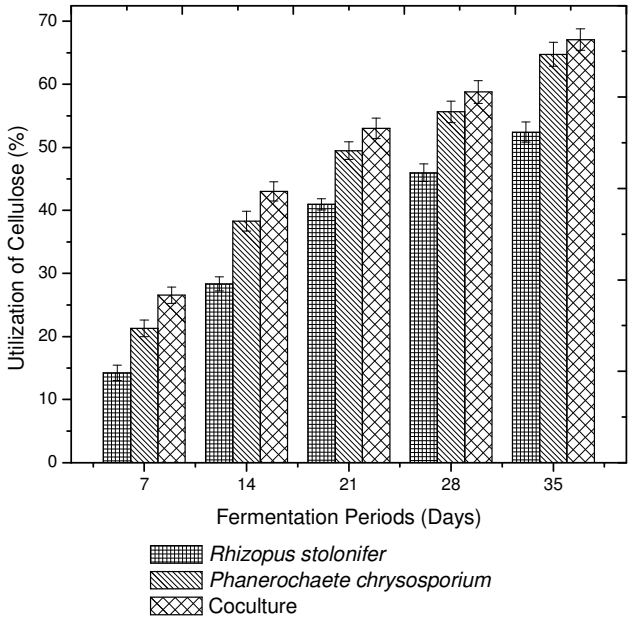

Figure 2. Utilization of cellulose (%) in coirwaste substrate during solid state fermentation with *R. stolonifer, P. chrysosporium* and coculture. Mean ± S.E indicated. The cellulose conversion significantly at probability level ($P < 0.05$).

Higher amount of cellulase production was observed in the growth of coculture and it was estimated by the activities of three cellulose components such as CMCase, filter paper activity and β-glucosidase assay (Figure 4, 5 and 6).

Coirwaste was used as lignocellulose materials for bioconversion. During the bioconversion, hemicellulose was effectively degraded by producing the enzyme xylanase. The increased amount of xylanase (16.4 IU/ml) was recorded after 28th days of incubation period (Figure 7). Xylan has interfered with the synthesis of cellulase particularly when the culture was grown in the hemicellulose substrate. Similar results have been reported earlier by Gamerith et al. (1992). Several hypotheses have reported the role of proteases in wood rotting fungi. Eriksson and Peterson (1982, 1988) indicated their possible implication in the release of lignolytic enzymes from the fungal cell wall. On the other hand, Dosoretz et al. (1990) postulated that one of the functions of the pro-

Table 4. Production of protein (mg/g) in coir waste supplemented with urea substrate during solid state fermentation with selected fungi.

Fungi	Fermentation periods (Days)					
	0	7	14	21	28	35
R. stolonifer	18.2 ± 1.2	19.8 ± 1.1	22.1 ± 1.4	24.1± 1.3	22.9 ± 1.3	21.0 ± 1.1
P. chrysosporium	-	20.2 ± 1.3	23.4 ± 1.2	24.5 ±1.5	26.4 ± 1.6	25.5 ± 1.7
Coculture	-	21.2 ± 1.2	24.3 ± 1.5	25.1± 1.6	27.5 ± 1.8	26.2 ± 1.9

Results are mean ± S.E of three replicates.

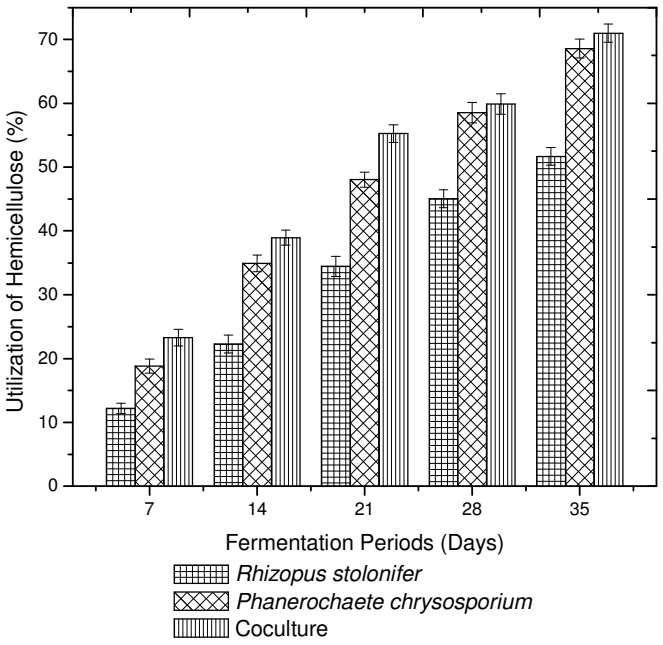

Figure 3. Utilization of hemicellulose (%) in coirwaste substrate during solid state fermentation with *R. stolonifer, P. chrysosporium* and coculture. Mean ± S.E indicated. The hemicellulose conversion significantly at probability level (P < 0.05).

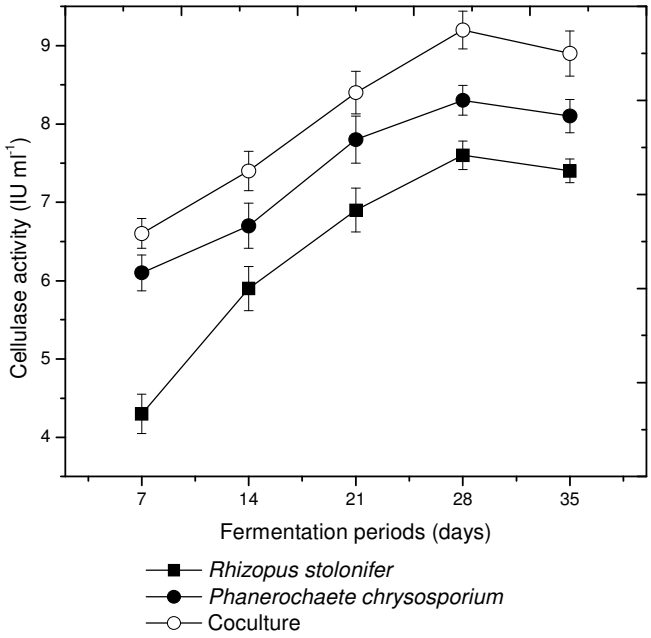

Figure 4. Cellulase enzyme activity (IU ml^{-1}) of coirwaste substrate and supplemented with urea substrate during solid state fermentation with *R. stolonifer, P. chrysosporium* and coculture. Mean ± S.E indicated. The cellulase activity significantly at probability level (P < 0.05).

teases produced by white rot fungi is to recycle nitrogen by break down of proteins released into the medium for cell autolysis. Furthermore several studies pointed out mediated degradation as a major cause of the decay of extracellular enzyme activities in the culture of white rot fungi (Dosoretz et al., 1990).

The pattern of protease enzyme production was different in both fungi. *P. chrysosporium* produced higher amount of protease (15.8 IU/ml) enzyme on 28th day of fermentation which was comparatively higher than *R. stolonifer.* But all the enzyme activity was higher in coculture studies. The protease production started at 7th day (8.8 IU/ml) of incubation period. However the higher amount of protease enzyme (17.3 IU/ml) production was observed by growth of co culture (Figure 8).

The lignocellulolytic enzyme activity was even detected within 7 days of intervals. Glucose suppressed the production of cellulose and xylanase but supported to the production of laccase. However, in the presence of lactose and cellobiose there was moderate aeration of different lignocellulolytic enzymes. Xylan has induced xylanase production but did not support for higher amount of cellulase enzyme production. Similar results have been reported earlier by Dhaliwal et al. (1991). Laccase oxidizes a wide range of substituted phenol (Roy et al., 1991). In the present study, the laccase activity increased gradually during fermentation and the maximum activity (5.1 IU/ml) was found to be in 28th days of fermentation carried out by coculture (Figure 9). Almost all the selected fungi could produce significant level of lignin peroxidase (LiP) during the fermentation period which was comparatively higher than laccase activity. Maximum amount of Lip activity (8.1 IU/ml) was observed on the 28th day of fermentation by using coculture method (Figure 10). But *R. stolonifer* produced very low level of activity (3.5 IU/ml) on the same fermentation period.

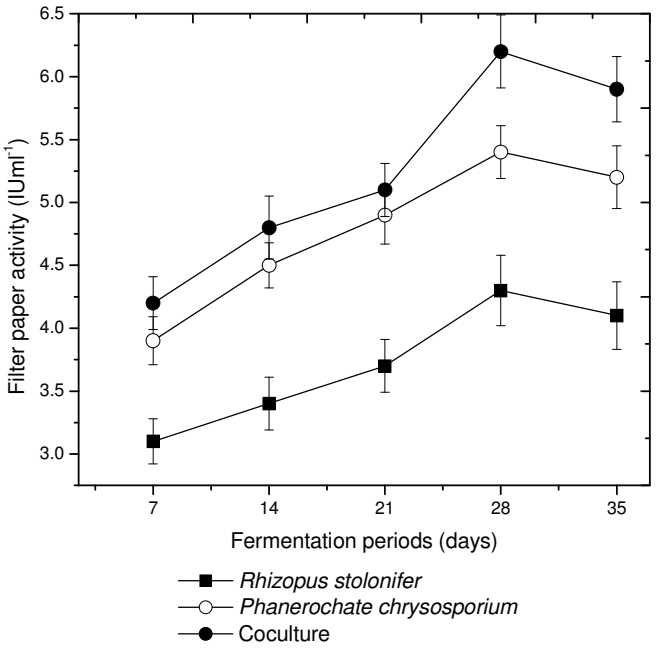

Figure 5. Filter paper enzyme activity (IU ml^{-1}) of coirwaste substrate and supplemented with urea substrate during solid state fermentation with *R. stolonifer, P. chrysosporium* and coculture. Mean ± S.E indicated. The filter paper activity significantly at probability level (P < 0.05).

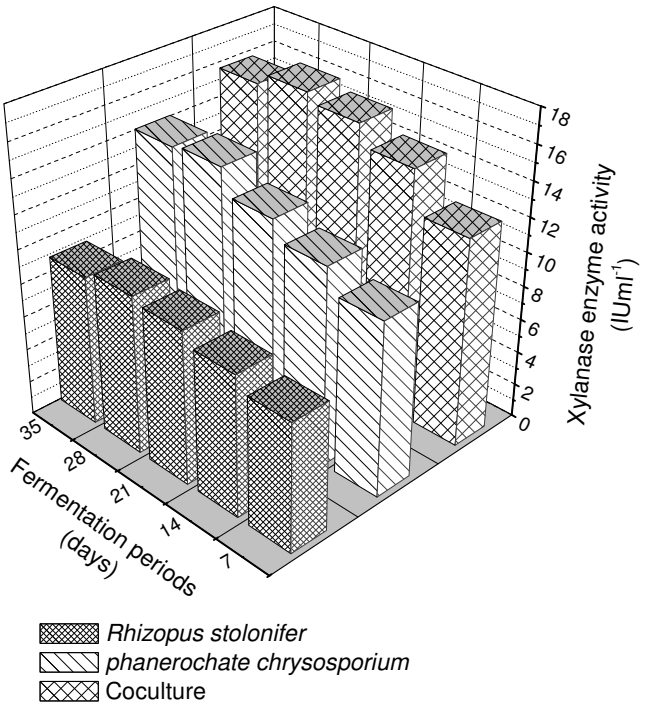

Figure 7. Xylanase enzyme activity (IU ml^{-1}) of coirwaste substrate and supplemented with urea substrate during solid state fermentation with *R. stolonifer, P. chrysosporium* and coculture. Mean ± S.E indicated. The xylanase enzyme activity significantly at probability level (P < 0.05).

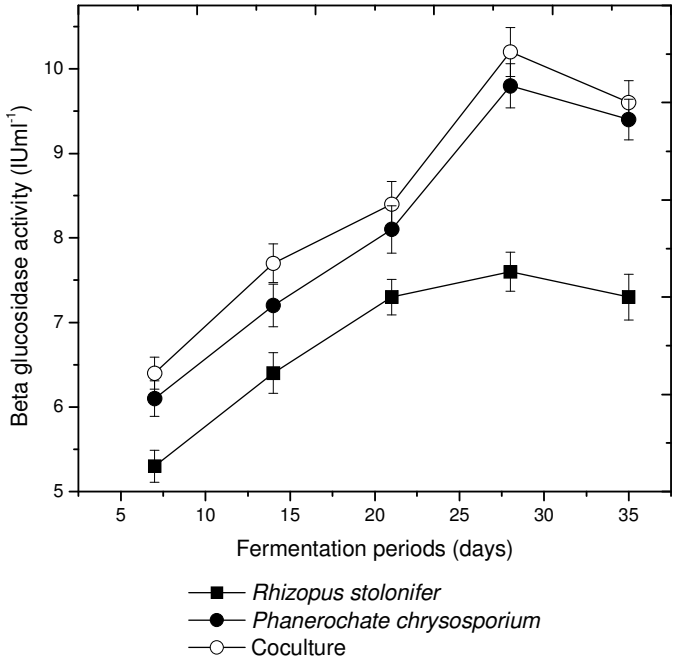

Figure 6. β-glucosidase enzyme activity (IU ml^{-1}) of coirwaste substrate and supplemented with urea substrate during solid state fermentation with *R. stolonifer, P. chrysosporium* and coculture. Mean ± S.E indicated. The glusidase activity significantly at probability level (P < 0.05).

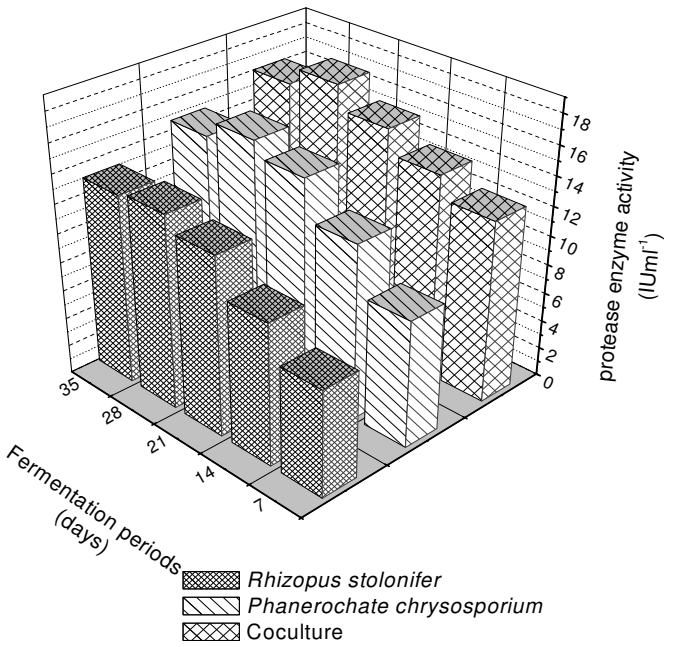

Figure 8. Protease enzyme activity (IU ml^{-1}) of coirwaste substrate and supplemented with urea substrate during solid state fermentation with *R. stolonifer, P. chrysosporium* and coculture. Mean ± S.E indicated. The protease enzyme activity significantly at probability level (P < 0.05).

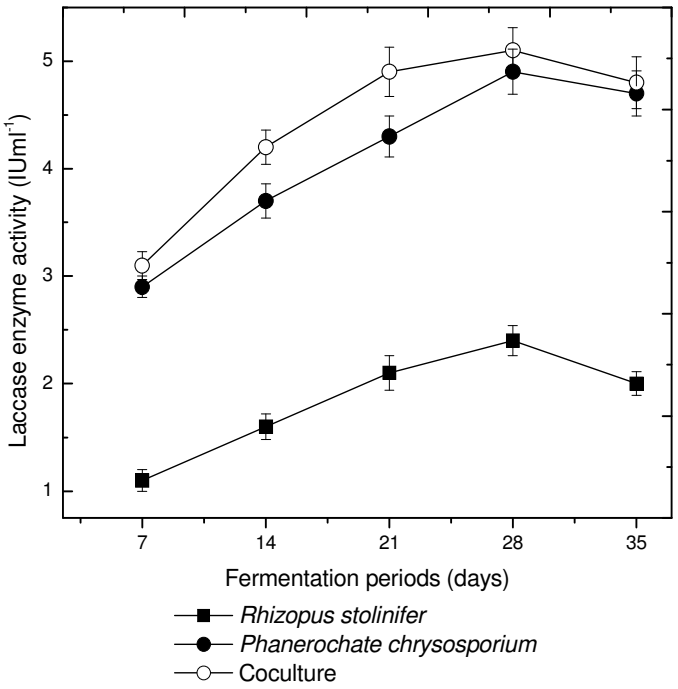

Figure 9. Laccase enzyme activity (IU ml^{-1}) of coirwaste substrate and supplemented with urea substrate during solid state fermentation with *R. stolonifer, P. chrysosporium* and coculture. Mean ± S.E indicated. The laccase enzyme activity significantly at probability level ($P < 0.05$).

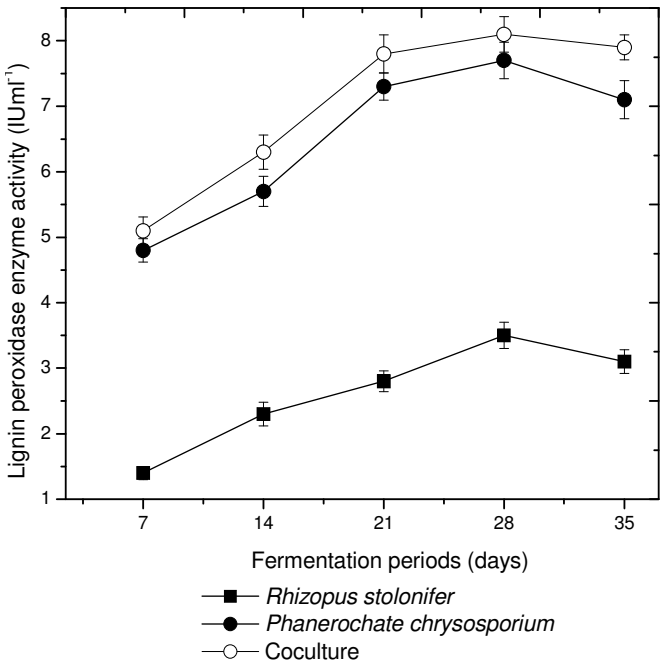

Figure 10. Lignin peroxidase enzyme activity (IU ml^{-1}) of coirwaste substrate and supplemented with urea substrate during solid state fermentation with *R. stolonifer, P. chrysosporium* and coculture. Mean ± S.E indicated. The lignin peroxidase enzyme activity significantly at probability level ($P < 0.05$).

Conclusion

This study concluded that lignocellulytic enzymes were produced from *P. chrysosporium* and *R. stolonifer* for lignocelluloses biodegradation. The combination of both cultures show better biodegradable activity with increased amount of lignolytic enzymes production than the individual fungus. Our present study provides simple information on lignolcellulytic enzyme production from *P. chrysosporium* and *R. stolonifer* and elucidates their potential for biodegradation of coir waste. Finally this SSF method can produce good and very cheap biofertilizer with higher nutritive effect to normal rural farmer people for the increasing yield of crop.

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