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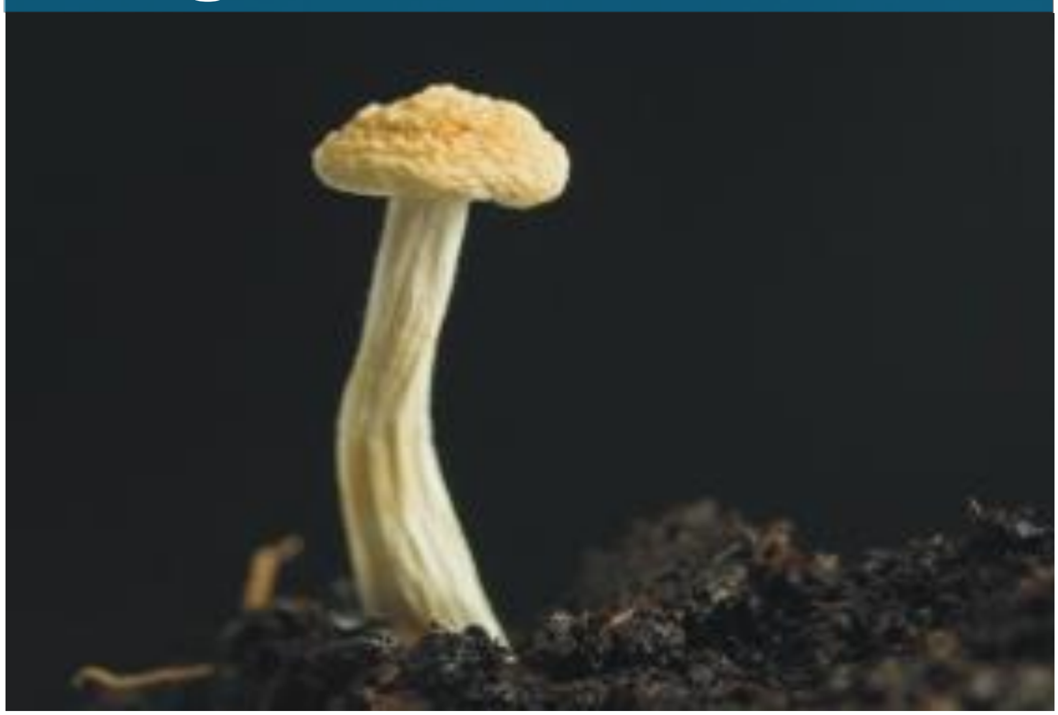

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Full Length Research Paper

Exploring for the possibility of utilizing *Pleurotus ostreatus* to manage *Eichhornia crassipes* in Zimbabwe

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The versatile nature of the commercial mushroom, *Pleurotus ostreatus* (Jacq.: Fr.) Kumm., provides basis for continued exploration of its biochemical processes during solid state fermentation on various lignocellulosic biomass as potential substrates. As a fungal organism, *P. ostreatus* feeds on lignocellulose by secreting various extracellular enzymes responsible for breaking down this organic polymer into smaller and simple compounds that the hyphae can absorb to develop into mycelia. In this study, *P. ostreatus* was assessed for its ability to grow on *Eichhornia crassipes* (Mart.) Solms-Laubach., or water hyacinth biomass and possibly, utilizing it as a substrate. *E. crassipes* is an aquatic herbaceous plant, often branded as the world's worst aquatic weed due to its invasive aggression, negative impact onto the aquatic ecosystems, and the cost usually associated with its control. When cultured onto the biomass of this nuisance weed followed by assessment by the agar plate-based clearing assay method and spectrophotometry, *P. ostreatus* demonstrated its practical ability to secrete cellulases, xylanases, pectinases, lignin peroxidases and laccases, thus showing its physiological capabilities to optionally utilize *E. crassipes* as a substrate. If properly optimized, this approach can be remarkably used as a sustainable and cost-effective strategy to manage *E. crassipes* in Zimbabwe.

Key words: *Pleurotus ostreatus*, *Eichhornia crassipes*, lignocellulosic biomass, lignocellulolytic enzymes, cellulases, xylanases, pectinases, lignin peroxidases, laccases.

INTRODUCTION

Eichhornia crassipes (Mart.) Solms-Laubach., or water hyacinth (Figure 1) is an aquatic flowering plant belonging to the family Pontederiaceae (Crow et al., 2000). The plant is native to the Amazon basin and often, a highly problematic invasive species outside its native range (Penfound and Earle, 1948). The hydrophyte is

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Figure 1. *Eichhornia crassipes* or water hyacinth. The image is of the weed growing in a fish pond at the Chinhoyi University of Technology in the town of Chinhoyi, Zimbabwe. Image credit; Nompumelelo Sibanda (March 2017).

frequently branded as the world's worst aquatic weed due to its invasive aggression, negative impact onto the aquatic ecosystems, and the cost usually associated with its control. Originally, a native of the Amazon basin, the weed has extensively spread throughout the tropical, sub-tropical and some other warmer temperate regions of the world since the late 1800s (Gutierrez et al., 2001; Villamagna and Murphy, 2010). The plant is typified as herbaceous, free-floating aquatic weed with erect aerial leaves, lilac flowers, and submerged roots. Free-floating individuals usually develop short bulbous petioles which are spongy, enabling the plant to float on the water's surfaces. Once growth is sufficient to cause crowding of individual plants, these petioles elongate and interweave, forming dense self-supporting mats that can cover the entire surfaces of dams and slow flowing rivers (Tham, 2012).

In Zimbabwe, *E*. *crassipes* was first recorded in the early 1900s and since then, the plant has widely spread throughout most parts of the country. This extensive distribution, as well as the resilience of the weed, is attributed to the highly eutrophic or nutrient-enriched state of the Zimbabwean waters and has led to the severe degradation of a number of aquatic ecosystems (Figure 2) (Chikwenhere, 1994; Cilliers et al., 2003). Negative effects associated with this weed's infestation include the suppression of local aquatic biodiversity, obstruction of river flows which may aggravate flooding and promote siltation, interference with water utilization for activities such as recreation and/or irrigation, and increased rates of evapotranspiration from water storages. *E*. *crassipes* infestation also poses a potential health risk in that the plant has been implicated in the creation of breeding habitats for mosquitos and/or their larvae that can cause malaria as well as other native disease-carrying vectors like bilharzia snails. As a result of these effects coupled with the limited availability of water resources in some parts of Zimbabwe (e.g. Chinhoyi), *E. crassipes* has been declared a category 1b weed in terms of the National Environmental Management: Biodiversity Act (10/2004): Alien and Invasive Species Regulations, 2014, which necessitates its control or eradication wherever possible (Magadza, 2003).

Combating *E*. *crassipes* infestations has drawn upon various management strategies, designed to reduce both the weed's spread and its biomass (Cilliers et al., 2003; Gutierrez et al., 2001). These include physical removal, the application of herbicides, utilization for commercial and subsistence purposes, and the importation and release of bio-control agents (Chikwenhere, 1994; Idrees et al., 2013; Ma et al., 2010; Moyo and Mapira, 2012). In most cases, however, the use of any of these strategies in isolation has had limited success, and in terms of physical and/or chemical interventions, they have proven to be both costly and unsustainable in the long-run (Cilliers et al., 2003; Villamagna and Murphy, 2010). This is especially true in Zimbabwe, where the management of the weed has been complicated by the eutrophic nature of the country's waters, and in terms of biological control, the highly seasonal and predominantly sub-tropical climate (Moyo and Mapira, 2012). However, whilst biological control still remains the most cost-effective and environmentally-friendly strategy for the sustainable control of this weed, recent research has sought how best to integrate and/or augment it with other

Figure 2. A section of Hunyani river fully clogged by the flourishing *E. crassipes*. The image was taken near the town of Chinhoyi in Zimbabwe. Image credit; Nompumelelo Sibanda (March 2017).

management strategies in order to achieve best results (Tham, 2012; Villamagna and Murphy, 2010).

In this reported study, we proposed use of this problematic weed as a possible substrate for the growth and sustainable cultivation of a common and commercially important mushroom, *Pleurotus ostreatus* (Jacq.: Fr.) Kumm. This commercial mushroom is a white-rot fungus, which belongs to the family Tricholomataceae (Ma et al., 2010; Saritha et al., 2012) and a diverse group of Basidiomycetes that attack the biologically stable polymer of plants, lignocellulose (Dorado et al., 2001; Jurado et al., 2011; Pandya and Albert, 2014). The ability of white-rot fungi to degrade plants essentially depends on the type and ecology of the fungus involved (Bisen et al., 2010; Tanesaka et al., 2004). Generally, *P*. *ostreatus* grows in conditions that would allow for the enzyme-based conversion reactions to take place, and such conditions include carbon source, substrate, temperature, light, pH, and humidity (Jurado et al., 2011; Kerem et al., 1992; Patel et al., 2009; Tien and Kirk, 1983). It is only when these conditions are well-established that mycelia are able to secrete the various sets of exo-, hydrolytic and oxidative enzymes that in turn breakdown the substrate into various products, which then the mycelia feed off to grow into fruiting bodies (Koshy, 2012; Téllez-téllez et al., 2013).

Thus, one main advantage of *P*. *ostreatus* and the rationale behind its particular choice for this reported study is its ease of culturing over a wide range of substrates that could either be of natural, industrial or agricultural origin (Howard et al., 2013). To this date, this mushroom has been successfully cultured on wheat straw, cotton seed cake, maize stover banana leaves, sugarcane bagasse and soya bean stover (Madhavi and Lele, 2009; Usha et al., 2014) and all these substrates being part of the agricultural and/or industrial waste, are found at relatively very minimal and/or no cost at all. Therefore, the probable successful cultivation of *P*. *ostreatus* on *E*. *crassipes* would on one hand, offer a viable strategy for the sustainable management of this problematic weed in local aquatic environments while on the other hand, providing prospects for the possible conversion of a low-value indigenous lignocellulosic-rich waste into products of high commercial value such as mushrooms and enzymes (Buswell et al., 1993; Villamagna and Murphy, 2010).

MATERIALS AND METHODS

Source of the test fungus

The *P*. *ostreatus* strain used in this study as the test fungus was purchased from Sylvan Africa (PTY) Ltd., which is a commercial supplier in Pretoria, South Africa. The fungus was supplied in form of a semi-dried spawn on ice and was later on kept at 4°C in the laboratory, for further use.

Viability testing of the test fungus

Growth viability of the purchased *P*. *ostreatus* was tested and determined by spread-plating the strain on 0.8% (*w/v*) agarose plates supplemented with either 0.2% (*w/v*) carboxymethylcellulose or 0.2% (*w/v*) birchwood xylan, followed by incubation at 37°C for 12 h in a 2001651 Incubat bench-top incubator (JP Selecta SA., Barcelona, Spain). Fungal growth was then observed as visible filamentous mycelia on agar plates.

Source of the substrate

E. crassipes biomass was harvested in black plastic bags from Hunyani river (Figure 2) near Chinhoyi town in the Southern African country of Zimbabwe and oven dried at 80°C for 18 h. The dried biomass was then transported to the Republic of South Africa for studies in the Plant Biotechnology Research Laboratory at North-West University. Permission to transport and study the biomass was sought and granted by the Department of Agriculture, Forestry and Fisheries, South Africa (Permit number: P0079761; Appendix Figure A1). To serve as control substrate, wheat straw was obtained from Molelwane Farm (Department of Crop Science, North-West University, South Africa) and was similarly oven dried at 80°C for 18 h before use.

Substrate preparation

The dried *E. crassipes* and its control biomass were chopped to 3 to 5 cm sizes with a bench-top grinder before transferring 200 g of the chopped biomass into a 500 ml Erlenmeyer flask, pre-filled with water (200 ml). The chopped biomass was then thoroughly soaked through submergence for 12 h before the submerging water was drained out to retain an average moisture content of ~60%. The biomass was finally sterilized in a SA-300VL autoclave (Sturdy Industrial Company Ltd., New Taipei City, Taiwan) at 121°C for 30 min, for further use as substrate.

Fungal cultivation and enzyme production

Under lamina flow conditions, each prepared substrate was separately inoculated with the *P. ostreatus* spawn at a 1% (*w/w*) inoculant to substrate ratio. Each inoculation was replicated three times (n = 3) before incubation in a TL-300 growth chamber (Lab Companion Jeio Tech, Seoul, Korea) at 30°C for the first 15 days with no illumination. This incubation period allowed for spawn runs to take place. After the spawn runs had completed and established, dark and light cycles of 12 h apiece at a relative humidity of ~80% were then introduced for another 15 days, to allow for substrate colonization.

Crude enzyme extractions

For each colonized substrate system, a crude enzyme extract of the *P. ostreatus* was obtained by incubating the substrate in 100 mM sodium citrate buffer; pH 5.0 at a 1:2 (*w/v*) mixture ratio and in the presence of 0.05% (*v/v*) Tween-80, with intermittent shaking for 30 min. The mixture was then first separated with a cheese cloth before being clarified at 4°C through centrifugation at 2500×*g* for 15 min in an LSE High Speed micro-centrifuge (Corning Inc., Amsterdam, Netherlands). The supernatant was collected and stored at -20°C as the crude enzyme extract.

Agar plate-based clearing assays

The probable ability of *P. ostreatus* to secrete a variety of lignocellulolytic enzymes when grown on *E. crassipes* as a potential substrate (and wheat straw as a control) was assessed by checking for presence of the following enzyme activities in the crude enzyme extract.

Cellulase activity

Cellulose activity was tested by loading 200 µl of the crude enzyme extract into wells of 0.8% (*w/v*) agarose plates supplemented with 0.2% (*w/v*) carboxymethylcellulose, followed by incubation at 37°C for 12 h. The plates were then stained for 10 min with 0.1% (*w/v*) Congo red, followed by de-staining with 1 M sodium carbonate for 10 min. Further de-staining with 10% (*v/v*) acetic acid was also undertaken to reveal zones of clearance.

Xylanase activity

Xylanase activity was tested by loading 200 µl of the crude extract into wells of 0.8% (*w/v*) agarose plates supplemented with 0.2% (*w/v*) birchwood xylan, followed by incubation at 37°C for 12 h. The plates were then stained with 0.1% (*w/v*) Congo red, followed by de-staining with 1 M sodium carbonate for 10 min, to reveal zones of clearance.

Pectinase activity

Pectinase activity was tested by loading 200 µl of the crude enzyme extract into wells of 0.8% (*w/v*) agarose plates supplemented with 0.2% (*w/v*) polygalacturonic acid, dissolved in sodium citrate buffer (100 mM) at pH 4.8. The plates were incubated at 37°C for 12 h followed by staining with 1% (*v/v*) iodine solution in 95% (*v/v*) ethanol for 30 min, to reveal zone of activity.

Lignin peroxidase activity

Lignin peroxidase activity was tested using the syringaldazine well test method as described by Pointing (1999). In brief, 200 µl of the crude enzyme extract were loaded into wells of 0.8% (*w/v*) agarose plates consisting of 0.2% (*w/v*) lignin basal medium, supplemented with 0.2% (*w/v*) glucose. The plates were incubated at 27°C in the dark for 5 to 10 days. After incubation, a few drops of 0.1% *(w/v)* syringaldazine in 95% (*v/v*) ethanol were added, followed by an immediate addition of a few drops of 0.5% *(w/v)* aqueous hydrogen peroxide to reveal color.

Laccase activity

Laccase activity was tested by loading the extract (200 µl) into wells of 0.8% (*w/v*) agarose plates supplemented with 0.5% (*v/v*) pyrogallol, dissolved in 100 mM sodium acetate buffer at pH 4.5. The plates were incubated at 37°C for 12 h, followed by staining with 1% (*v/v*) iodine solution in 95% (*v/v*) ethanol for 30 min, to reveal zones of activity.

Spectrophotometric enzyme activity assays

In order to consolidate the agar plate-based assay method, an alternative and more sensitive method of spectrophotometry was also used to assess for presence of activity of the same set of lignocellulolytic enzymes in the crude enzyme extract. With this alternative method, activities generated by the blank samples were considered negative controls while those that were generated by the crude extracts from wheat straw were considered positive controls.

Cellulase enzyme activity

Cellulase activity was tested in reaction mixtures containing 1 ml of the 1% (*w/v*) carboxymethylcellulose substrate in 50 mM sodium citrate buffer; pH 5.0 and 1 ml of the crude enzyme extract, followed by incubation at 50°C for 1 h. For each test sample, an appropriate blank, whereby the crude enzyme extract in the reaction mixture was omitted and replaced with an equal volume of sterile distilled water, was also prepared. After incubation, the reaction activity was terminated by adding 3 ml of the 3,5-dinitrosalicylic acid, mixing well, and boiling at 100°C for 5 min in a WS 27-2 Shel-Lab water bath (Sheldon Manufacturing Inc., Cornelius, Oregon). A total of 15 ml sterile distilled water was then added to each of the reaction mixtures, mixed well, followed by absorbance reading at 540 nm, using a Spectronic Helios Epsilon spectrophotometer (Thermo Electronic Scientific Instruments LLC., Middleton, Wisconsin). Alongside this, some carboxymethylcellulose standards (Supplementary Material) were prepared and used to plot a standard curve from where concentrations of the metabolized substrate were then estimated.

Xylanase enzyme activity

Xylanase activity was tested in accordance with the method of Miller (1959) whereby reaction mixtures containing 1 ml crude extract, 1 ml of 1% (*w/v*) birchwood xylan and 1 ml of 50 mM sodium acetate buffer; pH 5.0 were prepared and incubated at 50°C for 30 min. After incubation, 2 ml of the 3,5-dinitrosalicylic acid were added to the reaction contents to stop enzymatic activity. The contents were then mixed well before being boiled for 5 min in a water bath. A total of 15 ml sterile distilled was then added to the reaction contents, mixed well before absorbance at 540 nm was read. Appropriate blanks as is described for the cellulose activity assaying above were also prepared and ran alongside the experimental test samples to serve as controls. In addition to this, some birchwood xylan standards (Supplementary Material) were prepared and used to plot a standard curve from where concentrations of the metabolized substrate were then estimated.

Pectinase enzyme activity

Pectinase activity was tested in reaction mixtures containing 1 ml of 1% (*v/v*) polygalacturonic acid, 1 ml of the crude extract and 1 ml of 200 mM sodium acetate buffer at pH 4.5. The reaction mixtures were incubated at 37°C for 30 min and enzyme activity measured at 540 nm after hydrolysis of the polygalacturonic acid by the pectinases. In this system, appropriate blanks were also prepared and ran alongside the test samples as controls. In addition, some polygalacturonic acid standards (Supplementary Material) were prepared and used to plot a standard curve from where concentrations of the metabolized substrate were then estimated.

Lignin peroxidase enzyme activity

Lignin peroxidase activity was tested in reaction mixtures containing 1 ml of 2 mM veratryl alcohol, 1 ml of 10 mM tartrate buffer; pH 3.0 and 1 ml of the crude extract. The reaction mixtures were incubated at 25°C for 30 min. Enzyme activity was then determined at 340 nm by measuring the oxidation of veratryl alcohol to veratryl-dehyde by the peroxidases, in the presence of 0.27 mM hydrogen peroxide. In this system, appropriate blanks were also prepared and ran alongside the test samples as controls. Alongside this, some veratryl alcohol standards (Supplementary Material) were prepared and used to plot a standard curve from where concentrations of the metabolized substrate were then estimated.

Laccase enzyme activity

Laccase activity was tested in reaction mixtures containing 1 ml crude extract, 1 ml of 1 mM guaiacol and 1 ml of 10 mM sodium phosphate buffer at pH 5.5, and incubated at 40°C for 30 min. Enzyme activity was then measured at 460 nm after colour change and following the oxidation of guaiacol by the laccases in the crude extract. Alongside this, appropriate blanks were also prepared and ran alongside the test samples as controls. In addition, some guaiacol standards (Supplementary Material) were prepared and used to plot a standard curve from where concentrations of the metabolized substrate were then estimated.

Resolution and analysis of the total secreted crude enzyme extract proteins

The total protein content in the crude enzyme extract was resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and in accordance with the method of Laemli (1970). Briefly, 40 µl of the crude extract were suspended in 1X protein loading buffer (20% (*v/v*) glycerol, 100 mM Tris-HCL, 2% (*w/v*) sodium dodecyl sulphate (SDS), 0.1% (*w/v*) bromophenol blue and 20% (*v/v*) β-mercaptoethanol) and boiled for 5 min on an Accu-Block Digital dry bath (Labnet International Inc., Woodbridge, New Jersey). The samples were then loaded (for concentration purposes) onto a 5% (*v/v*) stacking gel (4.8% (*v/v*) acrylamide solution, 0.1% (*w/v*) SDS, 125 mM Tris-HCl, 0.05% (*w/v*) ammonium persulphate (APS) and 0.4% (*v/v*) tetramethylethylenediamine (TEMED)), before being resolved on a 12% (*v/v*) running gel (12% (*v/v*) acrylamide solution, 0.1% (*w/v*) SDS, 375 mM Tris-HCl, 0.05% (*w/v*) APS and 0.2% (*v/v*) TEMED). Actual protein resolution was achieved by running the loaded gel at 120 V for 1 h using an Mini Protean vertical gel system (Bio-Rad Laboratories Inc., Hercules, California) submerged in 1X protein running buffer (1.44% (*w/v*) glycine, 0.3% (*w/v*) Tris-HCl, 0.1% (*w/v*) SDS) and against an unstained standard molecular weight marker (Fermenters Int., Burlington, Canada). The resolved gel was then stained with a Coomassie staining solution (0.5% (*w/v*) Coomassie brilliant blue powder, 10% (*v/v*) ethanol, 10% (*v/v*) methanol and 10% (*v/v*) acetic acid) for 30 min, shaking at 100 rpm on an Ultra-Rocker platform (Bio-Rad Laboratories Inc., Hercules, California). The stained gel was then similarly de-stained with a de-staining solution (10% (*v/v*) ethanol, 10% (*v/v*) methanol, 10% (*v/v*) acetic acid). The de-stained and resolved gel was then visually analyzed for presence of the various protein bands against a standard molecular weight marker (Fermenters Int.,) and images captured by a Chemi-Doc Imaging system (Bio-Rad Laboratories Inc., Hercules, California).

Zymography of the cellulase and laccase enzymes

The dominant protein bands in the resolved SDS-PAGE gel were subjected to a specific in-gel enzyme activity assaying system, followed by a concerted evaluation of their associated activities. In this regard, two particular groups of enzymes (cellulases and laccases) were singled out and assessed.

Cellulase zymogram

This was undertaken according to the method of Téllez-téllez et al.

Figure 3. Testing for the viability status of *Pleurotus ostreatus* and its ability to grow on *E. crassipes*. (a) *P. ostreatus* cultured on microcrystalline cellulose (left plane) and birchwood xylan (right plane). (b) *P. ostreatus* growing on *E. crassipes* as a potential substrate (left plane) and wheat straw as a control substrate (right plane).

(2013) and Pointing (1999), whereby a 12% (*v/v*) polyacrylamide gel incorporated with 0.1% (*w/v*) carboxymethylcellulose was prepared, followed by an electrophoretic resolution of the total protein content in the crude enzyme extract against a standard molecular weight marker. After electrophoresis, the molecular weight marker was cut off the gel and separately stained with Coomassie brilliant blue while the remainder of the gel was separately washed; firstly, with a mixture (1:1) of 100 mM sodium phosphate buffer; pH 7.2 and 40% (*v/v*) isopropanol for 1 h, to remove SDS; and secondly, with sodium phosphate buffer only for an hour to remove isopropanol. The washed gel was then re-natured through submergence in a 1:1 mixture ratio of 50 mM sodium phosphate buffer and 1 mM EDTA at 4°C for 1 h. The re-natured gel was then incubated at 50°C for 1 h to allow the cellulase enzyme to degrade its carboxymethylcellulose substrate. After this, the gel was then stained for 30 min with 0.1% (*w/v*) Congo red, followed by de-staining for 30 min with 1000 mM sodium chloride. Furthermore, the gel was immersed in 0.5% (*v/v*) acetic acid for 1 h, to get a better clearing contrast at the areas of enzyme activity. Finally, the previously cut off molecular weight marker was re-aligned to the main gel and images captured by a Chemi-Doc Imaging system (Bio-Rad Laboratories Inc).

Laccase zymogram

This zymogram was generated under non-reducing, non-denaturing conditions, whereby a 6% (*v/v*) stacking gel and an associated 10% (*v/v*) resolving gel were prepared without SDS and β-mercaptoethanol in native Tris-HCl running buffer (1.44% (*w/v*) glycine, 0.3% (*w/v*) Tris-HCl) at pH 8.3, followed by resolution of the total protein fractions in the crude extract through electrophoresis. Laccase activity in the gel was then evaluated by submerging the resolved gel overnight in 5 mM guaiacol, prepared in 10 mM sodium acetate buffer; pH 5.6, followed by detection of colour developed as a result of the oxidation of guaiacol by the enzymes. The resultant gel images were then captured by a Chemi-Doc Imaging system (Bio-Rad Laboratories Inc).

Enzyme kinetics of the cellulase and laccase enzymes

Reaction set-ups with the linearly increasing concentrations (0.125, 0.25, 0.50 and 1.00 mM) of the substrate (carboxymethylcellulose for cellulases and guaiacol for laccases) were prepared, followed by measurement of enzyme activity (initial velocity) for each of the used substrate concentration as has already been described earlier. Using the obtained initial velocities and the used substrate concentrations for each enzyme, a Hanes-Woolf plot was then sketched, followed by determination of the reaction kinetics constants (K_m and V_{max}) for each of the assessed enzymes (Irving et al., 2011; Meier et al., 2010). From this sketch, K_m was determined as the negative value of the x-intercept ($x = -K_m$, when $y = 0$) of a linear fit of the plotted data while V_{max} was calculated from the y-intercept ($y = K_m/V_{max}$, when $x = 0$) of the same linear fit (Irving et al., 2011; Meier et al., 2010).

Statistical analysis

All enzyme assay data in this work are means of triplicate assays (n = 3) subjected to analysis of variance (ANOVA) (Super-Anova, Statsgraphics Version 7; Statsgraphics Corp., The Plains, VI, USA). Where ANOVA revealed significant differences between treatments, means were separated by the *post hoc* Student-Newman-Keuls (SNK) multiple range test ($p < 0.05$).

RESULTS

Testing for the viability status of the purchased *P. ostreatus* spawn and its ability to grow on *E. crassipes*

When the viability status of *P. ostreatus*, as a test fungus for the study was assessed, it emerged that this test fungus could both viably and vigorously grow on either the two test commercial substrates (carboxymethyl-cellulose and birchwood xylan) (Figure 3a) and the test experimental substrate (*E. crassipes*) (Figure 3b). On *E. crassipes*, *P. ostreatus* generated mycelial colonies that almost completely covered the whole substrate biomass, thus demonstrating its potential and/or physiological ability to utilize the tested experimental biomass as a substrate.

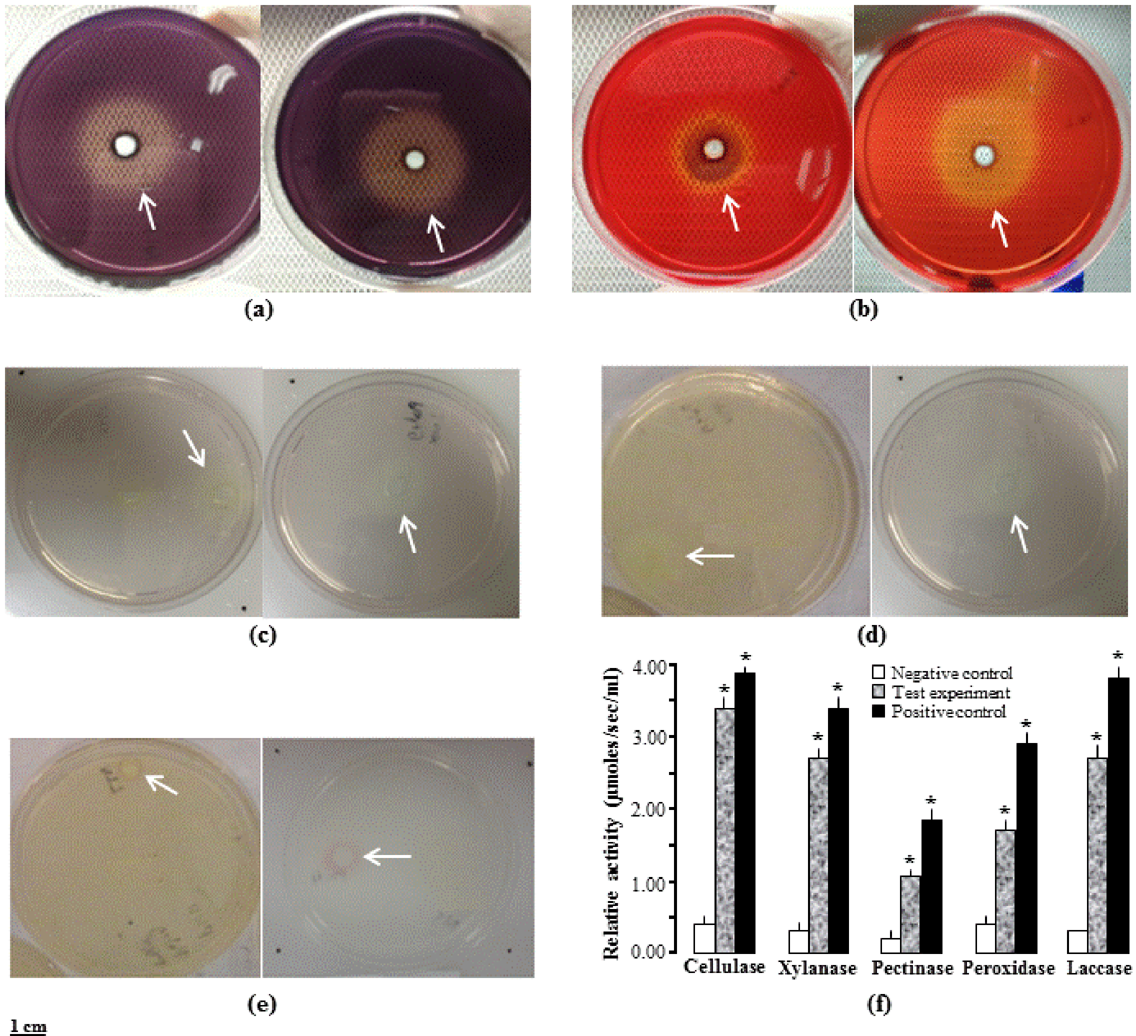

Figure 4. Testing the ability of *P. ostreatus* to secrete various lignocellulolytic enzymes when grown on *E. crassipes.* (a) Cellulase, (b) xylanase, (c) pectinase, (d) lignin peroxidase and (e) laccase activities of the extracellular enzyme extract secreted by *P. ostreatus* when grown on *E. crassipes* as a potential substrate (left planes) and wheat straw as a control substrate (right planes), and as is determined by the agar plate-based clearing assay method. Arrows mark ends of the zones of clearance and the scale relates to sizes of diameters of the observed zones of clearance. (f) Determination of the same enzyme activities by spectrophotometry, whereby activity was measured in the absence of enzymes (clear bars; negative control) and in the presence of enzymes secreted by the *P. ostreatus* when grown on *E. crassipes* (grey texture; test experiment) and wheat straw (black bars; positive control). Data are mean values (n = 3) and error bars show SE of the mean. Asterisks denote significant differences from the negative controls ($P < 0.05$) as determined by ANOVA and *post hoc* Student-Newman-Keuls multiple range tests.

Determining and validating the ability of *P. ostreatus* to secrete various lignocellulolytic enzymes when grown on *E. crassipes*

Using the agar plate-based clearing assay and spectrophotometric methods, it was ascertained that the *P. ostreatus* could viably secrete a whole cocktail of the lignocellulolytic enzymes during its growth on *E. crassipes* as a potential substrate (Figure 4). In the plate-based clearing assay method, enzymes secreted by microbes degrade polysaccharide compounds to generate clear halos on the solidified agar plates after addition of specific dyes and de-staining solutions (Figure 4a-e). In the spectrophotometric method, a 3,5-dinitrosalicylic acid detection system for reducing sugars is employed, which results in the generation of coloured compounds that are measurable and easily convertible into rates of activity, that is, 3.52, 2.97, 1.25, 1.98 and 2.99 µmole/s/ml for cellulases, xylanases, pectinases, lignin peroxidases and laccases respectively (Figure 4f).

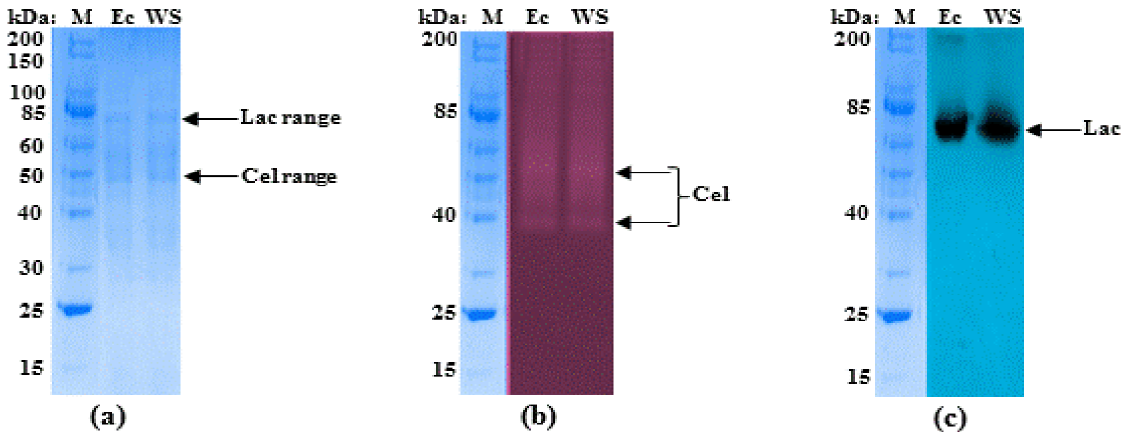

Figure 5. SDS-PAGE and zymogram analysis of the prominent protein fractions secreted by *P. ostreatus* when grown on *E. crassipes* (Ec) or wheat straw (WS). (a) An SDS-PAGE gel showing resolution of the secreted prominent protein fractions, (b) a zymography gel showing carboxymethylcellulose degradation by cellulases and (c) a zymography gel showing guaiacol decomposition by laccases. M represents the unstained molecular weight marker (Fermenters Int., Burlington, Canada) while arrows mark the gel resolving ranges of cellulases and laccases.

Resolution and activity assaying of the prominent enzyme fractions secreted by *P. ostreatus* when grown on *E. crassipes*

The various enzymatic protein fractions noted to be secreted by *P. ostreatus* when growing on *E. crassipes* were then attempted to be resolved by SDS-PAGE for probable further analysis (Figure 5). Apparently, it emerged that only fractions of the approximate molecular weight sizes of 50 and 80 kDa were resolved (Figure 5a), suggesting cellulases and laccases, respectively. Notably, a further analysis of the same gel by zymography then confirmed that the ~50 and ~80 kDa protein fractions were indeed cellulases (Figure 5b) and laccases (Figure 5c), respectively.

Reaction kinetics of the cellulase and laccase enzyme fractions secreted by *P. ostreatus* when grown on *E. crassipes*

After determining that, when grown on *E. crassipes*, *P. ostreatus* tends to secrete cellulases and laccases as its main protein components, the kinetic rates of these two most produced enzyme components were then assessed and ascertained via the Hanes-Woolf plot method (Figure 6). This was done in order to relate activities of these two major enzyme components to their counterparts in other known and/or related microbial systems. K_m was determined as the negative value of the x-intercept ($x = -K_m$, when $y = 0$) of the linear fit of the data set while V_{max} was calculated from the y-intercept ($y = K_m/V_{max}$, when $x = 0$) of the same linear fit. As is shown in Figure 6, a K_m value of 0.246 mM and a V_{max} value of 2 894.12 µmol/s for the cellulases were obtained (Figure 6a) while a K_m value of 0.145 mM and a V_{max} value of 1 479.59 µmol/s for the laccases were obtained (Figure 6b).

DISCUSSION

E. crassipes (Mart.) Solms-Laubach., or water hyacinth (Figure 1) is a prolific weed that has caused menace and problems in several fresh water bodies in the tropical and sub-tropical countries, including Zimbabwe (Figure 2) (Gutierrez et al., 2001). Despite its negative impacts as a weed, *E. crassipes* has been widely reported as a very useful commodity for various industrial and commercial applications; e.g. as a raw material for ethanol production (Manivannan et al., 2012; Ncube et al., 2012), as an excellent absorbent of various heavy metals present in polluted waters (Chikwenhere, 2001), as an essential component for phytoremediation (Bergier et al., 2012), as a raw material for the production of biogas (Bergier et al., 2012), as a bio-fuel (Tian et al., 2012), and as an excellent protein supplement in animal feeds (Singal et al., 2007). In Tanzania, this hydrophyte has since been shown to be a very good substrate for either the exotic mushroom, *P. ostreatus* (Çağlarırmak, 2007) or indigenous mushroom, *Pleurotus flabellatus* (Kivaisi et al., 2003; Murugesan et al., 2006). In Zimbabwe apparently, none of these efforts have ever been attempted. Therefore, this study was undertaken to assess if *P. ostreatus* could successfully grow on *E. crassipes* native to Zimbabwean waters and thus utilizing it as a substrate. Use of this weed as a substrate for *P. ostreatus* could serve as a cost-effective way of controlling it in local aquatic environments while at the

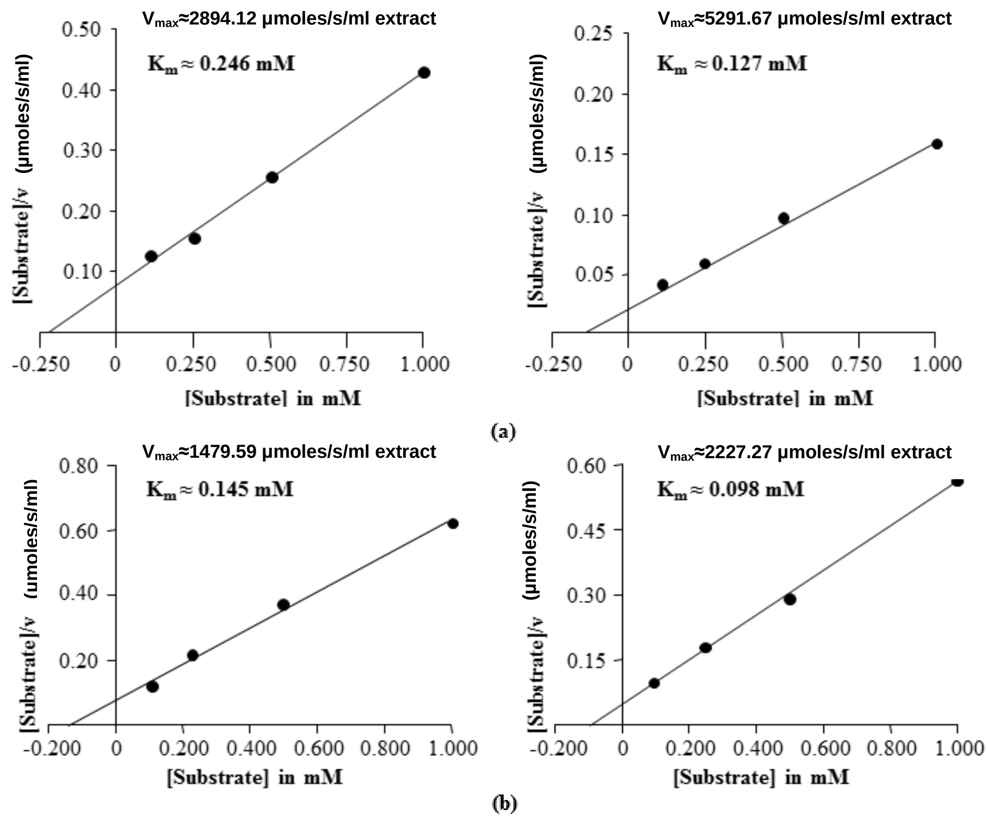

Figure 6. Reaction kinetics. Kinetic rates of the (a) cellulase and (b) laccase enzymes in the crude extracellular extract secreted by *P. ostreatus* when grown on *E. crassipes* (left planes) or wheat straw (right planes). K_m was determined as the negative value of the x-intercept ($x = -K_m$, when $y = 0$) of the linear fit of the data set while V_{max} was calculated from the y-intercept ($y = K_m/V_{max}$, when $x = 0$) of the same linear fit. All values obtained indicate means of three independent and fully representative experiments (n = 3).

same time, producing protein-rich foods for the surrounding communities and perhaps, also production of commercial enzymes for the national and/or international industries.

The *P. ostreatus* strain used in this study is an exotic mushroom that was commercially acquired through purchasing from the Sylvan Africa (PTY) Ltd., (Pretoria, South Africa). However, before this fungal strain could be used in the intended study, its growth viability was first checked and ascertained through culturing on two different substrates of commercial grade. As is shown in Figure 3a, the test fungus could both viably and vigorously grow on either carboxymethylcellulose or birchwood xylan. When the *P. ostreatus* was then cultured on *E. crassipes*, followed by assessment of its ability to grow on this test substrate, biomass colonization was relatively good with mycelia almost completely covering the whole substrate (Figure 3b). This could be as a result of the *P. ostreatus* secreting the various lignocellulolytic enzymes that then enabled it to grow and colonize the provided substrate. Generally, white rot fungi are known to be capable of secreting oxidases (laccases and peroxidases) that degrade lignin (Wesenberg et al., 2003), glucanases (exo- and endo-) that breakdown cellulose (Kuhad et al., 2011), xylanases that degrade hemicelluloses (Punniavan, 2012), and pectinases that breakdown pectins (Collins et al., 2005).

In nature, some white rot fungi produce the whole cocktail of lignocellulolytic enzymes while others produce only one or a few of them (Baldrian and Valášková, 2008; Maganhotto de Souza Silva et al., 2005). Therefore, in order to ascertain if the *P. ostreatus* was capable of secreting the whole cocktail of white rot fungal enzymes or part of it when growing on *E. crassipes*, its crude extracellular extract was tested for the various lignocellulolytic enzyme activities via the agar plate-based clearing assay method and spectrophotometry (Figure 4). Under the agar plate-based clearing assay method, the Congo red assay showed zones of clearance in diameters of over 2.83 cm for cellulases (Figure 4a) and 2.67 cm for xylanases (Figure 4b), demonstrating ability of the excreted enzyme extract to degrade carboxymethylcellulose and birchwood xylan, respectively. These revealed zones of clearance were not that much different from the ones previously generated by plant endophytes with massive capabilities to degrade cellulose and hemicellulose, which were >2 cm (Eichlerová et al., 2012). Such capabilities may be gained due to the adaptation abilities of fungi to their habitats, which are a whole set of lignocellulosic materials (Yopi et al., 2014). A related trend of clearance was also observed for the pectinases (Figure 4c), lignin peroxidases (Figure 4d) and laccases (Figure 4e), signifying ability of the excreted enzyme extract to degrade polygalacturonic acid, veratryl alcohol, and guaiacol, respectively. The same results as is reported earlier were also revealed by the spectrophotometric assaying technique (Figure 4f), a method that is alternative to the agar plate-based clearing assay method but more sensitive. These findings, therefore, showed that the *P*. *ostreatus* is capable of secreting the whole cocktail of white rot fungal enzymes when grown on *E. crassipes*, and thus able to utilize *E. crassipes* as an alternative substrate. Pleurotus species have previously been reported to have a unique ability to produce xylanases (Bhagobaty et al., 2007), carboxymethylcellulases, β-glucosidases, β–xylosidases, and extracellular lignocellulolytic enzymes, including laccases and lignin peroxidases (Elisashvili et al., 2015).

When the various fractions of the total protein content secreted by the *P*. *ostreatus* during its growth on *E. crassipes* were resolved by SDS-PAGE for further analysis, it emerged that the dominant protein fractions produced were most likely cellulases (~50 kDa) and laccases (~80 kDa) (Figure 5a). Cellulases are a multi-enzyme complex composed of various protein components with endoglucanase, exoglucanase and β-glucosidase activities that normally operate synergistically (Liming and Xueliang, 2004; Stajić et al., 2006). Of these protein components, cellobiohydrolase I (52.2 kDa) and cellobiohydrolase II (47.2 kDa) are the predominant ones (>90%) while endoglucanases and hemicellulases represent less than 10% (Da Vinha et al., 2011). On the other hand, laccases are glycosylated monomer or homodimer proteins generally composed of monosaccharides such as hexoamines, glucose, mannose, galactose, fructose, and arabinose (Gao et al., 2011). On SDS-PAGE, most laccases show mobility rates corresponding to molecular weight ranges of 60 to 100 kDa, of which 10 to 50% of that may be directly attributed to glycosylation (Leonowicz et al., 2005). In a previous study related to the present study, which involved the characterization and isolation of lacasse from *Aspergillus nidulans*, a band of 66 kDa was obtained (Xu et al., 2000).

Notably, when the same SDS-PAGE gel generated earlier (Figure 5a) was further subjected to a zymogram analysis, results obtained then showed that the resolved ~50 kDa proteins were indeed responsible for the degradation of carboxymethylcellulose (Figure 5b) while the ~80 kDa proteins were responsible for the decomposition of guaiacol (Figure 5c), thus firmly affirming our initial claim in the SDS-PAGE analysis (Figure 5a) that the ~50 kDa proteins were cellulases while the ~80 kDa proteins were laccases. Incidentally, the present work also managed to reveal a number of carboxymethylcellulose-degrading proteins, ranging from 40 to 50 kDa (Figure 5b), concurring with the fact that cellulases are multi-enzyme complex composed of various proteins such as endoglucanase I (46.0 kDa), II (42.2 kDa), IV (33.4 kDa), V (22.8 kDa) and VII (25.1 kDa); cellobiohydrolase I (52.2 kDa) and II (47.2 kDa); β-glucosidase I (75.3 kDa) and II (52.1 kDa); and β-glucosidase-1,4-glucanase (23.5 kDa) (Da Vinha et al., 2011). This outcome is closely linked to that of Elisashvili et al., (2015) who recorded carboxymethylcellulose-degrading proteins of 25, 50 and 100 kDa from three unnamed Indonesian endophytic fungi, isolated from medicinal plants (Yopi et al., 2014); and to that of Ncube et al. (2012), who reported molecular masses of 20 to 45 kDa for cellulases isolated from *Aspergillus niger* when the *Jatropha curcas* seed cake was used as substrate (Ncube et al., 2012). Overall, other studies that have been undertaken and reviewed independently, have also reported the molecular masses of fungal cellulases to be as low as 12 kDa and up to 250 kDa (Kuhad et al., 2011; Liming and Xueliang, 2004; Vivekanandan et al., 2014).

After determining that, when grown on *E. crassipes*, *P*. *ostreatus* tends to generate cellulases and laccases as its main protein components, the kinetic rates of these two most secreted enzyme components were then assessed and ascertained via the Hanes-Woolf plot method (Figure 6) (Irving et al., 2011; Meier et al., 2010). This was done in order to relate activities of these two major *P*. *ostreatus* enzymes to their counterparts in other known microbial systems. For the cellulases, a K_m value of 0.246 mM and V_{max} of 2 894.12 µmol/s were obtained (Figure 6a) while for the laccases, a K_m value of 0.145 mM and V_{max} of 1 479.59 µmol/s were obtained (Figure 6b). The kinetic values obtained for the cellulases are in close relation to those previously displayed by a recombinant CtCel5E protein from *Clostridium thermocellum*, whose K_m value was 2.1 mM and a V_{max} of 1 564.00 µmol/s (Vivekanandan et al., 2014; Zhang and Zhang, 2013). However, the kinetic values obtained for the *P*. *ostreatus* cellulases were notably, a bit better than those of the CtCel5E protein, probably due to two possible technical reasons. Firstly, the CtCel5E cellulase was a recombinant protein (Vivekanandan et al., 2014) while the *P*. *ostreatus* cellulases were not. Secondly, the origin of the CtCel5E cellulase was bacterial or prokaryotic (Vivekanandan et al., 2014) while that of the *P*. *ostreatus* cellulases was fungal or eukaryotic. For the laccases, on the other hand, it is generally reported that the typical K_m value for all laccases of fungal origin should be in the range 0.01 to 0.6 mM (Goller et al., 2002; Liu et al., 2011; Saito et al., 2003), which in this case, is in very good agreement with the 0.145 mM obtained for the *P*. *ostreatus* laccases. In addition, both the K_m and V_{max} values obtained for the *P*. *ostreatus* laccases are in further close agreement with those previously obtained by Vivekanandan et al. (2014), whereby the K_m and V_{max} values of a laccase isolated from *Aspergillus nidulans* were 0.052 mM and 2860.00 µmol/s, respectively (Vivekanandan et al., 2014). Notably, the reason why the kinetic rates for the *P*. *ostreatus* laccases were not as good as those for the laccase of Vivekanandan et al. (2014) could be due to the fact that our assays were carried out using a crude enzyme extract while that of the other group was carried out using an enzyme preparation that initially, had been highly purified via ultrafiltration, ammonium sulphate precipitation, ion exchange chromatography and gel filtration (Vivekanandan et al., 2014).

Overall, comparing *E. crassipes* to its control substrate (wheat straw), it is apparent that the control substrate was always performing better throughout the study. However, it is rather worth to note that the control substrate naturally has a higher biomass composition than the experimental substrate; that is, 30% cellulose, 50% hemicellulose, 20% lignin and 1% pectin for wheat straw (Howard et al., 2013; Saito et al., 2003) and 20% cellulose, 33% hemicelluloses, 10% lignin, and 1% pectin for *E. crassipes* (Avci et al., 2013). In addition, *E. crassipes* has always been reported to possess a very high adsorption capacity that makes it capable of taking up numerous nutrients, chemicals and metal substances, which may perhaps inhibit enzyme activity on its biomass (Idrees et al., 2013; Moyo and Mapira, 2012; Reddy and D'Angelo, 1990; Saha et al., 2014; Tham, 2012; Usha et al., 2014). However, even though the general production of mushrooms has always been undertaken using wheat or rice straw, the overall performance of *E. crassipes* as a substrate in this study (0.75-0.98 folds) strongly proposes as a very good alternative.

Finally, by summing up all findings of this study, it is conceivable that the *E. crassipes* native to Zimbabwe can be viably utilized as a substrate of *P*. *ostreatus*, which if properly optimized, the approach can then be used as a sustainable and cost-effective way of managing *E. crassipes* in the country. More so, the effective utilization of *E. crassipes* as a substrate of *P*. *ostreatus* can also be tailor made towards the production of protein rich mushrooms for local communities and a whole cocktail of the white rot lignocellulolytic enzymes for various applications in the industry and/or commercial systems. In this regard, the present study therefore, strongly recommended for a further optimization of its findings so that *P*. *ostreatus* can be viably utilized for the sustainable and cost-effective management of *E. crassipes* in Zimbabwe.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

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APPENDIX

Import permit P0079761.

agriculture, forestry & fisheries

Department: Agriculture, Forestry and Fisheries REPUBLIC OF SOUTH AFRICA

Page 1

Directorate Plant Health

Permit No. P0079761

PERMIT FOR THE IMPORTATION OF CONTROLLED GOODS

In terms of the provisions of section 3(1) of the Agricultural Pests Act, 1983 (Act 36 of 1983) and subject to the conditions stated here under, authorisation is hereby granted to:

NOMFUMELELO SIBANDA Tel No: 774 004202

PRIVATE BAG 7724

CHINHOYI, ZIMBABWE

00263

to import into the Republic the following controlled goods: AQUATIC PLANTS

AQUATIC PLANTS 50 KG

Name and address of foreign supplier: ZIMBABWE

Conditions: 1. AS ATTACHED

Port of Entry: BEIT BRIDGE

O R TAMBO INTERNATIONAL AIRPORT

Import authorised from: 2016/11/10 TO 2017/11/10

IMPORTANT: This permit does not exempt the holder from the provisions of any other Act, ordinance or agreement.

DEPARTMENT OF AGRICULTURE FORESTRY AND FISHERIES DIRECTORATE PLANT HEALTH 2016 -11- 10 DIRECTORATE PLANT HEALTH PO Box 0024, ARCADIA, 0007

Date Executive Officer

Reference Number: 8/16/262

INQUIRIES: TEL: (012)319 6100 (Sooman Matsa) FAX: (012)319 6370

11. Take precautions at all times to prevent the escape and introduction of any pest(s), which may be present in/on the samples into the RSA.

12. No cultures, sub-cultures or specimens of the imported sample(s) may be given to any other person or be used for any work outside the facility mentioned in 3. of this condition for any reason whatsoever, without the written consent of the Director.

13. **All imported sample(s) must be destroyed by autoclaving/incineration after completion of the laboratory analysis and notify the Manager, (for attention: Ms Rorisang Mahlakoana: Fax 012 319 6101) immediately thereof in writing.**

14. If any of the above-mentioned conditions are not complied with or are violated, the material shall be destroyed, at the importer's expense.

THE FOLLOWING UNDERTAKING TO BE COMPLETED BY IMPORTER OR HIS AUTHORISED AGENT:

I the undersigned, ____________________

I D No: ____________________

am fully aware of the above-mentioned conditions and understand that should I contravene or fail to comply with any of the above conditions I shall be guilty of an offence and be liable for prosecution under the Agricultural Pests Act, 1983 (Act No. 36 of 1983).

SIGNED: ____________________ DATE: ____________________

PERMIT NO.: ____________________

DEPARTMENT OF AGRICULTURE FORESTRY AND FISHERIES DIRECTORATE PLANT HEALTH 2016 -11- 10 DIRECTORATE PLANT HEALTH PO Box 40024, ARCADIA, 0007

agriculture, forestry & fisheries

Department: Agriculture, Forestry and Fisheries REPUBLIC OF SOUTH AFRICA

PHYTOSANITARY REQUIREMENTS FOR THE IMPORTATION OF REGULATED ORGANISM (S) FOR RESEARCH PURPOSE

1. The consignment must be inspected at the point of entry and found free from contaminants.
2. Quarantine label to be affixed to the parcel;
3. The imported **AQUATIC WEED[maximum 50 KG]** sample (s), shall only be handled at **PLANT BIOTECHNOLOGY RESEARCH LABORATORY** facility
4. Import sample(s) shall be addressed to **Prof. OZINIEL RUZVIDZO / Ms. TSEGOFATSO DIKOBE**
   Name of institution / company: **UNIVERSITY OF NORTH WEST**
   Postal address: **CHINHOYI UNIVERSITY OF TECHNOLOGY P. BAG 7724 CHINHOYI ZIMBABWE**
5. The importer shall be responsible for custom clearance;
6. The container(s) shall be opened and the material handled in the facility at **PLANT BIOTECHNOLOGY RESEARCH LABORATORY**
7. Name of Laboratory facility: **PLANT BIOTECHNOLOGY RESEARCH LABORATORY**
   Physical address: **PLANT BIOTECHNOLOGY RESEARCH LABORATORY NORTH WEST UNIVERSITY MAFIKENG CAMPUS 1 UNIVERSITY DRIVE MMABATHO 2735**
8. Responsible laboratory technician: **Prof. OZINIEL RUZVIDZO / Ms. TSEGOFATSO DIKOBE**
   Tel no: **018 389 2289**
9. Destroy all packing material and wrapping by incineration or autoclaving.
10. Due to the foreign status of the sample(s) all experimental material shall be marked as potentially dangerous to the South African agricultural industry.

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DIRECTORATE: PLANT HEALTH

GENERAL INFORMATION TO IMPORTER/PERMIT HOLDER/AGENT

AGRICULTURAL PESTS ACT, 1983 (ACT NO. 36 OF 1983)

Subject to the provisions of section 3 of the agricultural pests Act, the importation of plants, plant products and other controlled goods is subject to a permit.

A copy of the permit and conditions should be communicated to the foreign supplier, by the importer/permit Holder/ Agent.

The national plant protection (NPPO) of the exporting country must issue a phytosanitary certificate, complying with the conditions of the South African permit. Each consignment must be accompanied by an original phytosanitary certificate.

Should the NPPO of the exporting country not be able to comply with the conditions of the permit, export cannot proceed. For assistance in this regard, the importer/permit holder should contact this Directorate.

Controlled goods can only be imported through a prescribe port of entry, except where determined otherwise by the Executive Officer.

On arrival, each consignment with relevant documentation must be presented (by the importer/permit holder/agent) to the Executive Officer for inspection at the port of entry. Goods may not be removed from the port of entry without the written authorization of the Executive Officer.

Please note:

i. where any other place than the port of entry has been determined or when goods are imported via a courier service, the importer/permit holder/agent must on arrival, present the goods to the Executive Officer;
ii. if brought in per passenger, the material must first be declared at the customs control point before presenting it to the Executive Officer. The red line to be taken at the customs control point.

Please take note of the expiry date of the permit. A permit expires on the date indicated on the permit. Should you wish to proceed with a similar import, please apply at least 30 days prior to the expiry date of the permit, for a new permit.

Please accept the above as guideline, but take note that all stipulations of the Agricultural Pests Act should be complied with. Authorization in terms of this Act does not exempt the holder from the provisions of any other Act, ordinance or agreement.

Other Acts that may be relevant: Plant Improvement Act & GMO Act.

For assistance please contact:

Permit Office:

Mr. Bonie Kgomo	Tel: (012) 319 6130
Mr. Emmanuel Liabara	Tel: (012) 319 6207
Ms. Priscilla Baloyi	Tel: (012) 319 6396
Email:	PlantHealthPermits@daff.gov.za

Figure A1. Import permit. The permit was sought and granted by the Department of Agriculture, Forestry and Fisheries, South Africa to transport and undertake research with the Zimbabwean *E. crassipes* at the North-West University.

Related Journals: