Improving Ruminal Degradibility Of Oil Palm Fronds Using Enzyme Extracts From White Rot Fungi

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Abstract

Background: Oil palm fronds (OPF) when pretreated with white rot fungi (WRF) shows increased rumen degradability but with significant biomass loss. Thus, effects of pre-treated OPF with enzyme extracts from WRF on rumen degradability were studied in vitro. The enzyme extracts were prepared by inoculating OPF with three WRF, i.e Ceriporiopsis subvermispora, Lentinula edodes and Ganoderma lucidum, for 15, 30 and 45 days with either ammonium sulphate, (NH4)2SO4 and sodium nitrate, NaNO3 added to the culture media for each inoculation period. After preparation of enzyme extracts, the enzyme activities were determined. OPF was then pre-treated with enzyme extracts in a citrate buffer (pH 5.0) in a forced air oven at 40 oC during 5 days. Further, the in vitro rumen degradation of OPF pre-treated with enzyme extracts, with respect to the short chain fatty acid (SCFA) production, was determined after 24 h incubation. Activity of lignolytic (laccase and MnP), cellulolytic (CMCase and avicelase) and hemicellulolytic (xylanase) enzymes were measured in all of the extracts irrespective on the inoculation period.

Results: Treatment of OPF with enzyme extracts from G. lucidum after 45 days of inoculation showed a numerical increase (13%) in total SCFA and apparently rumen degradable carbohydrates (ARDC) after 24 h in vitro incubation, without any loss of biomass. However, this increase was not clearly correlated to results of the enzyme assays.

Conclusion: This study indicates pre-treatment of OPF with enzyme extracts from specific WRF to be promising to enhance the ruminal degradability of OPF without simultaneous loss of biomass.

1. Background

The high lignin-(hemi)cellulose complex in the lignocellulosic agricultural by-products cause a serious impairment of their degradability by rumen microbes [1]. Thus, upgrading such lignocellulosic by-products could be of interest to increase their nutritional value for livestock production. Cereal straws (e.g. from rice, oat, wheat) as well as oil palm fronds (OPF) are examples of lignocellulosic by-products. Oil palm fronds, consisting of leaf and petioles, are generally regarded as agriculture waste from the oil palm industry and widely used in ruminant diets in Malaysia [2] despite their considerable lignin content (about 200 g/kg DM, e.g.[3]).

White rot fungi (WRF) have been reported as the most effective basidiomycetes to degrade lignin and improve degradability of fibrous material such as wheat straw [4],[5], rice straw [6] and oat straw [7]. Biological pre-treatment of OPF with WRF also showed promising results to breakdown lignocellulosic bounds in OPF, thereby increasing *in vitro* rumen apparent degradability of carbohydrates by up to 31% [3]. Although lignin degradation by WRF might stimulate rumen fermentation, there are still major drawbacks of this approach due to non-selective degradation of lignin, which was accompanied by cellulose and hemicellulose losses, and hence decreased carbohydrate availability for ruminal microbial degradation [8]. Treatment of wheat straw with enzymatic extracts from fungal strains rather than with

the fungi itself might minimize DM losses [4]. In that study, enzymatic extracts isolated from four fungal strains (i.e. 2 strains of *Trametes versicolor, Bjerkandera adusta* and *Fomes fomentarius),*, enhanced lignin degradation and increased rumen neutral detergent fibre (NDF) degradability of wheat straw, up to 13% as compared with non-treated wheat straw.

However, effectiveness of WRF to degrade lignin and improve rumen fermentation is substrate-specific as well as dependent on conditions of the solid state fermentation (e.g. origin of the additional N source, duration of the solid state fermentation). Hence, in the current study, enzyme extracts were screened for their effectiveness to improve rumen degradability of OPF. These extracts were prepared using WRF which showed potential to improve rumen degradability of OPF [3] and the effectiveness of the extracts was compared with that of the fungi through a mass balance approach. Lignolytic, cellulolytic and hemicellulolytic enzyme activities of the extracts were also determined to assess whether they are linked to changes in rumen degradability of OPF.

2. Methods

2.1 Substrates

Oil palm fronds were provided by the Malaysian Agricultural Research and Development Institute (MARDI), Selangor, Malaysia. The fresh OPF were chopped to 1–2 cm length and air dried before being inoculated with WRF.

2.2 Fungal strains

Three WRF strains, i.e Ceriporiopsis. subvermispora CBS 347.63 (purchased from The Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands), Lentinula. edodes UF 20911 and Ganoderma. lucidum UF 20706 (isolated from decaying plant material collected in the North of Portugal and characterized by Dias [9]) were used to obtain the enzymatic extracts. Fungi were cultured on potato dextrose agar plates and incubated at 28^{o}C for 10 days.

2.3 Inoculation of OPF with WRF to produce enzyme extracts

Inoculation of OPF with WRF to produce enzyme extracts were performed in quadruplicate under solid state fermentation, as described by [10]. Briefly, the enzymes were extracted in 250 ml Erlenmeyer flasks with culture media content (each flask contained OPF, 15 g; glucose, 22.5 mg and 45 ml deionized water). The control flasks only contained these substrates whereas additionally, either of two nitrogen sources (ammonium sulphate, $(NH_4)_2SO_4$,2.4 mM or sodium nitrate, $NaNO_3$, 2.4 mM) were added in treatments and referred to as NH_4-medium and NO_3-medium, respectively, throughout this study. The addition of nitrogen source is to stimulate the fungi's growth, thus, enhancing their enzyme production. Before inoculating OPF with WRF, the flasks were autoclaved at 121 ^{o}C for 20 minutes. After the cooling process, three 10 mm agar plugs, taken from each agar plate with fungi, were added to each flask under sterile conditions. Flasks were then incubated at 28 ^{o}C and OPF inoculated with one of the fungi was harvested

after 15, 30 and 45 days of inoculation. Afterwards, contents of the culture flasks were suspended in 150 ml of deionized water and incubated on a rotary shaker (100 rpm) at 28 ^{o}C for 3 h. Extracts were filtered (Whatman GF/A) and 0.06 g polyvinyl polypyrrolidone (PVPP) was added before being centrifuged at 12 000 x g for 10 min. The supernatant were recovered and used for enzyme activity determination and pre-treatment of OPF.

2.4 Determination of enzymatic activities

Enzymatic activities were determined on quadruplicate samples using a Helios UV-vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Lignolytic enzyme activities such as laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP) were monitored using 0.1 to 0.4 ml supernatant of the culture samples and the respective buffered substrate in 1.5 ml total reaction volume. Laccase activity was determined according to [10] by measuring the oxidation of 2.0 mM 2,2'-azino-bis–3-ethylbenzthiazoline–6-sulphonic acid (ABTS) buffered with 100 mM citrate-phosphate (pH 4.0) and the formation of an ABTS cation radical was monitored at 420 nm (ε_{420} = 36.0 mM^{-1} cm^{-1}). Manganese peroxidase activity was determined according to the modified method of Heinfling et al. (1998) by the formation of Mn^{3+}-tartrate (ε_{238} = 6.5 mM^{-1} cm^{-1}) from 0.10 mM $MnSO_4$ using 100 mM tartrate buffer (pH 5.0) and 0.10 mM H_2O_2. Lignin peroxidase activity was monitored at pH 3.0 according to [11] and the formation of veratraldehyde was monitored at 310 nm (ε_{310} = 9.3 mM^{-1} cm^{-1}). For cellulolytic enzyme assays, activities of carboxymethylcellulase (CMCase) and Avicel digesting cellulase (avicelase) were measured according to the IUPAC recommendations [12], using substrate solutions of 10 g/l carboxymethylcellulose and 10 g/l avicel (cellulose microcrystalline) in a 50 mM citrate buffer (pH 4.8) at 50 C for 30 min and 3 h, respectively. The reducing sugars released were determined by dinitrosalicylic acid (DNS), using glucose as a standard. As for hemicellulolytic enzyme, xylanase, the activity was determined under similar conditions as for CMCase, except that 10 g/l of xylan solution was used as the substrate. The release of reducing sugars were determined by DNS, using xylose as a standard [10].

2.5 Pre-treatment of OPF with enzyme extracts from WRF

Quadruplicates of the enzyme extracts recovered from inoculation of OPF with WRF were pooled for each inoculation time (15, 30 and 45 days) and used for pre-treatment of OPF. Pre-treatment of OPF with each of the enzyme extracts were performed in triplicate. A total of 70 ml citrate buffer (50 mM; per liter of distilled water: 10.5 g $C_6H_8O_7.H_2O$ and 14.7 g $C_6H_5Na_3O_7.2H_2O$; pH 5.0) were added to 11 g OPF in 250 ml Erlenmeyer flasks. The flasks were autoclaved at 121 ^{o}C for 20 minutes. After cooling, 20 ml enzymatic extracts, 6.7 ml $MnSO_4$ and 1 ml H_2O_2 were added to the flasks. The flasks were then put in a forced air oven at 40 C for 5 days. Every 24 h, 1 ml H_2O_2 were added to each flask to stimulate the production of MnP. After pre-treatment, the contents of the flasks were transferred to a plastic container for freeze-drying. The OPF residues obtained after freeze-drying were used to determine DM losses and to perform in vitro ruminal incubations. Besides pre-treatments with three WRF, OPF pre-treated with non-enzyme extracts (citrate buffer only) were included as a control.

2.6 In vitro rumen incubation and analysis

In vitro ruminal incubations (24 h) were carried out to provide evaluation of the degradability of pre-treated OPF with enzyme extracts. The in vitro batch incubations were performed by including the three replicates per treatment (as described in 2.5) in glass gastight serum flasks as described by [13]. Briefly, 0.250 g of pre-treated OPF were added to each flask. Flasks containing 0.250 g of non-treated OPF were also included as negative control. One milliliter of distilled water was added and the flasks were flushed four times with CO_2 before the addition of rumen fluid. Rumen fluid collection was done from three fistulated Texel sheep, fed with good quality hay at maintenance level, and was collected before the morning feeding before being immediately transferred into thermos flasks to the lab. The fistulated sheep were treated according to the guidelines of the ethical commission of the Institute for Agricultural and Fisheries Research (ILVO) of the Flemish Government, Belgium (EC 2009/114). The rumen fluid obtained from the animals was mixed, homogenized and filtered through a sieve with a pore size of 1 mm under continuous CO_2 flushing. A mixture of 5 ml strained rumen fluid and 20 ml of phosphate/bicarbonate buffer (per liter of distilled water: 3.58 g $Na_2HPO_4.12H_2O$; 1.55 g KH_2PO_4; 0.124 g $MgCl_2.6H_2O$; 8.74 g $NaHCO_3$ and 1 g NH_4HCO_3, flushed with CO_2 for 30 min and adjusted to pH 6.8) were added to each flask. The flasks were put in the batch culture incubator (Edmund Bühler Gmbh, Hechingen, Germany) at 39 C for 24 h. At 0 h of incubation, 2 ml of rumen fluid were collected for short chain fatty acid (SCFA) analysis after acidifying with 0.2 ml 2-ethylbutyric/formic acid (10 mg/ml). After 24 h of incubation, the incubation flasks were removed from the incubator and placed immediately in an ice bath to stop microbial activity. Flasks were then opened, pH measured and 2 ml of incubation content were recovered for SCFA after acidifying with 0.2 ml 2-ethylbutyric/formic acid (10 mg/ml) and centrifuged for 15 min at 15 000 x g. The supernatant was recovered and analysed for SCFA by gas liquid chromatography on a Shimadzu 2010 (Shidmadzu Corporation's Hertogenbosch, The Netherlands) according to [14]. Net production of SCFA was calculated by subtracting the amounts at the 0 h time point from amounts found after 24 h of incubation. Apparently rumen degradable carbohydrates (ARDC) were calculated as:

ARDC (mg) = (Acetate/2 + Propionate/2 + Butyrate) x 162/1000,

with 162 as the assumed molecular weight (g) of 1 mol of fermented carbohydrates (Demeyer 1991). Acetate, propionate and butyrate were expressed as net micro molar productions.

2.7 Statistical analysis

All data were statistically analyzed using SPSS (2006). All the parameters measured were evaluated separately using a general linear model (univariate). For enzyme activities, the parameters measured were used to assess the main and interaction effect of fungi ($A_{i = 1-3}$), inoculation day ($B_{j = 1-3}$) and medium ($C_{k = 1-3}$), according to $Y_{ijk} = \mu + A_i + B_j + C_k + AB_{ij} + AC_{ik} + BC_{jk} + ABC_{ijk} + \xi_{ijk}$ where Y_{ijk} is the response; μ the overall mean; A_i the effect of fungi (C. subvermispora, L. edodes or G. lucidum); B_j the effect of inoculation day (15, 30 or 45 days); C_k the effect of medium (no addition N source, NH_4 medium or NO_3 medium); AB_{ij} the interaction between fungi and inoculation day; AC_{ik} the interaction between fungi and

medium; BC_{jk} the interaction between inoculation day and medium; ABC_{ijk} the interaction between fungi, inoculation day and medium; and ξ_{ijk} the residual error. As for the in vitro incubation, the parameters measured were used to assess whether pre-treatment with enzyme extracts improved rumen degradability of OPF, through the comparison of OPF pre-treated with each of the enzyme extracts with OPF pre-treated with the citrate buffer (control) by a Dunnet post-hoc test, according to $Y_{ij} = \mu + D_i + \xi_{ij}$ where Y_{ij} is the response; μ the overall mean; D_i the effect of OPF pre-treated with enzyme extracts; and ξ_{ij} the residual error. Effect with $P<0.05$ were considered statistically significant.

3. Results

3.1 Enzymatic activities of the enzyme extracts

Enzymatic activities of the extracts obtained from inoculation of OPF with any of the three WRF are presented in Figure 1 and 2. In the current study, activity of the lignolytic enzymes, laccase and MnP was detected (Figure 1 and 2, respectively), whereas LiP was not detected for any of the fungi over any of the inoculation periods tested. Among the three fungi, *L. edodes* and *C. subvermispora* showed the highest and lowest activity of laccase, respectively (Figure 1 and Table 1). Laccase activity developed gradually over the incubation period showing the highest activity after 45 days of inoculation (Figure 1 and Table 1). A higher laccase activity was observed for N-supplemented media (Table 1), irrespective of the N-source used. On the contrary, the MnP activity decreased over the inoculation period. For *G. lucidum,* duration of the inoculation affected laccase and MnP only to a limited extent showed the lowest MnP activity among the three fungi tested (Figure 2 and Table 1). Again, N supplemented to the media generally increased MnP activity (Table 1).

All fungi extracts showed cellulolytic enzyme activities such as CMCase (endoglucanase) and avicelase (exoglucanase). Carboxymethylcellulase activity in *C. subvermispora* extracts was highest (Figure 2a and Table 1). The activity of CMCase differed over the inoculation period with the highest and lowest activity being observed after 15 and 30 days of inoculation, respectively (Figure 2a and Table 1). Lower CMCase activity was observed for N-supplemented media, irrespective of the N-source (Table 1). As for avicelase (Figure 2b), its activity was very low, as compared with CMCase. The highest and lowest activity of avicelase was observed for *C. subvermispora* and *L. edodes,* respectively (Table 1). Nevertheless, the production of avicelase activity was relatively stable over the incubation period except for a slight decrease observed after 30 days of inoculation (Table 1). With respect to the type of medium, the extract obtained after fungi inoculation in a NO_3-enriched medium showed a lower activity, although the difference is minor and generally, activities of this enzyme are low. The hemicellulolytic enzyme, xylanase, was also produced during the inoculation period by all fungi, with *L. edodes* showing the highest activity. The production of xylanase was increased after 45 days inoculation compared to 15 and 30 days inoculation (Figure 2c and Table 1). The lower xylanase activity was observed for N-supplemented media, irrespective of the N-source (Table 1).

In general, no differences were observed in enzyme activities between two N sources which stimulated lignolytic and reduced fibrolytic enzyme activity, respectively (Table 1). Nevertheless, interaction effects indicate this might vary according to inoculation day and fungal strain (Table 1). For instance, enzyme activities in extracts of *G. lucidum* only showed minor differences between inoculation media, particularly for the longer inoculation period, except for laccase activity.

3.2 In vitro rumen degradation of OPF pre-treated with enzymatic extracts

Oil palm fronds pre-treated with enzyme extracts obtained after 15, 30 and 45 days with inoculation of C. subvermispora, L. edodes and G. lucidum did not significantly change total SCFA production and ARDC, as compared with OPF pre-treated with citrate buffer (control; Table 2). Nevertheless, OPF pre-treated with enzyme extracts obtained after 45 days of inoculation with G. lucidum showed a numerical increase by 13% in total SCFA production and ARDC as compared with the control. This increase was shown for the enzyme extract from G. lucidum inoculated in media containing NO_3 or without extra N source. As for the individual SCFA production, the enzymatic treatment generally resulted in decreased acetate and increased propionate and butyrate proportion of pre-treated OPF as compared with the control.

4. Discussion

4.1 Enzyme activities of the extracts

The production of lignolytic enzymes by WRF when inoculated with fibrous material has been reported by several authors [15], [4], [16]. Laccase and MnP are considered to be the most common lignolytic enzymes produced by WRF [17], [18] which is in line with the lignolytic enzymes determined in the current study. Nevertheless, laccase and MnP activities reported in the current study (e.g *L. edodes)* were higher as compared with other studies using the same protocol (0.144 and 0.160 U/ml, respectively) through inoculation of wheat straw during 15 days by *Trametes versicolor* [4]. Furthermore, in the current study, a higher activity of laccase as compared to MnP was observed, which is in contrast with other studies (e.g [19], [20]) in which 10 times higher levels of MnP activity were reported than laccase activity after 28 days inoculation of wheat straw with WRF (e.g *Nematoloma frowardii)*.. These differences mainly might be due to the production of enzyme extracts in different growth media and using different strains of fungi. Indeed, variation in production of lignolytic enzymes (e.g. laccase and MnP) between fungal strains was also observed in the current study with *L. edodes* being the highest producer, followed by *G. lucidum* and *C. subvermispora.* In the current study, LiP was not detectable in the enzyme extract. Some former studies reported some WRF to produce all three lignolytic enzymes (e.g laccase, MnP and LiP) while others produce only one or two of them [20].

The higher endoglucanase (CMCase) as compared with exoglucanase (avicelase) activity indicated a common feature of cellulolytic enzyme activity [21], [4]. [10] reported 10 times higher CMCase than avicelase activity in *Irpex lacteus* cultivated on wheat straw, which is in line with the current study (10 to 20 times higher). The higher xylanase activity as compared with CMCase suggested a possible

preference of the currently studied WRF to degrade hemicellulose rather than cellulose [6]. Accordingly, in a study with six fungal species, hemicellulose content was much lower than cellulose in fungi-treated wheat straw as compared with untreated wheat straw [22].

4.2 Effects of pre-treated OPF with enzyme extracts on in vitro rumen degradability

A numerical increase of 13% in total SCFA production and ARDC for OPF pre-treated with enzyme extracts of *G. lucidum* obtained after 45 days of inoculation in NO_3-medium or in medium without extra N-source did not correlate with differences in enzyme activities: lignolytic enzyme activities in these extracts were lower as compared with activities measured in extracts obtained from the other fungal strains tested in the current study. However, also in other studies no direct relationship between lignolytic enzyme activities and the extent of degradation was found, e.g. for wheat straw [23]. Eventually, the improved OPF degradability was induced by enzymes present in the extract of which the activity was not measured. Several studies have reported that other enzymes such as β-glucosidase, feruloyl and acetyl esterases could play an important role during the degradation of lignocellulosic materials such as wheat straw [24]. [25] showed that feruloyl esterases have the ability to hydrolyze lignin-hemicellulose bounds, improving further exposure of structural carbohydrates to cellulolytic enzymes.

In our former study [3], we showed the NDF and ADF content of OPF decreased when colonized with WRF. These decreases in fiber fractions are consistent with degradation of lignin which is part of ADF and NDF, which then could allow accessibility of the structural carbohydrates by cellulolytic and hemicellulolytic bacteria and further enhance the degradability of OPF in the rumen. The decrease of cellulose indicates non-selective degradation by fungi of both lignin and cellulose [26]. Nevertheless, a decrease in cellulose content due to WRF treatment is one of the major drawbacks of this approach as it results in a biomass loss. This is illustrated in our previous study [3]. where a 10% and 31% increase in rumen degradability as provoked by *G. lucidum* and *L. edodes,* respectively, were associated with a net loss of degradable biomass (250 and 200 g/kg DM for *G. lucidum* and *L. edodes,* respectively). An overview of degradability and mass balance from non-treated OPF and OPF pre-treated with fungi is presented in Table 3 and 4, respectively.

As illustrated in Table 4, OPF pre-treatment with *G. lucidum* does not result in a net increase in rumen degradable biomass despite the improvement in rumen degradability. For biomass losses at an order of magnitude of 20%, an increase in degradability of more than 30% is required (e.g LEW9) to ensure a net gain of degradable biomass. Even then, improvement in degradable biomass is limited. Therefore, pre-treatment of OPF with enzyme extracts from WRF seems promising to overcome the biomass loss problem. Indeed, [4] showed that enzymatic extracts from WRF enhanced lignin degradation and further increased rumen NDF degradability of wheat straw (up to 13%), with minimal DM losses. Their improvement in degradability by pre-treatment with enzyme extract from WRF is in agreement with the current study, where the pre-treatment of OPF with an enzyme extract of *G. lucidum* showed an increase in OPF degradability by up to 13%. This improvement was higher than OPF pre-treated with *G. lucidum (*10%; [3]) with an advantage of not losing biomass.

Mass balances for this pre-treatment approach are shown in Table 5, including the recovery of the OPF which was used for the production of the enzyme extract. For this OPF, degradability characteristics of fungal pre-treated material [3] were assumed. Nevertheless, this recovery would not be required when an efficient enzyme production on a larger scale is applied, requiring relatively less OPF. To assess the mass balance of the OPF pre-treated with enzyme extracts, both the biomass required for enzyme extraction as well as minor losses occurring during pre-treatment of OPF with enzyme extract were considered. The current lab scale enzyme production and OPF pre-treatment required 14 and 86% of the OPF biomass, respectively (Table 5).

To produce the enzyme extract, 15 g OPF and 195 ml water was used in each flask. As we had 4 replicates, the total amount of OPF and water used was 60 g and 780 ml, respectively. After filtration, the enzyme extracts from the four replicates were pooled and only 20 ml were used for pre-treatment of OPF with enzyme extract, which represented the use of 1.5 g of OPF (20 ml/780 ml = 2.5% x 60 g = 1.5 g OPF was needed to produce 20 ml enzyme extract), for the total treatment of 11 g of OPF, which represents 14% extra OPF needed to produce the enzyme extract (20 ml enzyme extract needed to pre-treat 11 g OPF from which OPF material with a 13% higher in degradability was reached).

When a possible recovery of this biomass is considered with a degradability improvement of 10% (as obtained for direct fungal treatment of OPF, [3]), a 9% gain of degradable biomass was calculated (Table 5), which is higher as compared to the increase that was reached by the best strategy with direct WRF pre-treatment of OPF in our former study (e.g +4.8% by LEW9). Moreover, given the relative great sensitivity of the direct fungal treatment (e.g inoculation period is very critical), a more standardized treatment with an enzyme extract (produced under more standardized (industrial) conditions) might be of interest. Moreover, the advantage of minimized DM losses is mainly of interest when upscaled enzyme production would be optimized and would require only minimal amounts of biomass.

5. Conclusion

Pre-treatment of OPF with enzyme extracts from selected WRF seemed to be promising to enhance the ruminal degradability of OPF without simultaneously resulting in loss of biomass. Nevertheless, further study is needed to clarify the relationship between the enzyme activities and the pattern of ruminal degradability of lignocellulosic materials.

Abbreviations

oil palm fronds (OPF), white rot fungi (WRF), apparently rumen degradable carbohydrates (ARDC), short chain fatty acids (SCFA), manganese peroxidase (MnP), lignin peroxidase (LiP), carboxymethylcellulase (CMCase).

Declarations

Ethics approval and consent to participateNot applicable.

Consent for publicationNot applicable.

Availability of data and materialsThe data analysed and materials used during the study are not publicly available due to study participant confidentially but are available from the corresponding author upon request.

Competing interest The authors declare that there is no conflict of interest regarding the publication of this paper

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Authors' contributions HA involved in running the experiment, analyzing data and manuscript preparation, AF involved in manuscript preparation, ML and VF involved in experimental work, BR, DA, FI, YM and RM are the research collaborator which involved in data analysis and manuscript preparation.

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Tables

Table 1: Overview of enzymatic activities recovered in extracts obtained after treatment of OPF for 15, 30 and 45 days with *Ceriporiopsis subvermispora, Lentinula edodes* and *Ganoderma lucidum* (averages per fungi, per treatment period and per media.

Source	Enzyme activities (U/ml)				
	Laccase	MnP	CMCase	Avicelase	Xylanase
Fungi (A)					
Ceriopsis subvermispora	0.023[a]	0.188[b]	0.131[b]	0.009[c]	0.159[a]
Lentinula edodes	0.509[c]	0.254[c]	0.107[a]	0.006[a]	0.205[b]
Ganoderma lucidum	0.259[b]	0.072[a]	0.110[a]	0.007[b]	0.159[a]
SEM	0.007	0.004	0.003	0.000	0.005
Treatment period, day (B)					
15	0.113[a]	0.225[c]	0.168[c]	0.008[b]	0.135[a]
30	0.290[b]	0.188[b]	0.074[a]	0.005[a]	0.136[a]
45	0.388[c]	0.101[a]	0.105[b]	0.007[b]	0.253[b]
SEM	0.007	0.004	0.003	0.000	0.005
Medium (C)					
NH_4-medium	0.289[b]	0.176[b]	0.105[a]	0.007[b]	0.166[a]
NO_3-medium	0.282[b]	0.178[b]	0.111[a]	0.006[a]	0.155[a]
No N source-medium	0.220[a]	0.162[a]	0.131[b]	0.007[b]	0.203[b]
SEM	0.007	0.004	0.003	0.000	0.005
Significance[z]					
Fungi (A)	***	***	***	***	***
Treatment period (B)	***	***	***	***	***
Medium (C)	***	**	***	***	***
A*B	***	***	***	***	***
A*C	***	*	***	***	***
B*C	***	***	***	***	***
A*B*C	***	***	***	***	***

[a, b, c] Values within a column and within factor fungi, treatment period or medium bearing the same superscripts are not significantly different (P>0.05)

[Z] *P<0.05; **P<0.01; ***P<0.001

Table 2a: Effect of pretreated OPF with enzymatic extracts recovered after 15 days treatment with *Ceriporiopsis subvermispora (CS), Lentinula edodes (LE)* and *Ganoderma lucidum (GL)* on total SCFA (µmol/incubation), relative proportions of individual SCFA (mmol/mol total SCFA) and ARDC (g/g OPF) after 24 h incubation

Treatment	Treatment period, day[x]	N-source in media	Parameters				
			Total SCFA	Acetate	Propionate	Butyrate	ARDC
OPF*+CS*	15	$(NH_4)_2SO_4$	852	734	171	58.1	0.282
		$NaNO_3$	852	737	170	56.6	0.282
		-	828	734	171	57.7	0.274
OPF*+LE*		$(NH_4)_2SO_4$	890	731	174[a]	59.0	0.295
		$NaNO_3$	933	730	174[a]	58.8	0.309
		-	885	725[a]	176[a]	60.8[A]	0.293
OPF*+GL*		$(NH_4)_2SO_4$	834	723[a]	175[a]	61.5[a]	0.276
		$NaNO_3$	838	725[a]	175[a]	60.3	0.277
		-	842	730	172[A]	60.1	0.279
OPF + citrate buffer	None	-	892	742	163	56.9	0.294
		SEM	31.3	3.57	2.34	0.964	0.010

[A] 0.1<P<0.05 trend; [a] P<0.05 significantly different between OPF pretreated with enzymatic extracts and citrate buffer (control); [x] treatment period=duration of OPF treated with WRF to produce enzyme extracts; SCFA=short chain fatty acid; ARDC=apparently rumen degradable carbohydrates

Table 2b: Effect of pretreated OPF with enzymatic extracts recovered after 30 days treatment with *Ceriporiopsis subvermispora (CS), Lentinula edodes (LE)* and *Ganoderma lucidum (GL)* on total SCFA

(µmol/incubation), relative proportions of individual SCFA (mmol/mol total SCFA) and ARDC (g/g OPF) after 24 h incubation

Treatment	Treatment period, day[x]	N-source in media	Parameters				
			Total SCFA	Acetate	Propionate	Butyrate	ARDC
OPF*+CS*	30	$(NH_4)_2SO_4$	814	737	167	57.6	0.269
		$NaNO_3$	836	732	173	57.0	0.276
		-	864	733	172	57.4	0.285
OPF*+LE*		$(NH_4)_2SO_4$	896	726[a]	178[A]	60.4[a]	0.297
		$NaNO_3$	866	726[a]	175	60.0[a]	0.287
		-	876	732	173	59.2[a]	0.290
OPF*+GL*		$(NH_4)_2SO_4$	870	728[A]	174	60.1[a]	0.288
		$NaNO_3$	877	725[a]	176	61.3[a]	0.291
		-	829	729	170	60.8[a]	0.274
OPF + citrate buffer	None	-	902	745	165	55.1	0.298
		SEM	25.9	4.42	3.19	0.828	0.009

[A] 0.1<P<0.05 trend; [a] P<0.05 significantly different between OPF pretreated with enzymatic extracts and citrate buffer (control); [x] treatment period=duration of OPF treated with WRF to produce enzyme extracts; SCFA=short chain fatty acid; ARDC=apparently rumen degradable carbohydrates

Table 2c: Effect of pretreated OPF with enzymatic extracts recovered after 45 days treatment with *Ceriporiopsis subvermispora (CS), Lentinula edodes (LE)* and *Ganoderma lucidum (GL)* on total SCFA (µmol/incubation), relative proportions of individual SCFA (mmol/mol total SCFA) and ARDC (g/g OPF) after 24 h incubation

Treatment	Treatment period, day[x]	N-source in media	Parameters				
			Total SCFA	Acetate	Propionate	Butyrate	ARDC
OPF*+CS*	45	$(NH_4)_2SO_4$	850	732	170	58.7	0.281
		$NaNO_3$	899	733	172	57.9	0.297
		-	878	725[a]	178[a]	59.5	0.291
OPF*+LE*		$(NH_4)_2SO_4$	902	732	174[a]	59.0	0.300
		$NaNO_3$	847	728[A]	173[A]	59.8[A]	0.280
		-	855	724[a]	176[a]	60.6[a]	0.283
OPF*+GL*		$(NH_4)_2SO_4$	775	713[a]	178[a]	64.5[a]	0.256
		$NaNO_3$	1028	723[a]	176[a]	61.6[a]	0.341
		-	1031	733	175[a]	58.3	0.342
OPF+ citrate buffer	None	-	906	746	163	55.9	0.300
		SEM	44.8	4.50	2.54	1.07	0.019

[A] 0.1<P<0.05 trend; [a] P<0.05 significantly different between OPF pretreated with enzymatic extracts and citrate buffer (control); [x] treatment period=duration of OPF treated with WRF to produce enzyme extracts; SCFA=short chain fatty acid; ARDC=apparently rumen degradable carbohydrates

Table 3: Overview of degradability and mass balance of non-treated OPF

	Non-treated OPF
Original material (g/kg DM)	1000
Degradability (%)*	29
Degradable material (g/kg DM)	290

*Source: [13]

Table 4: Overview of degradability and mass balance of OPF pretreated with fungi

	OPF pretreated with fungi	
	GLW9	LEW9
Original material (g/kg DM)	1000	1000
DM loss after pretreatment (g/kg DM)	250	200
OPF left for animals to feed after pretreatment (g/kg DM)	750	800
Improvement in degradability after pretreatment (%)*	+10	+31
Degradability after pretreatment (%)	32	38
Degradable material after pretreatment (g/kg DM)	240	304
Relative change to in degradable material to non-treated OPF (%)**	-17.2	+4.8

GLW9 and LEW9=*Ganoderma lucidum* and *Lentinula edodes* inoculated during 9 weeks

*Source: Rahman et al. (2011)

**Source: Table 3

Table 5: Overview of degradability and mass balance of fungal pretreated OPF after preparation of enzyme extract (EE) (a), and after pretreatment with EE from *Ganoderma lucidum* (b)

	Preparation of EE (a)	Pretreatment with EE (b)
Original material (g/kg DM)	140	860
DM loss (g/kg DM)	250	13.2
OPF left to feed to animals (g/kg DM)	105	849
Improvement in OPF degradability (%)	+10	+13
Degradability of OPF (%)	32	33
Degradable material (g/kg DM)	33.6	280.2
Complete process of pretreatment of OPF with EE from *Ganoderma lucidum* = OPF recovered after enzyme preparation (a) + OPF pretreated with EE (b)		
Original material (g/kg DM) = (a)+(b)	1000	
Total degradable material (g/kg DM) = (a)+(b)	314	
Relative change in degradable material to non-treated OPF (%)	+8	

Figures

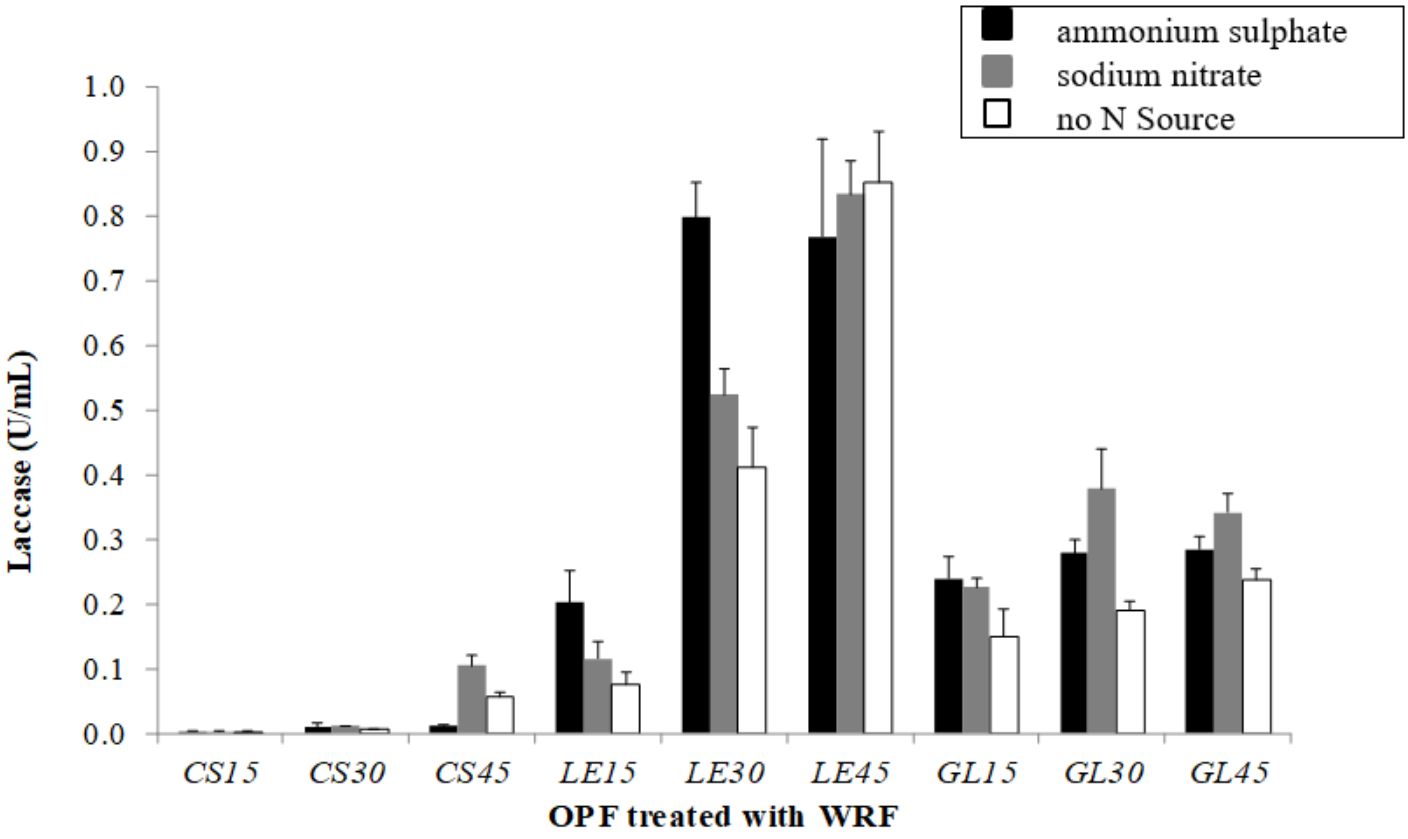

Figure 1

Laccase activity from enzyme extracts after inoculation of OPF for 15, 30 and 45 days with Ceriporiopsis subvermispora (CS), Lentinula edodes (LE) and Ganoderma lucidum (GL) in media with (NH4-medium and NO3-medium, respectively) and without (control) supplementation of a N source.

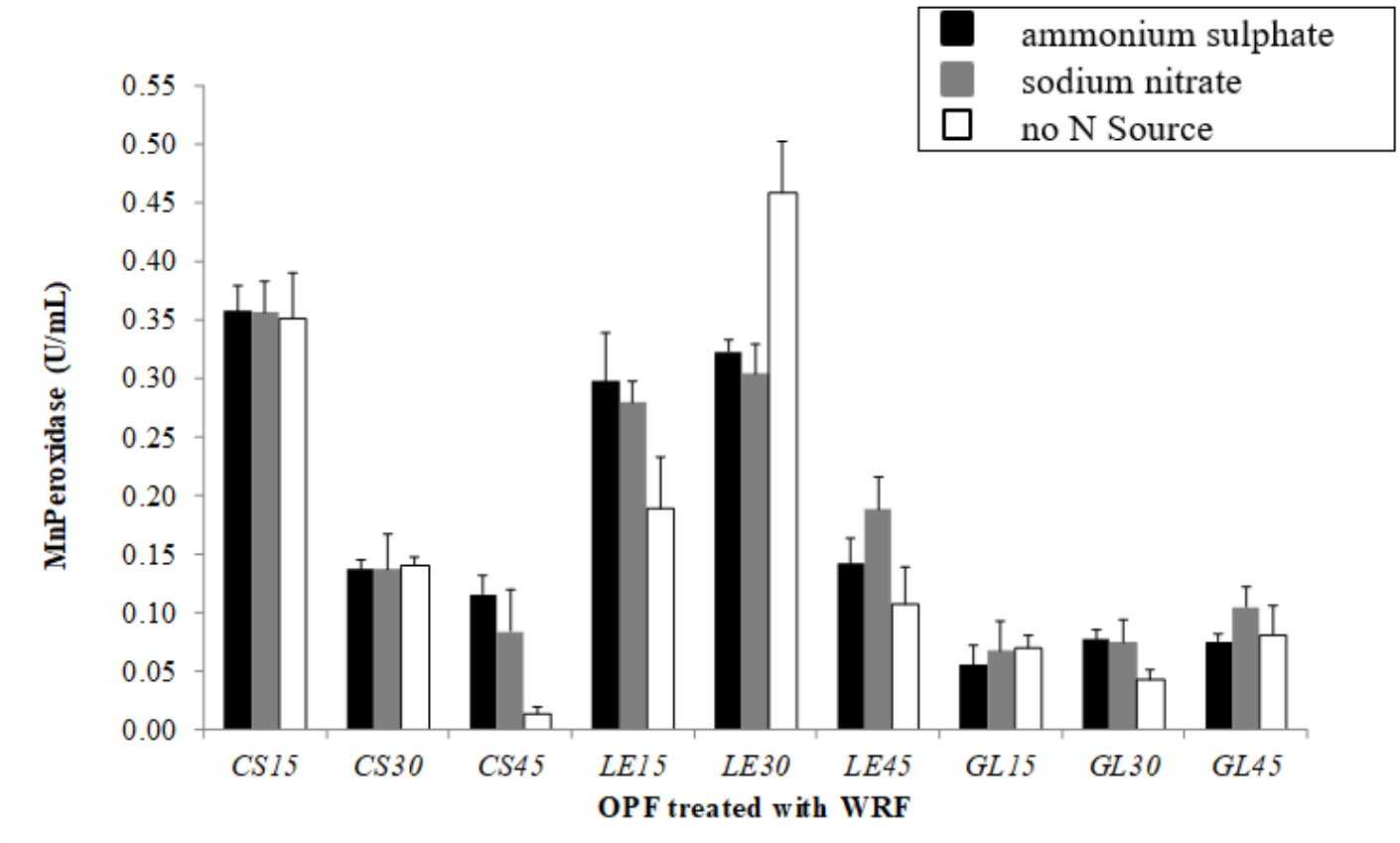

Figure 2

MnPeroxidase activity from enzyme extracts after inoculation of OPF for 15, 30 and 45 days with Ceriporiopsis subvermispora (CS), Lentinula edodes (LE) and Ganoderma lucidum (GL) in media with (NH4-medium and NO3-medium, respectively) and without (control) supplementation of a N source.

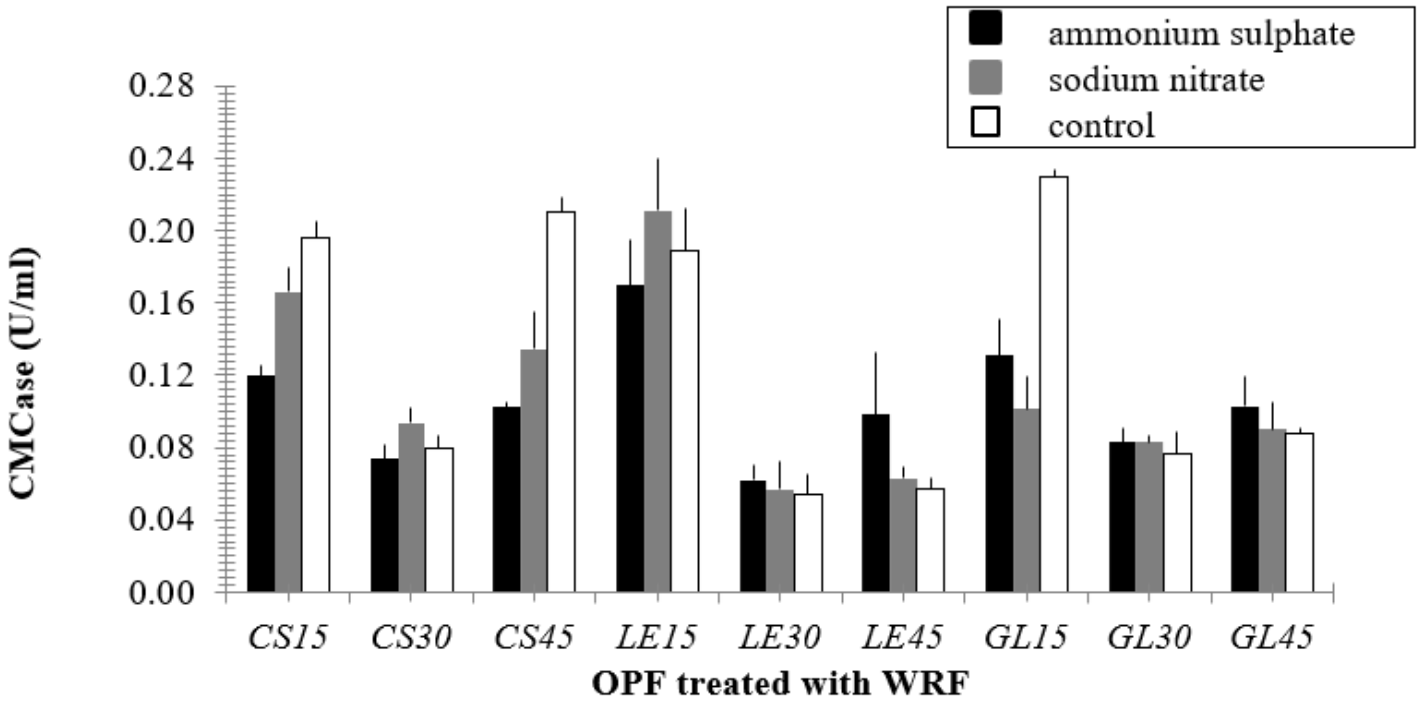

Figure 3

CMcase enzyme activities of enzyme extract after inoculation of OPF for 15, 30 and 45 days with Ceriporiopsis subvermispora (CS), Lentinula edodes (LE) and Ganoderma lucidum (GL) in media with (NH4-medium and NO3-medium, respectively) and without (control) supplementation of a N source.

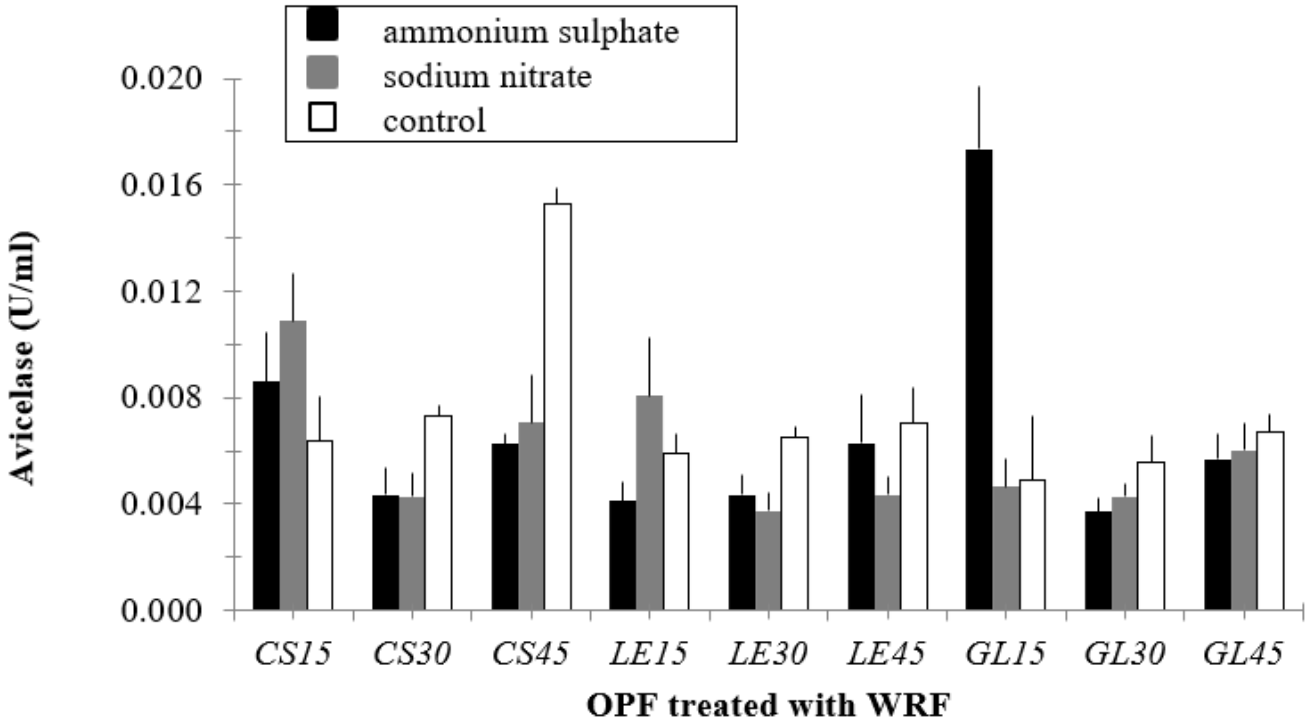

Figure 4

Avicelase enzyme activities of enzyme extract after inoculation of OPF for 15, 30 and 45 days with Ceriporiopsis subvermispora (CS), Lentinula edodes (LE) and Ganoderma lucidum (GL) in media with (NH4-medium and NO3-medium, respectively) and without (control) supplementation of a N source.

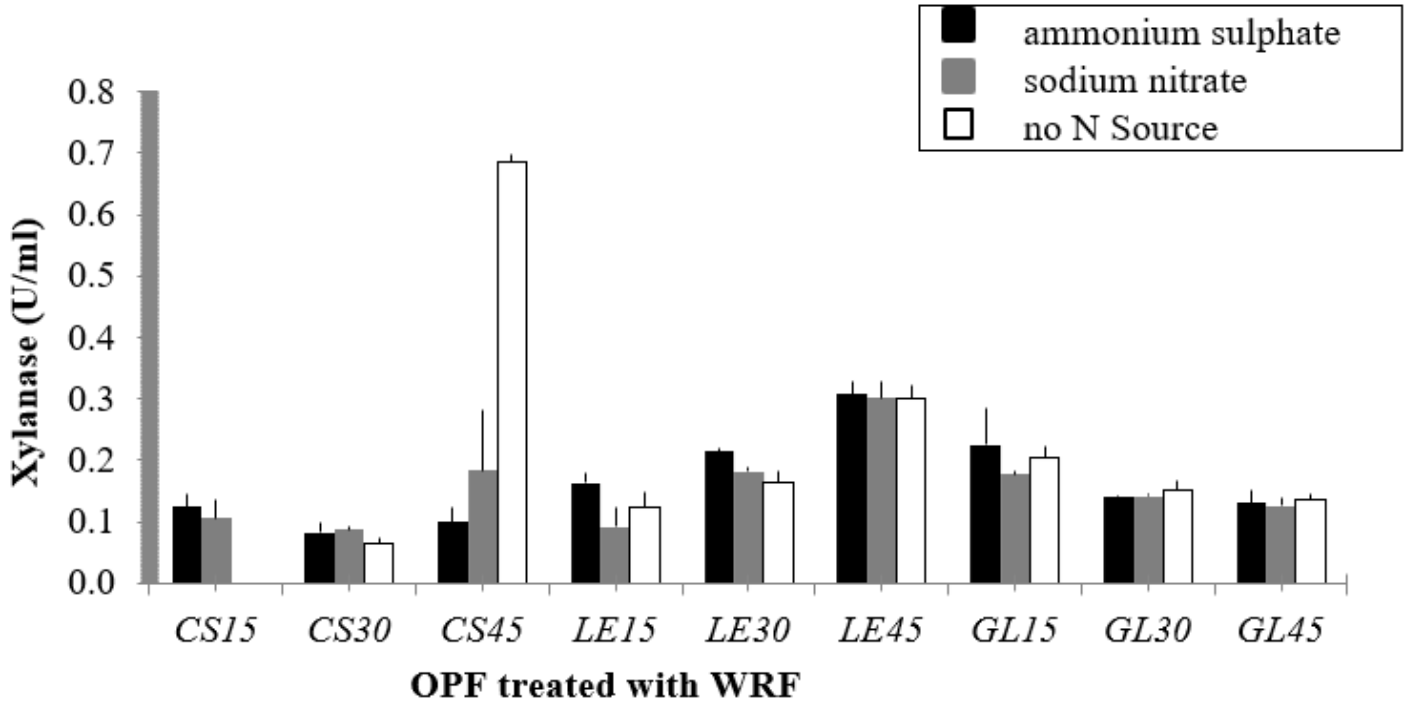

Figure 5

Xylanase enzyme activities of enzyme extract after inoculation of OPF for 15, 30 and 45 days with Ceriporiopsis subvermispora (CS), Lentinula edodes (LE) and Ganoderma lucidum (GL) in media with (NH4-medium and NO3-medium, respectively) and without (control) supplementation of a N source.