



Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: <http://www.keaipublishing.com/en/journals/aninu/>

Original Research Article

Optimizing bio-physical conditions and pre-treatment options for breaking lignin barrier of maize stover feed using white rot fungi



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ARTICLE INFO

Article history:

Received 27 May 2016

Received in revised form

5 August 2016

Accepted 17 August 2016

Available online 25 August 2016

Keywords:

Crop residues

Lactobacillus plantarum

Lignin biodegradation

Mycelial growth

ABSTRACT

The greatest limitation to utilization of maize stover by ruminants as a feed is the high concentration of lignin, which limits fibre digestibility. However, ruminants can effectively utilize maize stover if its nutritive value is improved using white rot fungal species. This study was designed to determine optimal bio-physical conditions for mycelial growth and select the most ideal fungal species and pre-treatment options for improving nutritive value of maize stover. Four popular edible *Pleurotus* fungal species (viz. *Pleurotus florida*, *Pleurotus ostreatus*, *Pleurotus sajor caju* and *Pleurotus pulmonarius*) were subjected to varying temperatures, pH levels, hydrogen peroxide (H₂O₂) concentration and illumination to establish the extent of mycelial growth rate. Inclusion of H₂O₂ was used to determine optimal levels for preservation and prevention of contamination from other indigenous microbiota. Effects of pre-treatment options on chemical composition and nutritive value of maize stover were also examined. Mycelial growth rate of *Pleurotus* species on potato dextrose agar (PDA) varied ($P < 0.05$) with temperature, pH level and H₂O₂ concentration following a quadratic trend. Optimal temperature, pH and H₂O₂ concentration for mycelial growth on PDA were 25 °C, 5 and 0.01 mL/L, respectively. Under the different bio-physical conditions, *P. sajor caju* had the highest mycelia density and growth rate. Chemical composition of solid-state fermented maize stover differed ($P < 0.05$) among the *Pleurotus* species. Maize stover fermented with *P. sajor caju* had the highest crude protein (CP) of 86.6 g/kg DM, *in-vitro* dry matter digestibility (IVDMD) of 731 g/kg DM, *in-vitro* organic matter digestibility (IVOMD) of 670.4 g/kg DM and metabolizable energy (ME) of 10.0 MJ/kg DM but with the lowest lignin (sa) of 50 g/kg DM. At 25 °C, *P. sajor caju* had the highest mycelial growth rate on PDA and highest lignin (sa) breakdown in the maize stover substrate. It was, therefore, selected as the most ideal fungal species for improving nutritive value of maize stover. Pre-treatment of maize stover with *Lactobacillus plantarum* and molasses under anaerobic condition for 7 days before inoculation with *P. sajor caju* resulted into a substrate with the highest ($P < 0.05$) CP (96.6 g/kg DM), IVDMD (752.3 g/kg DM), IVOMD (687.2 g/kg DM) and ME (10.2 MJ/kg DM). However, neutral detergent fiber exclusive of residual ash (NDFom) and lignin (sa) fractions decreased ($P < 0.05$) as a result of subjecting maize stover to pre-treatment with *L. plantarum* and molasses prior to fermentation with *P. sajor caju*.

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Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.



Production and Hosting by Elsevier on behalf of KeAi

<http://dx.doi.org/10.1016/j.aninu.2016.08.009>

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Therefore, pre-treatment of maize stover with *L. plantarum* and molasses for 7 days prior to fermentation with *P. sajor caju* for 14 days in darkness at 25 °C offered the greatest potential for breaking the lignin barrier.

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1. Introduction

Although the history of lignin and cellulose chemistry is old with voluminous amounts of literature (Barton, 1988), the recalcitrant nature of lignin still limits the availability of nutrients to ruminants. This undermines the utilization of cereal stover and straws as ruminant feeds. Lignin is a phenolic compound of high molecular weight that adds rigidity to cell wall structure but limits the digestibility and availability of cell wall contents to rumen microbes (Chaudhry, 1998). Maize stover is rich in carbohydrates but its voluntary intake by ruminants is limited due to high levels of ligno-cellulosic bondages, which increase with plant maturity. Consequently, many farmers burn maize stover despite its potential as a source of energy for ruminants.

Although ruminants are known to have a highly effective digestive system for fibre, they are incapable of extracting sufficient energy and protein from maize stover to facilitate growth, production and reproduction (Montañez-Valdez et al., 2015). This is because the cell wall contents of maize stover, particularly cellulose and hemicelluloses, are locked into a complex polymeric compound, which is exquisitely constructed to resist biological and chemical hydrolysis (Mahesh and Mohini, 2013). Linkages between lignin with cellulose and hemicellulose inhibit accessibility of rumen microbial enzymes to the structural carbohydrates and, therefore, locking significant amounts of potential energy (Tengerdy and Szakacs, 2003).

Use of white rot fungi as a biological means to liberate carbohydrates from the ligno-cellulosic bondage has been widely reported as a promising, affordable and environmentally acceptable approach to increasing nutritional value of straws (Chaudhry, 1998; Tengerdy and Szakacs, 2003; Graminha et al., 2008). Furthermore, earlier studies indicated that pre-treatment of crop residues with a consortia of fungi working in a synergistic and syntropic association with other fungi, yeast such as *Saccharomyces cerevisiae* or bacteria (*Lactobacillus plantarum*) would be preferable for better solid state fermentation (Wan and Li, 2012; Owen et al., 2012; Darwish et al., 2012). Darwish et al. (2012) indicated that maize stover treated with *Pleurotus ostreatus* and *S. cerevisiae* at 28 °C for 7–28 days improved CP content from 36 to 118 g CP/kg DM. Meanwhile hemicellulose, cellulose and lignin were reported to decrease with increasing incubation time of fermentation process from 260.1 to 141.7 g/kg DM, 410.0 to 200.8 g/kg DM and 114.9 to 58.4 g/kg DM, respectively, when treated with a combination of white rot fungi and yeast. Similarly, Chen et al. (1995) observed that solid state fermentation of maize stover for 28 day at 27 °C with *Cyathus stercoreus*, *Phledia brevispora* and *Phanerochaete chrysosporium* increased *in-vitro* dry matter digestibility (IVDMD) from 409 g/kg (un-treated maize stover) to 514 g/kg DM (treated with *P. brevispora*) and 523 g/kg DM (treated with *C. stercoreus*) but growth of the white rot fungi *P. chrysosporium* resulted into reduced IVDMD from 409 to 298 g/kg DM. However, Fazaeli (2007) indicated that IVDMD of wheat straw treated with *Pleurotus florida* at 22 ± 5 °C for 17 days increased from 281 to 403 g/kg while when treated with *P. ostreatus* IVDMD increased from 281 to 370 g/kg DM. Both *P. florida* and *P. ostreatus* decreased lignin content of fermented wheat straw from 82 g/kg DM to 74 and 72 g/kg DM, respectively.

Ability of white rot *Pleurotus* fungal species to secrete exogenous enzymes that liberate and degrade lignin and yet preserve cellulose (Shirma and Arora, 2015) offers an attractive opportunity to harness the fungal species that secrete oxidative enzymes during mycelial colonisation (Dashtban et al., 2010). However, there is a paucity of information on the appropriate *Pleurotus* species, optimum bio-physical conditions and pre-treatment options that farmers in the tropics can use to improve the nutritive quality of fibrous crop residues including maize stover as livestock feed. The objectives of this study were, therefore, to: 1) determine bio-physical conditions that farmers can use to optimize mycelial growth rate and effectiveness of selected *Pleurotus* species, 2) identify the best *Pleurotus* species that can be used under these condition and 3) determine pre-treatment options that can improve the effectiveness of solid state fermentation of maize stover with the best *Pleurotus* species.

2. Materials and methods

2.1. Preparation of experimental fungal materials

The study was conducted in the animal science laboratory at the College of Agricultural and Environmental Sciences (CAES), Makerere University, Uganda. Pure stock commercial cultures of 4 white rot *Pleurotus* species (*Pleurotus* spp.) including *P. florida*, *P. ostreatus*, *P. sajor caju* and *P. pulmonarius* were procured from a Belgium based mushroom culture company (MYCELIA). The 4 fungal species are the most popular edible commercial mushrooms in Uganda. An agar block of pure stock culture per species was aseptically transferred to a sterilized 90 mm diameter petri dish containing sterile potato dextrose agar (PDA) (Formedium Hunstanton, England). The aseptic conditions of the petri dishes were achieved by sterilization at 121 °C for 15 min in an autoclave. Three petri dishes per fungal species were randomly inoculated with the pure stock culture and sealed with a parafilm to allow uniform mycelial ramification at 25 °C. After 7 days of fungal mycelial growth and ramification, a sterile cork borer was used to cut out round mycelial discs of 8 mm diameter. The discs were used as secondary mycelia of the pure cultures for each of the test *Pleurotus* spp.

2.2. Determination of fungal growth

The effects of varying temperature, pH, substrate-sterilization with H₂O₂ and illumination regimes on fungal mycelial growth of the four *Pleurotus* spp. were investigated using petri dishes containing sterile PDA substrates. The outside bottom of each petri dish was dissected into four quarters using vertical and horizontal axes and labeled as r1, r2, r3 and r4, respectively (Fig. 1). The secondary mycelial discs of the different *Pleurotus* spp. were transferred after 7 days of mycelial growth to the centre (intersection point) of the petri dishes. Mycelial growth was measured along each radius of the petri dish.

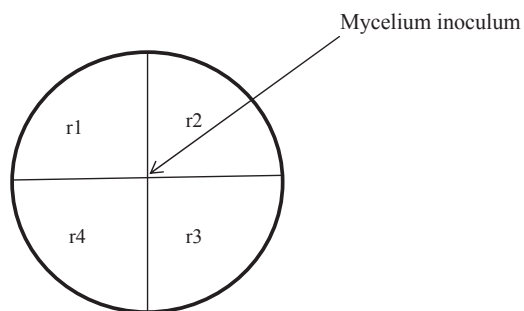


Fig. 1. Illustration of the 4 axes along which radial mycelial growth was measured.

2.2.1. Effects of temperature on fungal mycelial growth

The effects of temperature on mycelial growth rate of the *Pleurotus* spp. were evaluated at 4 levels including 20, 25, 30 and 35 °C. In this experiment, pH was fixed at 5.0 and H₂O₂ concentration at 0.01 mL/L (vol/vol). Secondary mycelial discs of 7 days old from each of the 4 *Pleurotus* spp. were inoculated in triplicates into the petri dishes for each test temperature. The 12 test petri dishes for each fungal species were randomly allocated to each of the 4 incubators maintained at different temperatures. Precaution was taken to ensure that each incubator contained twelve petri dishes that comprised 3 dishes for each *Pleurotus* spp. examined at the assigned temperature.

2.2.2. Effects of pH on fungal mycelial growth rate

The effects of pH on mycelial growth rate of the *Pleurotus* spp. was evaluated at 5 pH levels including 4.0, 5.0, 6.0, 7.0 and 8.0. In this experiment, temperature was fixed at 25 °C and H₂O₂ concentration at 0.01 mL/L (vol/vol). The different pH levels were obtained by adding varying proportions of 1.0 mol/L acetic acid and 1.0 mol/L sodium hydroxide solutions to the PDA media. Adjustments to pH 7 and pH 8 were achieved using calcium carbonate (CaCO₃). Using a pH meter (Activon ACE69 model), which had been calibrated prior to the experiment with a two point buffering system at pH 4 and 8, the pH of the unadjusted medium was initially established at room temperature (25 °C). To minimize the impact of temperature on pH readings of the media, the buffer solution used to standardize the pH meter was kept in a water bath at 25 °C. Because of the difficulty of having to maintain pH of agar media at the pH extremes of 4 and 8, adjustments to pH 4 and pH 8 were achieved after sterilization. This was possibly because of dissociation rate of the media to H⁺ and OH⁻ ions, which are temperature-dependent. However, agar media for pH 5, 6 and 7 were stable and pH adjustments were achieved prior to sterilization. Same methodological procedures as already indicated in Section 2.2.1 were followed to assess the effects of pH on the rate of mycelial growth for the different *Pleurotus* spp.

2.2.3. Effects of substrate sterilization with H₂O₂ on fungal mycelial growth rate

The effects of chemical sterilization of PDA with H₂O₂ on mycelial growth rate were investigated at 5 varying concentrations including 0.01, 0.032, 0.1, 0.32 and 1.0 mL/L (Zharare et al., 2010). In this experiment, pH was fixed at 5.0 and temperature at 25 °C. Inclusion of H₂O₂ was mainly used to determine optimal levels for preservation and prevention of contamination from other members of the microbiota that lack the peroxidase-mediated antimicrobial system. Each of the four *Pleurotus* spp. was tested in triplicates for each H₂O₂ concentration. The 5 H₂O₂ concentrations of the media were established at logarithmic intervals using the procedure reported by Zharare et al. (2010) in the equation: $ar^{n-1} = b$. Where: 'a'

is the lowest concentration of H₂O₂ (0.01 mL/L) above zero (control), 'b' is the highest concentration of H₂O₂ lower than 10.0 mL/L fixed at 1.0 mL/L, 'n' is the number of concentration (i.e., $n = 5$) within the range of a - b and 'r' is the constant obtained by solving the equation $0.01r^4 = 1.0$.

2.2.4. Effects of substrate illumination on fungal mycelial growth rate

In this experiment, the effects of illumination on mycelial growth rate were evaluated at 2 conditions including presence and absence of light. Irrespective of illumination conditions, all the PDA substrates in the petri dishes were kept at pH 5 and sterilized with H₂O₂ (0.01 mL/L [vol/vol]). Under lighted conditions, twelve inoculated petri dishes comprising of 3 petri dishes for each of the 4 *Pleurotus* sp. were placed in an incubator with artificial light of 322.92 lumens/m² maintained at 25 °C. The dark conditions were obtained by placing twelve inoculated petri dishes comprising of 3 petri dishes per *Pleurotus* sp. each wrapped with a sterile black piece of paper first and then with an aluminum foil and incubated at 25 °C. Visual examination of mycelial density was carried out following the procedure reported by Kanmani et al. (2009).

2.2.5. Effects of pH and temperature on mycelial growth rate

In this experiment, effects of pH on mycelial growth rate of *Pleurotus* spp. as affected by varying temperatures on the 5th day of mycelial colonization was evaluated at 5 pH levels of 4.0, 5.0, 6.0, 7.0 and 8.0. The temperature was varied at 20, 25, 30 and 35 °C but H₂O₂ concentration was fixed at 0.01 mL/L (vol/vol). Secondary mycelial discs of 7 days old from each of the 4 *Pleurotus* spp. were inoculated in triplicates into the petri dishes for each test temperature and pH. The 12 test petri dishes for each fungal species at each pH level were randomly allocated to each of the 4 incubators maintained at different temperatures.

2.3. Calculation of mycelial growth rate

Mycelial growth rate of the different fungal species on PDA was calculated based on the zone of the agar in the petri dish ramified by mycelium. Fungal species with the highest mycelial growth rate was selected as the most suitable for biological treatment of maize stover. The average mycelial growth rate (mm²/day) was determined using the formula suggested by Lonergan et al. (1993): $M = \{[G(d6) - G(d5)] + [G(d5) - G(d4)] + [G(d4) - G(d3)] + [G(d3) - G(d2)]\}/4 \dots \dots (i)$, where: M = average mycelial growth rate per day (mm²/day) for each *Pleurotus* spp.; G (d6) was the average mycelial growth (in mm) on day 6; G (d5) was the average mycelial growth (in mm) on day 5; G (d4) was the average mycelial growth (in mm) on day 4; G (d3) was the average mycelial growth (in mm) on day 3; G (d2) was the average mycelial growth (in mm) on day 2.

To compute area of ramification for respective *Pleurotus* spp. on PDA petri dishes, mean radius of mycelial growth (mm²/day) was incorporated into the formula πr^2 ($\pi = 3.14$). *Pleurotus* sp. with the highest mycelial growth rate was selected as the most suitable for biological treatment of maize stover.

2.4. Sampling and analysis of maize stover fermented with *Pleurotus* spp.

Sample substrates tested were obtained from open pollinated maize (Longe 5), which was planted at a spacing of 0.75 m between rows and 0.25 m between plants within a row in 4 blocks each of one hectare in different locations. Sloping nature of the land from uphill to the valley formed the basis for blocking. In establishment and management of the maize crop, all recommended agronomic

practices for maize production in the area were followed in each of the blocks. Diammonium phosphate (DAP) fertilizer was applied at a rate of 100 kg/ha at planting time and urea was applied when the plant reached 0.7 m at a rate of 120 kg/ha. Weed clearing was manually accomplished using a hand hoe and sickle, however, no irrigation was practiced. Harvesting of maize was done per plot per block after 120 days when maize grain was about 175 g/kg DM moisture content. Each block was divided into 8 plots and each plot had at least 12 stubbles of maize stover randomly harvested in 2 batches.

Four bags were used per plot; each accommodating 3 chopped maize stover stubbles, making a total of 32 bags for a block and a total of 128 bags for the 4 blocks. Maize stover for each bag was separately chopped using a hand forage chopper. The chopper was thoroughly cleaned after every four bags that were obtained per plot. This exercise was repeated to get 2 batches each of 128 bags from the 4 blocks. One batch of 128 bags was stored in a cool dry place for later use on the pre-treatment options prior to fermentation with the appropriate fungal species. From one of the batches, all the chopped maize stover harvested from each plot was packed in labeled sisal bags and soaked in a solution of H₂O₂ at a concentration of 0.01 mL/L of distilled water for 24 h in steel water tanks (200 cm × 100 cm × 80 cm size). Although H₂O₂ is commonly used for preservation and prevention of contamination of substrate with indigenous microbiota, it was also used to delignify and remove hemicellulose to facilitate accessibility of hydrolytic enzymes to cellulose across all the treatments. The wet samples were drained and sun-dried to a moisture content of 650–700 g/kg DM determined by squeeze test procedures as suggested by [Elisashvili et al. \(2008\)](#).

After H₂O₂ treatment, maize stover samples (10 kg DM/bag) were transferred into a black polyethylene bags (35 cm × 40 cm; 100 µm thickness) and inoculated with 7-day-old secondary mycelial discs (10 discs per bag). Each of the 4 popular commercial *Pleurotus* spp. (viz; *P. florida*, *P. sajor caju*, *P. pulmonarius* and *P. ostreatus*) was randomly assigned to ferment 1 of the 4 H₂O₂-treated maize stover substrate from each plot. Each *Pleurotus* sp. was inoculated on 8 sample bags of maize stover from each of the 4 blocks. From each of the 32 bags randomly allocated to a single fungal sp., samples of the original maize stover treated with H₂O₂ were also transferred into black polyethylene bags without fungal inoculation and designated as the control. Thereafter, the control bags were subjected to similar conditions as those inoculated with the different *Pleurotus* spp. After inoculation, the bags were tightly sealed with nylon ropes to allow controlled aeration but with a headspace to ensure gaseous exchange. Limited aeration was earlier recommended for effective selectivity of lignin degradation over cellulose and hemicellulose by white rot fungi ([Wan and Li, 2012](#)). The substrate was then subjected to solid-state fermentation for 14 days at 25 °C in a dark room. During the second week of solid-state fermentation, when mycelial running started, all the sides of the bags were punched with pinholes to provide aeration necessary for the highly oxidative lignin degradation process ([Fazaali, 2007](#); [Wan and Li, 2012](#)). Thereafter, the substrate was sun-dried to terminate colonization and then preserved in cotton-sewn bags for subsequent laboratory analyses.

Analyses of dry samples were done in triplicates for dry matter (DM), crude protein (CP) and ash according to procedures of 930.15, 954.01 and 942.05, respectively ([AOAC, 1995](#)). Neutral detergent fiber (NDF), exclusive of residual ash (NDFom), was determined without addition of α-amylase or sodium sulphite according to the method (973.18; [AOAC, 1995](#)). The same sample was sequentially analysed for acid detergent fiber exclusive of residual ash (ADFom) and lignin (sa) was determined by solubilization of cellulose with sulphuric acid (12N) according to the method of [Robertson and Van](#)

[Soest \(1981\)](#). The concentration of hemicellulose was calculated as the difference between NDFom and ADFom analysed sequentially on the same sample, while cellulose was computed as the difference between ADFom and lignin (sa). *In vitro* digestibility was estimated as the proportions of dry matter (IVDMD) and organic matter (*in vitro* organic matter digestibility [IVOMD]) disappearance using [Tilley and Terry method \(1963\)](#) as modified by [Goering and Van Soest \(1970\)](#). Metabolizable energy (ME MJ/kg DM) values were estimated from IVOMD data using the equation: $ME_{(IVOMD)} = 0.81 \times 0.184 \times IVOMD$ ([ARC, 1980](#)).

2.5. Pre-treatment of maize stover prior to fermenting with *P. sajor caju*

Effects of four pre-treatment options of maize stover prior to fermentation with *P. sajor caju* were randomly selected and investigated. The pre-treatment options included 1) steaming chopped maize stover in an autoclave at 121 °C for 5 min used as the control, 2) thoroughly mixing and soaking the chopped maize stover with molasses solution (11 g/kg DM) for 7 days, 3) thoroughly mixing and soaking chopped maize stover in *L. plantarum* solution (10³ cfu/mL) for 7 days and 4) thoroughly mixing and soaking chopped maize stover in a mixture of *L. plantarum* (10³ cfu/mL) and molasses (11 g/kg DM) for 7 days. Each of the 4 pre-treatment options was conducted in triplicates. The substrates were kept under anaerobic conditions by tightly tying the sample bags with a nylon rope for 7 days before inoculating it with *P. sajor caju* and fermenting for 14 days.

The *L. plantarum* was obtained by harvesting it from yellow apple bananas following procedures as outlined by [Dawson et al. \(1990\)](#). Bioinformatics analysis of putative extracellular proteins of the lactic acid-forming bacterium was carried out in a molecular laboratory and *L. plantarum* was identified as a domain composition of the sample ([Teusink et al., 2006](#)).

2.6. Statistical analysis

Data to establish optimal growth performance of the *Pleurotus* spp. on PDA was analysed as a completely randomized design using mixed model procedures of [SAS \(2002\)](#). The model included the different bio-physical conditions as fixed factors, while the four *Pleurotus* spp. were considered as random variables. Effects of bio-physical conditions on mycelial growth rate with increasing temperature, pH levels and H₂O₂ concentration were examined using linear and quadratic contrasts. The data on mycelial growth rate of the different *Pleurotus* spp. on the 5th day and the relationship between pH levels, temperature and how this influenced mycelial growth rate were illustrated using graphical means.

The effects of substrate illumination on mycelial growth rate were subjected to a *t*-test while data on the effects of fermenting maize stover with 4 different *Pleurotus* spp. on chemical composition, and effect of pre-treatment option on nutritive value were subjected to a one-way analysis of variance using a completely randomized block design. The treatment combinations were replicated 4 times as represented by the number of blocks but repeated 8 times in each of the 4 blocks. The total number of observations were: 4 (fungal species tested on each of the 4 stover bags per plot) × 8 (plots per block) × 4 (blocks) × 3 run replicates in the laboratory. After averaging the 8 plot results for the respective white rot fungal species from each block and the laboratory run replicates, the remaining 16 observations were randomly distributed in the 4 blocks and subjected to separation of the treatment means using Duncan's multiple range test at a significance level of $P < 0.05$.

3. Results

3.1. Mycelial growth rate

Effects of bio-physical conditions on mycelial growth rate of *P. florida*, *P. sajor caju*, *P. pulmonarius* and *P. ostreatus* are presented in Table 1. Increasing temperature from 20 °C increased mycelial growth rate of the *Pleurotus* spp. ($P < 0.05$) at a decreasing rate with the highest growth observed at the intermediate temperature. The optimum mycelial growth rate was observed with *P. sajor caju* at 25 °C. Moreover, *P. sajor caju* maintained the highest mycelial growth rate across all the four incubation temperatures.

Mycelial growth rate of all *Pleurotus* spp. increased quadratically with increasing levels of pH apart from that of *P. sajor caju* that only showed tendencies ($P = 0.06$) (Table 1). The highest mycelial growth rate of 1,133.74 mm²/day was attained at pH 5 and was recorded among petri dishes with *P. sajor caju*. At pH 5, *P. ostreatus* had the lowest mycelial growth rate of 979 mm²/day compared to the other species.

The mean mycelial growth rate on PDA sterilized with different concentrations of H₂O₂ varied among the white rot fungal species in the order of *P. sajor caju* > *P. pulmonarius* > *P. florida* > *P. ostreatus* (Table 1). As H₂O₂ concentration increased above 0.01 mL/L, an inverse relationship appeared between mycelial growth rate and H₂O₂ concentration across all the *Pleurotus* spp. At the highest H₂O₂ concentration (1.0 mL/L), mycelial growth rate of *P. pulmonarius* was the lowest.

The relationship between pH and mycelial growth rate for all the *Pleurotus* spp. exhibited a quadratic trend and was also graphically shown to depend on temperature (Fig. 2). The optimal temperature was 25 °C in all *Pleurotus* spp. and mycelial growth rate of *P. sajor caju* was almost 2 times higher than that of *P. florida* under similar

bio-physical conditions. Furthermore, mycelial growth rate of *P. sajor caju* was observed even beyond pH 8 at 25 °C.

Mycelial growth rates of *P. sajor caju* and *P. pulmonarius* were influenced ($P < 0.05$) by illumination (Table 2). Generally, all *Pleurotus* spp. in petri dishes incubated in the darkness had relatively higher mean mycelial growth rate than those incubated under light. Also very thick mycelium was recorded in petri dishes incubated in the dark condition while thin mycelium was observed in plates incubated under light conditions.

3.2. Chemical composition of maize stover fermented with *Pleurotus* spp.

Chemical compositions and *in-vitro* digestibility of maize stover sterilized with 0.01 mL/L H₂O₂ concentration at 25 °C, pH 5 and inoculated with *Pleurotus* spp. for 14 days are presented in Table 3. There were differences ($P < 0.05$) in DM, CP, NDFom, ADFom and lignin (sa) among maize stover samples fermented with different *Pleurotus* spp. Maize stover substrate fermented with *P. sajor caju* had the highest CP (86.6 g/kg DM) but with the lowest ADFom (419 g/kg DM) and lignin (sa) (50 g/kg DM) content compared to substrates fermented by other fungal spp. and the control. Although *P. sajor caju* exhibited superior mycelial growth rate on both PDA and maize stover substrate, it resulted into the highest ($P < 0.05$) dry matter loss of fermented maize stover. However, fermenting maize stover with *P. florida* resulted into the lowest DM loss. The highest IVOMD (670.4 g/kg DM) and, consequently, ME (10.0 MJ/kg DM) were also observed in maize stover substrate fermented with *P. sajor caju*. Based on the results of mycelial growth rate at 25 °C on PDA, rate of lignin (sa) breakdown and ME content of maize stover, *P. sajor caju* was identified as a more viable white rot fungi for further evaluation during pre-treatment of maize stover.

3.3. Pre-treatment options of maize stover fermented with *P. sajor caju*

Chemical composition of pre-treated maize stover varied ($P < 0.05$) with pre-treatment options (Table 4). Chopped maize stover pre-treated with *L. plantarum* and molasses before inoculation with *P. sajor caju* had the highest CP content (96.6 g/kg DM). The NDFom and lignin (sa) contents of maize stover varied ($P < 0.05$) with the pre-treatment options. Pre-treating maize stover with *L. plantarum* and molasses prior to inoculation with *P. sajor caju* resulted into the highest reduction in lignin (sa) from 61.0 to 45.01 g/kg DM. Subsequently, IVDMD, IVOMD and ME content of maize stover significantly improved ($P < 0.05$) to 752.3, 687.2 g/kg DM and 10.2 MJ/kg DM, respectively.

4. Discussion

4.1. Bio-physical conditions for mycelial growth

Although bio delignification of maize stover with *Pleurotus* spp. often results in increased IVOMD (Karunanandaa and Varga, 1996), identifying the best fungal species and optimizing its bio-physical and pre-treatment conditions is essential for better mycelial growth rate and lignin break down. Arguably, mycelial growth rate is one of the ways of comparing colonization ability and enzyme production potential of different fungal species under different bio-physical conditions (Novotný et al., 2004). Mycelial growth rates of *Pleurotus* spp. spawned on PDA media subjected to varying temperature, pH level, H₂O₂ concentration and illumination varied among species but were similar to earlier reported values (Khushairi and Zainol, 2011; Dulay et al., 2012). Quadratic

Table 1
Effect of bio-physical conditions¹ on mycelial growth rate of *Pleurotus* spp.²

Treatment	Mycelial growth rate, mm ² /day			
	<i>Pleurotus florida</i>	<i>Pleurotus sajor caju</i>	<i>Pleurotus pulmonarius</i>	<i>Pleurotus ostreatus</i>
Temperature, °C				
20	655.6	888.6	755.4	540.3
25	970.4	2,082.8	1,918.1	1,256.4
30	638.2	658.6	422.0	635.9
35	199.3	542.2	205.9	188.0
SEM	71.0	71.5	71.8	71.4
Linear	<0.001	0.01	0.04	0.03
Quad	0.03	0.04	0.04	0.04
pH level				
pH 4	980.9	992.8	962.7	913.1
pH 5	992.8	1,133.7	1,119.1	979.4
pH 6	571.1	638.2	638.2	638.2
pH 7	305.7	418.8	418.8	307.7
pH 8	195.3	307.7	307.7	195.3
SEM	72.2	72.2	72.0	72.0
Linear	0.23	0.24	0.17	0.86
Quad	0.01	0.06	<0.001	<0.001
H ₂ O ₂ , mL/L of distilled water				
0	563.5	915.1	913.1	418.8
0.01	561.5	1,133.7	1,107.4	571.1
0.032	418.8	563.5	561.6	396.0
0.1	307.7	195.3	202.0	211.2
0.32	202.0	181.5	174.2	173.4
1.0	151.8	174.5	139.2	163.0
SEM	70.7	71.1	71.1	70.7
Linear	0.2	0.14	0.2	0.2
Quad	0.02	<0.001	<0.001	0.03

¹ Temperature, pH level, H₂O₂ concentration.

² *Pleurotus florida*, *Pleurotus sajor caju*, *Pleurotus pulmonarius*, *Pleurotus ostreatus* (n = 12).

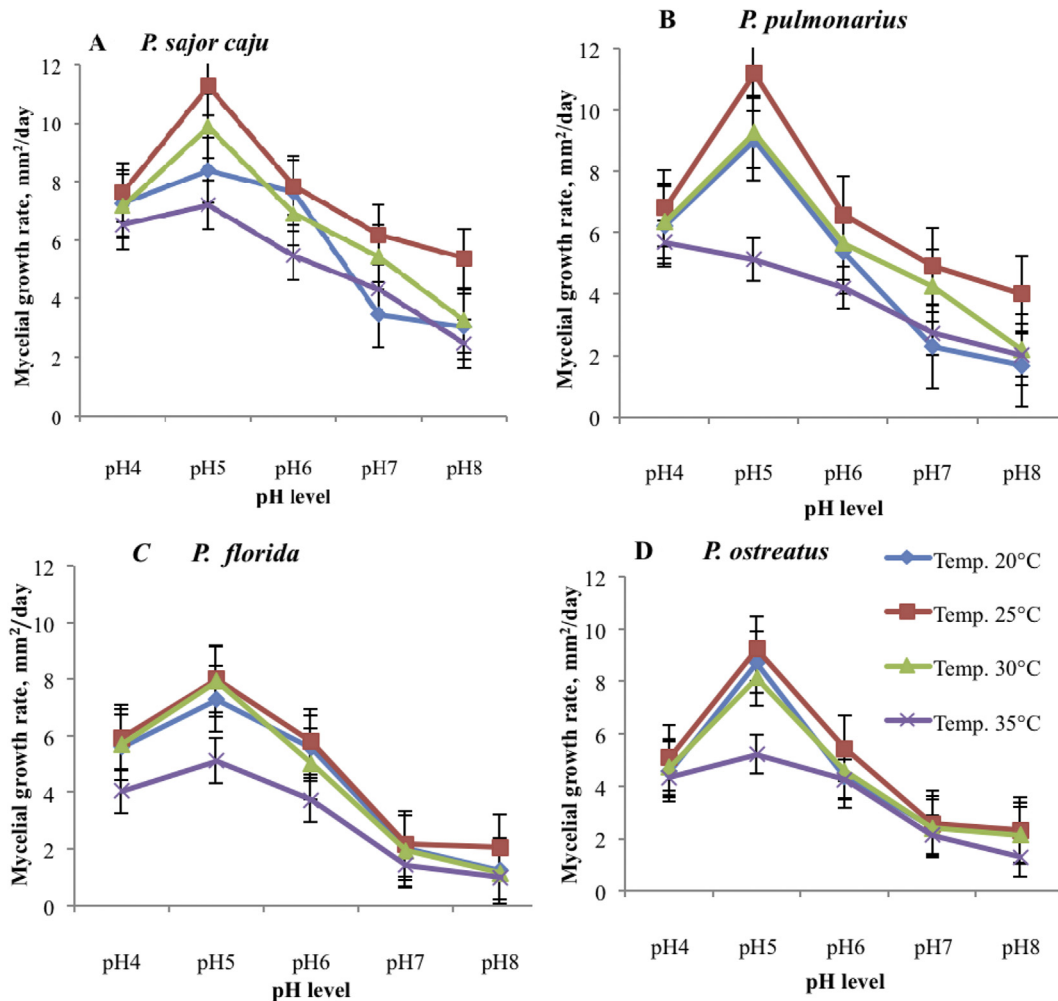


Fig. 2. Mycelial growth rate of the fungal species (A) *P. sajor caju* (B) *P. pulmonarius* (C) *P. florida* and (D) *P. ostreatus* on the 5th day of incubation on potato dextrose agar (PDA) under varying temperatures and pH.

Table 2
Effect of illumination on mycelial growth rate of *Pleurotus* spp.

Item	Mean mycelial growth rate, mm ² /day			
	Light	Dark	SEM	P-value
<i>Pleurotus florida</i>	1,170.2	1,176.0	11.09	0.08
<i>Pleurotus sajor caju</i>	2,038.4	2,067.7	10.26	0.02
<i>Pleurotus pulmonarius</i>	2,053.4	2,087.2	10.29	0.02
<i>Pleurotus ostreatus</i>	1,178.9	1,187.2	10.82	0.06
Mycelial density ¹	+	++		

¹ + thin mycelial density, ++ thick mycelial density (Kanmani et al., 2009).

mycelial growth rate observed across all *Pleurotus* spp. with varying temperature, pH levels and H₂O₂ concentration suggest that these biophysical parameters have an inhibitory effect on mycelial growth beyond optimal levels of 25 °C, pH 5, and 0.01 mL/L, respectively. The observed mycelial growth rate of 542.2 mm²/day in petri dishes inoculated with *P. sajor caju* at 35 °C possibly indicates its tolerance and resilience to higher tropical temperatures compared to other *Pleurotus* spp. Relationships in Fig. 2 further demonstrate that pH effects on mycelial growth rate heavily depends on temperature for all the *Pleurotus* spp. Moreover, the highest growth rate of *P. sajor caju* on PDA at pH 5 and 25 °C on the 5th day of fermentation agrees with earlier results (Dulay et al., 2012). Mycelial growth rate of *Pleurotus* spp. on PDA under light and dark conditions were higher than what was earlier reported

(Dulay et al., 2012). However, presence of light had negative influence on mycelial growth rate and vigor, especially, for *P. sajor caju* and *P. pulmonarius*. This is consistent with earlier findings by Khushairi and Zainol (2011) who indicated superiority in mycelial growth rate of *Pleurotus* spp. in darkness. This is an indication that farmers may need black polyethylene bags of the right gauge to cut off light for better colonization ability of *P. sajor caju* when fermenting maize stover as a feed for ruminants.

4.2. Chemical composition of fermented maize stover

The profile of chemical components (Table 3) of maize stover fermented with different *Pleurotus* spp. were in agreement with earlier studies (Chen et al., 2009; Montañez-Valdez et al., 2015) and depicts inherent variations among *Pleurotus* spp. Badarinaa et al. (2013) reported that cellulose, hemicelluloses and lignin are the main sources of carbon and energy for the growth of *Pleurotus* spp. Therefore, mycelial proliferation by all the *Pleurotus* spp. on maize stover was possibly due to their saprophytic ability to solubilize structural carbohydrates and their ability to extract fibre bound nitrogen for their metabolism and growth. Different *Pleurotus* spp. are known to differ in their ability to secrete both hydrolytic and oxidative extracellular enzymes (Rodríguez Couto and Sanroman, 2005; Olfati and Peyvast, 2008; Mahesh and Mohini, 2013). Based on results of this study, it is possible that changes in DM, CP and

Table 3
Chemical composition of fermented maize stover substrate with *Pleurotus* spp.

Chemical composition	<i>Pleurotus</i> spp.					SEM	P-value
	Control	<i>Pf</i>	<i>Ps</i>	<i>Pp</i>	<i>Po</i>		
DM, g/kg	913.0 ^a	887.0 ^b	865.0 ^c	877.0 ^b	883.0 ^b	7.9	0.02
CP (N × 6.25), g/kg DM	54.2 ^b	81.2 ^a	86.6 ^a	85.9 ^a	80.1 ^a	6.0	0.03
NDFom, g/kg DM	772.0 ^a	625.0 ^b	619.0 ^b	612.0 ^b	627.0 ^b	6.4	0.02
ADFom, g/kg DM	458.0 ^a	426.0 ^b	419.0 ^b	425.0 ^b	427.0 ^b	6.9	0.04
Lignin (sa), g/kg DM	83.0 ^a	65.0 ^b	50.0 ^c	53.0 ^c	67.0 ^b	5.9	0.02
Cellulose, g/kg DM	375.0 ^a	360.0 ^c	372.0 ^a	369.0 ^b	361.0 ^c	4.7	0.01
Hemicellulose, g/kg DM	314.0 ^a	198.0 ^b	200.0 ^b	187.0 ^c	201.0 ^b	4.3	0.01
IVDMD, g/kg DM	385.5 ^a	698.2 ^b	731.0 ^c	717.3 ^c	682.3 ^b	4.9	0.01
IVOMD, g/kg DM	310.6 ^a	638.9 ^b	670.4 ^c	656.9 ^c	618.6 ^b	6.7	<0.001
ME, MJ/kg DM	4.6 ^a	9.5 ^b	10.0 ^b	9.8 ^b	9.2 ^b	0.9	<0.001

DM = dry matter; CP = crude protein; NDFom = neutral detergent fibre not assayed with α -amylase expressed exclusive of residual ash; ADFom = acid detergent fibre expressed exclusive of residual ash; Lignin (sa) = determined by solubilization of cellulose with sulphuric acid; IVDMD = *in-vitro* dry matter digestibility; IVOMD = *in-vitro* organic matter digestibility; ME = metabolizable energy; *Pf* = *Pleurotus florida*; *Ps* = *Pleurotus sajor caju*; *Pp* = *Pleurotus pulmonarius*; *Po* = *Pleurotus ostreatus*.

^{a,b,c} Least square mean in the same row followed by different superscript differ ($P < 0.05$).

Table 4
Effect of pre-treatment options on nutritive value of maize stover substrate fermented with *P. sajor caju*.

Nutritive value	Pre-treatment options ¹				SEM	P-value
	SMS	Mol	Lp	Lpm		
DM, g/DM	887.0 ^a	883.0 ^a	877.0 ^a	865.0 ^b	7.92	0.05
CP, g/kg DM	81.2 ^b	80.1 ^b	95.9 ^a	96.6 ^a	7.69	0.04
NDFom, g/kg DM	625.0 ^a	627.0 ^a	602.0 ^b	591.0 ^c	9.87	0.03
ADFom, g/kg DM	407.0 ^a	419.0 ^a	412.0 ^a	398.0 ^a	7.38	0.07
Lignin (sa), g/kg DM	61.0 ^b	68.0 ^a	51.1 ^c	45.0 ^c	7.66	<0.001
Cellulose, g/kg DM	346.0 ^b	351.0 ^b	361.0 ^a	353.0 ^b	3.52	0.04
Hemicellulose, g/kg DM	218.0 ^a	208.0 ^b	190.0 ^c	193.0 ^c	5.87	0.03
IVDMD, g/kg DM	698.1 ^c	602.4 ^c	728.4 ^b	752.3 ^a	6.99	0.02
IVOMD, g/kg DM	649.3 ^b	615.8 ^c	666.5 ^b	687.2 ^a	9.81	<0.001
ME, MJ/kg DM	9.7 ^{ab}	9.2 ^b	9.9 ^a	10.2 ^a	0.53	0.05

DM = dry matter; CP = crude protein (N × 6.25); NDFom = neutral detergent fibre not assayed with α -amylase expressed exclusive of residual ash; ADFom = acid detergent fibre expressed exclusive of residual ash; Lignin (sa) = lignin determined by solubilization of cellulose with sulphuric acid; IVDMD = *in vitro* dry matter digestibility; IVOMD = *in vitro* organic matter digestibility; ME = metabolizable energy; SMS = steamed maize stover.

^{a,b,c} Least square means in the same row within chemical composition with different superscript differs significantly ($P < 0.05$).

¹ SMS, Steamed maize stover at 121 °C for 5 min; Mol, molasses, 11.4 g/L added in 2 L of water to form a pre-treatment solution into which maize stover was soaked for 7 days; Lp, *L. plantarum*, 10³ cfu/mL added to maize stover and fermented for 7 days; Lpm, mixture of molasses, 11.4 g/L and *L. plantarum*, 10³ cfu/mL mixed together for pretreatment of maize stover by soaking for 7 days.

fibre components of maize stover substrate are influenced by different enzymes secreted by the different *Pleurotus* spp. Hemicellulose and lignin are the 2 cell wall structural components that are metabolized during primary growth stage of the fungi in almost the same proportions (Montañez-Valdez et al., 2015). Therefore, a significant reduction of lignin (sa) from 83.0 g/kg DM in the control to 50.0 g/kg DM in maize stover fermented with *P. sajor caju* indicates increased fibre solubility and improved nutritive value of maize stover. Moreover, it is documented that mycelial growth rate and substrate colonisation are the most critical parameters that influence bio-degradation of ligno-cellulosic materials (Rodrigues et al., 2008). Furthermore, Pérez et al. (2002) reported that the ability of *Pleurotus* spp. to produce extracellular lignin-modifying enzymes such as phenol oxidases (i.e., laccase) or heme peroxidases (i.e., lignin peroxidases; manganese peroxidases and versatile peroxidases) varies among white rot fungi.

Maize stover substrate fermented with *P. sajor caju* for 14 days at 25 °C had the highest amounts of CP (86.6 g/kg DM) and exhibited superiority of IVDMD (731.0 g/kg DM) and IVOMD (670.4 g/kg DM). This was possibly due to supremacy in

colonisation ability of *P. sajor caju* that allowed lignin breakdown and transportation of nitrogen and iron in form of heme peroxidases needed to modify ligno-cellulosic complexes through depolymerisation of cellulose and lignin oxidation using the Fenton system (Fe²⁺ and H₂O₂). Therefore, the dominance exhibited by *P. sajor caju* in mycelial growth rate on PDA and colonization ability that resulted into the highest DM loss of the maize stover compared to other *Pleurotus* spp. was possibly an indication of its unique lignolytic system. This is in agreement with earlier findings of Rahman et al. (2011) who indicated that preferential degradation of lignin depends on fungal species. This is also consistent with earlier results obtained by Chen et al. (1995) who observed increase in IVDMD, of maize stover treated with *Phlebia brevispora* at 27 °C for 28 days, from 409.0 to 514.0 g/kg DM. Similarly, Fazaeli (2007) indicated that IVDMD of wheat straw treated with *P. florida* at 22 ± 5 °C for 17 days increased from 281.0 to 403.0 g/kg DM while when treated with *P. ostreatus*, IVDMD increased from 281.0 to 370.0 g/kg DM. Such feedstock improvement was far lower than values obtained in the current study under similar conditions possibly because of the different fungal species, substrates and days of solid-state fermentation. Generally, irrespective of the *Pleurotus* spp. used, white rot fungi decreased lignin (sa), ADFom and NDFom contents of fermented maize stover but increased CP content, IVDMD and IVOMD, which was consistent with earlier results observed in wheat straw and maize stover (Chen et al., 1995; Adamović et al., 1998; Fazaeli, 2007). However, the relatively high ADFom observed in maize stover substrates fermented by the *Pleurotus* spp. was probably due to chitin content of the fungal mycelia known to interfere with digestibility in ruminants (Hendriks and Zeeman, 2009). The high chitin content ascribed to fungal mycelia may lead to a rise in phenolic intermediates during lignin metabolism by *P. sajor caju* thus reducing the *in vivo* digestibility and utilisation of fermented maize stover (Owen et al., 2012). This, therefore, necessitates evaluation of fermented maize stover substrate using both *in-vitro* and *in-vivo* experiments to validate the high IVDMD and IVOMD in this study. Owen et al. (2012), however, contends that lignin-degradation products are unlikely to be harmful to rumen microbes because lignases produced by white rot fungi are active *in vitro*, but arguably concedes that a consortia of fungi, yeast and bacteria would be preferable for solid state fermentation.

4.3. Pre-treatment of maize stover prior to fermentation with *P. sajor caju*

Effects of the different pre-treatment options on enhancement of the nutritive value of maize stover fermented with *P. sajor caju* is

consistent with earlier observations by Peréz et al. (2002) which indicated that enhancement of nutritive value of maize stover fermented with white rot fungi requires multi-step process that may be synergistic in order to optimise enzymatic hydrolysis and oxidation. If ruminants are to optimally utilize maize stover as a feed, biomass pre-treatment with heat, fungal enzymes, physical chopping or use of additive is one of the additional steps needed for effective liberation of cellulose and hemicelluloses from lignin (Wan and Li, 2012; Shirma and Arora, 2015). Since pre-treatment of maize stover with *L. plantarum* and molasses before fermentation with *P. sajor caju* improved CP content from 81.2 to 96.6 g/kg DM, IVDM from 698.1 to 752.3 g/kg DM and IVOMD from 649.3 to 687.2 g/kg DM, it is possible that homofermentation of water soluble carbohydrates to lactic acid provided a conducive environment for fungal colonisation. This is consistent with the findings of Gupta et al. (2011), which showed increased ME content when hay was pre-treated with yeast and *L. plantarum*. The *L. plantarum* inoculant is not only known to produce cellulolytic enzymes, such as cellulases, cellobiohydrolase, endoglucanase and exoglucanase that act on the surface of insoluble ligno-cellulosic complexes but it is also known to produce enzymes that hydrolyse pectin (Malik et al., 2015). Therefore, pre-treatment of maize stover with *L. plantarum* and molasses possibly synergized the action of *P. sajor caju* to biodegrade lignin to its lowest value of 45.0 g/kg DM by initiating production of a series of enzymes including pectin-hydrolysing enzymes. Such pectinases hydrolyse pectin that binds hemicellulose to cellulose microfibrils and lignin, thereby unlocking the lignin bondage. A series of hydrolytic enzymes have also been reported to play an important role of providing easily digestible hemicellulose-derived sugar such as xylose, mannose, galactose, rhamnose and arabinose as main source of carbon for selective ligninolysis needed for fungal metabolism and growth (Shirma and Arora, 2015). The hydrolytic enzymes produced by *L. plantarum* possibly accelerated homofermentation of water-soluble carbohydrates to lactic acid and acetic acid, which shortens the time for pH stability. The low pH provides a favourable environment for mycelial growth and high colonization ability of *P. sajor caju* resulting into higher solid state fermentation reflected by the elevated IVDM, IVOMD and ME. Although Wan and Li (2012) argue that pretreatment process is quintessential in overcoming biomass recalcitrance, the role of lignolytic enzymes during the early stage of solid-state fermentation needs further elucidation. Furthermore, no evidence of correlation between presence of oxidative enzyme and lignin degradation by *Ceriporiopsis subvermisporea* was reported during the early stages of fungal colonization (Wan and Li, 2012). Possibly, pre-treatment of maize stover with *L. plantarum* and molasses induced formation of not only H₂O₂ but also a series of low molecular mass intermediates within the glyoxylate cycle that include glyoxylic acids, oxalic acid, and several unsaturated fatty acids, which initiate depolymerisation of crystalline cellulose and facilitate the penetration of lignin-degrading enzymes. Moreover, such low molecular mass agents have been suggested as a mechanism by which delignification and cellulose depolymerisation is induced (Msimango and Fon, 2016) by the Fenton system. It is, therefore, argued that mineralization of lignin occurs after lignin modification in early stages of pre-treatment, which is indicative of a series of syntropic and synergistic reactions in the lignin degradation (Wan and Li, 2012). Consequently, improved diffusion of the oxidative enzymes possibly explained why a consortium of *P. sajor caju* working in concert with *L. plantarum* and molasses resulted into the highest IVDM, IVOMD and ME but with lowest lignin (sa) and NDFom. The importance of *P. ostreatus* working in a consortium with *Saccharomyces cerevisiae* when used for solid state fermentation of maize stalks for 14 day at 28 °C was earlier illustrated with the improved

IVDM and IVOMD from 153.5 to 480.0 g/kg DM and 292.5–720.0 g/kg DM, respectively, coupled with reduction of lignin from 114.9 to 58.4 g/kg DM (Darwish et al., 2012).

Although *L. plantarum* is a facultative organism that can also use oxygen for respiration, it has no electron transport chain (ETC) to convert oxygen to water but instead it ultimately metabolises it to H₂O₂ (Ranjit and Kung, 2000), which is used as a weapon to exclude other competing microorganisms on the substrate that would otherwise be contaminants. Molasses in consortium with *L. plantarum* is, therefore, used as a source of carbon to the bacterium and in production of organic H₂O₂, which is essential in the Fenton system necessary for manufacturing extracellular phenolic ligninases such as Lignin peroxidase and in cellulose depolymerisation (Novotný et al., 2004). Pre-treatment of maize stover with *L. plantarum* and molasses, therefore, negates the need by farmers to treat maize stover with inorganic H₂O₂ to sterilize the substrate. However, if applied in excessive quantities, molasses may supply extra nitrogen, sulphur and readily available carbon that may stimulate release of enzymes by *P. sajor caju* that hydrolyse cellulose and hemicellulose at the expense of selective lignin oxidation by peroxidases (Tripathi and Yadav, 1992; Rahman et al., 2011). Therefore, biological pre-treatment of maize stover with a combination of *L. plantarum* and molasses prior to inoculation with *P. sajor caju* enhances lignin bio-degradation and enriches nutritive value of maize stover. However, before promoting the innovation, *in vivo* and *in situ* studies with ruminants are recommended. For safety of ruminants from mycotoxins, care should be taken to ensure that the maize stover pre-treated with *L. plantarum* and molasses prior to solid-state fermentation with *P. sajor caju* is not of dark colour and should be devoid of foul smell due to production of butyric acid.

5. Conclusion

Pre-treatment of maize stover with a mixture of *L. plantarum* and molasses prior to fermentation with *P. sajor caju* offers a promising option for breaking lignin barrier and improving its nutritive value. The potential of *P. sajor caju* to break down lignin barrier at a temperature of 25 °C, pH 5, and in absence of light was demonstrated. However, to rule out anti-nutritional effects that may arise from high fungal chitin and phenolic intermediates of lignin break down, there is need for *in vitro* and *in vivo* experimentation to establish performance of dairy and beef cattle.

Acknowledgments

The authors are grateful to East African Agricultural Productivity Project (EAAP), National Agricultural Research Organization (NARO) and the National Livestock Resources Research Institute (NaLIRRI) for the financial support during the progress of this work. We also acknowledge the efforts of Mr. S. Kyeyune and Mr. E. Mutebi Senior Laboratory Technicians, Department of Agricultural Production, College of Agricultural and Environmental Sciences, Makerere University.

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