Antioxidant potentials of culturable endophytic yeasts from *Phragmites australis* Cav. (Trin) ex Steud. from copper-contaminated mining site in Mankayan, Benguet

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Three endophytic yeasts were isolated from the lower stem and roots of *Phragmites australis* Cav. (Trin) ex Steud., observed to be dominant along the banks of the tailings pond 5A of the Lepanto Consolidated Mining Co. in Mankayan, Benguet. These yeast isolates were tested for their antioxidant properties *in vitro* to establish the possible contribution of these endophytes to the survival of their hosts in this inhospitable environment. All fungal yeast isolates displayed significantly higher antioxidant activities than the sterile potato dextrose broth that served as the control. Notable isolates were Yph3, which displayed the highest antioxidant activity using the 1,1-diphenyl-2-picrylhydrazyl assay, and *C. parapsilosis* Yph5, which yielded the highest ascorbic acid equivalent per mg dry weight using the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay. *C. parapsilosis* Yph5 also produced the highest amount of phenolic compounds measured as gallic acid equivalents. All three yeast isolates were observed to exhibit similar ascorbate peroxidase activities. On the other hand, *D. hansenii* Yph4 showed the highest catalase activity. It is clear from this study that the different endophytic yeasts have different mechanisms for quenching reactive oxygen species. This may be an indication that *in situ*, they perform synergistic and complementary roles in the antioxidative system of their host plant *P. australis*.

INTRODUCTION

The production of antioxidants by microbial symbionts of plants is well-documented (Hamilton and Bauerle 2012). It is postulated that the production of antioxidant compounds by endophytic microorganisms has beneficial consequences to the host especially when the host is currently experiencing stress, *e.g.*, drought, light, metal stress. Abiotic stresses commonly cause increased production of reactive oxygen species (ROS). These ROS may lead to unspecific oxidation of proteins and membrane lipids, or may cause DNA injury (Ravindran et al. 2012). Copper (Cu), though an essential element, at elevated levels can directly produce ROS (Gamalero et. al. 2009). For heavy metal (HM) tolerant metallophytes, therefore, innate antioxidant activity and any contributing antioxidant capacity of symbiotic organisms are welcome adaptations to these stressful conditions.

Phragmites australis Cav. (Trin) ex Steud. plants were observed to have luxuriant though patchy populations along the banks of the mine tailings pond 5A of the Lepanto Consolidated Mining Co. in Mankayan, Benguet. The tailings pond was found to be high in Cu at 305 ppm. This high concentration of Cu together with the other associated HM possibly poses HM-induced oxidative stress to these plants. It is therefore not far-fetched to assume that these plants have adaptive characters to mitigate oxidative stress due to HM.

The genus *Phragmites* is a rhizomatous plant of the family Poaceae with a wide geographical distribution. It generally grows in wetlands, where it may flourish and become the dominant plant species. This plant can withstand extreme edaphic conditions (Ye et al. 2003). Currently, the genus is considered to be monospecific (*P. australis*), although a thorough taxonomic work on this taxon is still needed (www.iucnredlist.org).

There are many researches on *P. australis* studying its involvement in pollution. This plant, being a riparian species, is typically studied in relation to organic and heavy metal pollutants of various water bodies. Because of their fibrous root systems with large contact areas, *P. australis* plants generally have the ability to accumulate larger concentrations of pollutants in plant organs than those in the surrounding water (Bonanno and Lo Giudice 2010). Ait Ali et al. (2004) mention that this plant is tolerant to high Cu concentrations in the soil. They tested its *in vitro* response to 15.7 mM Cu in solution culture and found that the plant is indeed tolerant to these conditions. They also discovered that the roots accumulate higher amounts of metal than the shoot. Ianelli et al. (2002) also found that *P. australis* is capable of accumulating large amounts of Cd^{2+}. Because of the perceived tolerance of *P. australis* to elevated amounts of pollutants, they have been widely used in constructed wetlands for the treatment of industrial wastewaters containing metals (Ye et al. 2003).

Fungal endophytism in *P. australis* has been studied, but a similar study has never been done in the Philippines, nor in Southeast Asia. Studies by Pelaez et al. (1998), Wirsel et al. (2001), Ernst et al. (2011) and Neubert et al. (2006) had all been done in Europe. Their results showed that as many as 89% of the isolates are Ascomycetes. Very few basidiomycetes and zygomycetes have been discovered to occur in European representatives of this plant.

In this study, three endophytic yeasts were successfully isolated from the submerged portions of *P. australis* in the sediments of the tailings pond. Studies have shown that fungal endophytes contribute to the overall oxidative-stress tolerance in host

Figure 1. Photo showing the population of *P. australis* that was sampled at the banks of tailings pond 5A, LCMC.

plants (White and Torres 2010). Thus, the antioxidant potentials of these three endophytic yeasts were investigated.

Both enzyme and non-enzyme antioxidants are known to contribute to the observed antioxidant potentials of organisms. Examples of non-enzyme antioxidants include phenolics, flavonoids, tannins and anthocyanins. According to Liu et al. (2007), phenolics and flavonoids show positive correlations with radical-scavenging activity. Superoxide dismutase, catalase, glutathione transferase and ascorbic peroxidase are a few examples of enzyme antioxidants. These enzymes contribute by scavenging the different ROS, thus protecting the cells and organelles from tissue dysfunction (Dixit et al. 2011). Specifically, catalase acts to remove H_2O_2 by degrading it to water and oxygen (Desikan et al. 2004). In fungi, this enzyme's location is currently being established and are postulated to be housed in the Woronin bodies (Schliebs et al. 2006). Similarly, ascorbate peroxidase is involved in the metabolism of H_2O_2 via the ascorbate-glutathione cycle (Desikan et al. 2004). H_2O_2 is intricately involved in the interconversion of ROS such as the superoxide radical and the hydroxyl radical.

In this study, the three yeast isolates were screened for their antioxidant activity. The following are the specific objectives: (1) to detect and quantify the antioxidant activity of the yeast isolates as ascorbic acid equivalents using the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assays; (2) to detect and quantify the phenolic compounds of each isolate as gallic acid equivalents; and (3) to detect and quantify the ascorbate peroxidase and catalase activities and express those in enzyme units.

METHODS

Isolation of Endophytic Yeasts

Endophytic yeasts were isolated from fifteen (15) healthy looking *P. australis* plants that were observed to be the dominant plant species occurring at the tailings pond 5A of the Lepanto Consolidated Mining Co. (LCMC) in Mankayan, Benguet. The plants were initially washed at site with water to remove adhering sediments. The plants were then transported to the University of the Philippines Baguio for further processing and for the isolation of the fungal endophytes. In the laboratory, the plant tissues were thoroughly washed with tap water to remove remaining debris. Representative segments of the roots and the lower stems were cut and subjected to surface sterilization following one of the protocols suggested by Stone et al. (2004). The following was the sequence and duration of the steps taken during surface sterilization: 95% ethanol for 1 min, 10% NaOCl for 5 min, then 95% ethanol for 30s. The segments were rinsed in sterile water three times in sequence then blotted dry on sterile absorbent paper. The effectiveness of the surface sterilization method was tested with the plating of 100 ml of the last rinse on potato dextrose agar (PDA) plates. The surface-sterilized segments were incubated at 30°C on PDA with Chloramphenicol to inhibit bacterial growth, and with Rose Bengal to slow down the growth of filamentous fungi especially the vigorous ones. The plates were incubated for 7 days. Each colony that grew from the segments was re-isolated in PDA slants.
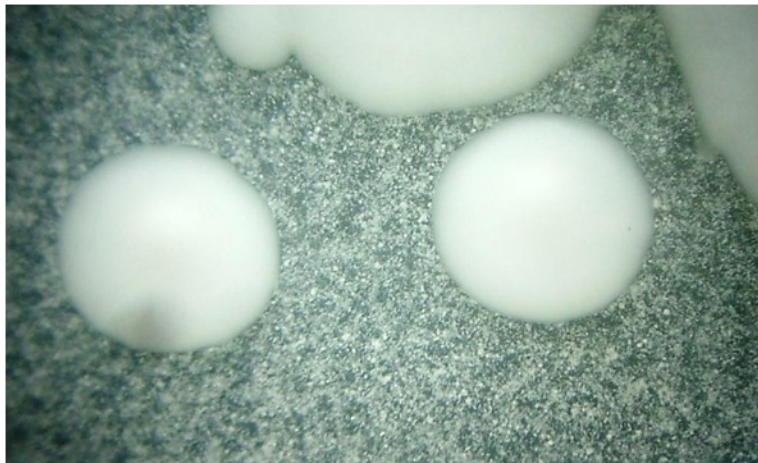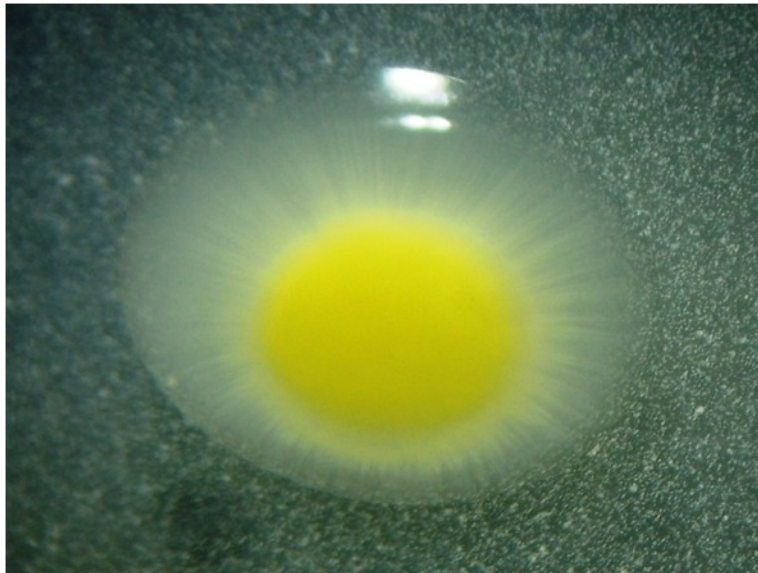
Figure 2. Colony morphology of the yeast isolates on Pikovskaya's agar. (Top) Yph3; (Middle) *D. hansenii* Yph4; (Bottom) *C. parapsilosis* Yph5.

Fermentation Set-up

Pure cultures of the yeast isolates were grown in potato dextrose broth for seven days at 30^0C in a shaker incubator set at 100 rpm. After 7 days, 1 ml aliquots were dispensed in sterile microcentrifuge tubes and centrifuged at 5000 rpm for 5 min to pellet the yeast cells. The supernatant was separated and tested using the various antioxidant assays. The yeast pellet was dried in an oven dryer set at 60^0C until weight was constant to get the dry weight (DW) of the yeasts.

Colony and Cell Morphology

Colony morphology was documented using a stereomicroscope (Swift SM100). The scanning electron microscope (JEOL JSM-610 LV) of the Physics Department of the College of Science, University of the Philippines Baguio, was used to take electron micrographs of cell morphology.

Molecular Identification and Phylogenetic Analysis of the Yeast Isolates

Genomic DNA isolation. Small aliquots (1.5 ml) of broth cultures of the yeast isolates were centrifuged to pellet the cells. The cells were then suspended in 50 ml of 33.5 mM KH_2PO_4, pH 7.5. Lyticase was added (20 ml of 2.5 U/ml) and incubated at 37 °C for 1 hr. The spheroplasts were spun down at 5000 x g for 10 min at 4°C. The succeeding steps followed the protocol of the Vivantis GF-1® bacterial DNA extraction kit. The isolated DNA was suspended in 100 µl of the elution buffer and tested for purity in 1% agarose gel.

Molecular identification. The endophytic yeast isolates were identified by sequencing the internal transcribed region (ITS) of the 18S rDNA, using universal primers ITS-1 (5'TCC GTA GAA CCT GCG G-3') for the forward primer and ITS-4 (TCC TCC GCT TAT TGA TAT GC') for the reverse primer. The PCR mixture (36 µl) contained 12.5 µl of the Vivantis PCR Master Mix, 0.75 µl $MgCl_2$, 1 l each of the forward and reverse primers, 14.75 µl of PCR water, and 1 µl of gDNA. The reaction cycle consisted of a pre-denaturation phase of 5 min at 95°C, followed by 35 cycles of 30s at 95°C, 1 min at 60°C and 1 min at 72°C. Final extension phase of 6 min at 72°C was done. The PCR products were run on 1% agarose gel to check the generation of the amplification products of the desired length which is about 550 bp. The PCR products were sent to the 1st Base Sequencing Facility in Singapore for cleaning and subsequent sequencing using ITS-1 as the sequencing primer.

The Basic Local Alignment Sequence Tool search program using nucleotides (BLASTn) available in the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov) was used to look for nucleotide sequence homology of the 18S ITS (1/4) regions of the fungal isolates with the sequences in the GenBank database. Isolates whose sequences had a similarity greater than 97% were considered as belonging to the same species as that in the database. This 3% difference used to define species boundaries appears to correlate well with differences among known endophytic species (Arnold and Lutzoni 2007). In instances where the similarity is 95-97%, only the genus was accepted. A sequence with a similarity below 95% was treated as unidentified (Sanchez-Marquez et al. 2008). Morphological examination was used to clarify ambiguities and to confirm the results of the sequence similarity searches.

Total Antioxidant Capacity Assays

The assay for the production of antioxidants was done using two of the several standard procedures for the estimation of radical scavenging capacity. One assay was the ABTS test and the other was the DPPH test, with three replicates for each test and for each week-old yeast isolates in potato dextrose broth incubated at 30^0C. The control for each of the tests is an aliquot of a sterile potato dextrose broth.

The ABTS test evaluates the disappearance of the radical cation of diammonium 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) which is oxidized from ABTS by the potassium peroxodisulfate. This radical has a strong absorption in the range of 600-750 nm. It reacts with proton donors, such as phenolics, being converted into a non-colored form of ABTS (Posmyk et al. 2009). For this experiment, 6 ml each of 7.2 mM ABTS and 2.6 mM potassium persulfate in methanol were prepared. Three ml of each of these solutions were mixed together and incubated at room temperature for 16 hrs in the dark. This was then diluted with methanol until absorbance at 734 nm was at 1.1. From this solution, 1.9 ml were withdrawn and added to 1 ml of the yeast broths that was previously incubated for 7 days. This final mixture was incubated for 2 hrs at room temperature and the absorbance measured at 734 nm with methanol as blank. Antioxidant activity was quantified using a standard curve of ascorbic acid. The generated regression equation was $y = 348.12x - 90.733$ ($R^2 = 0.9839$). Ascorbic acid was chosen as the standard among other equally useful standards because the antioxidant capacity expressed as such is familiar and easy to understand (Kim et al. 2002).

On the other hand, DPPH is a molecule containing a stable free radical. In the presence of an antioxidant which can donate an electron to DPPH, the purple color typical of free DPPH radical decays. There would be a decrease in absorbance at 517 nm that could be followed spectrophotometrically if antioxidant activity occurs (Posmyk et al. 2009). For this experiment, 75 µl of the yeast broths were added to 1.5 ml of the DPPH solution. The reaction mixture was incubated at 37^0C for 30 min. The absorbance was then measured at 517 nm with methanol as blank.

Non-Enzyme Antioxidants: Soluble Phenols Assay

Phenolic compounds produced extracellularly were assayed using the methods of Ravindran et al. (2012) and Liu et al. (2007). In brief, the following were done: sample broths were centrifuged at 5000 rpm for 3 min to separate the mycelia; 660 µl of the supernatant were added to 1 ml dH_2O and 300 µl of Folin Ciocalteu reagent; the mixture was incubated in the dark for 2 hrs at room temperature; absorbance was measured at 760 nm. Gallic acid equivalents were computed using a standard curve of gallic acid, the standard frequently used in Folin Ciocalteu assays for phenolic acids. The generated regression equation was $y = 1376.9x + 8.2349$ ($R^2 = 0.9999$).
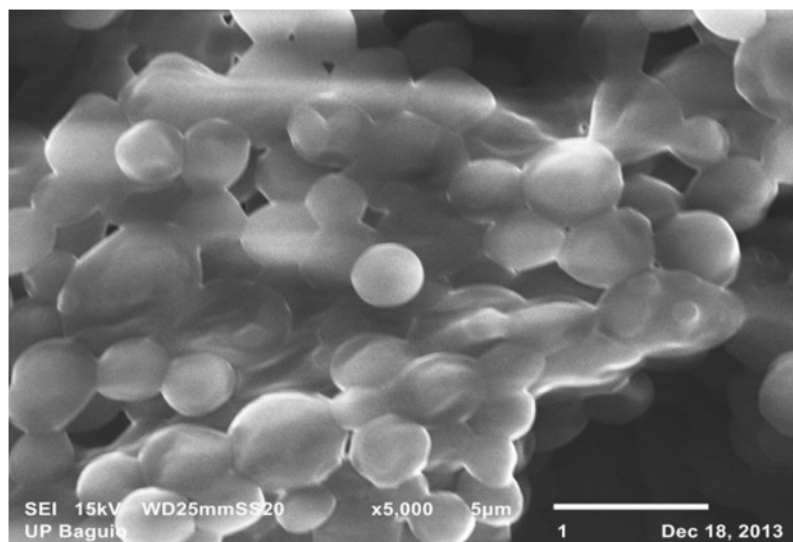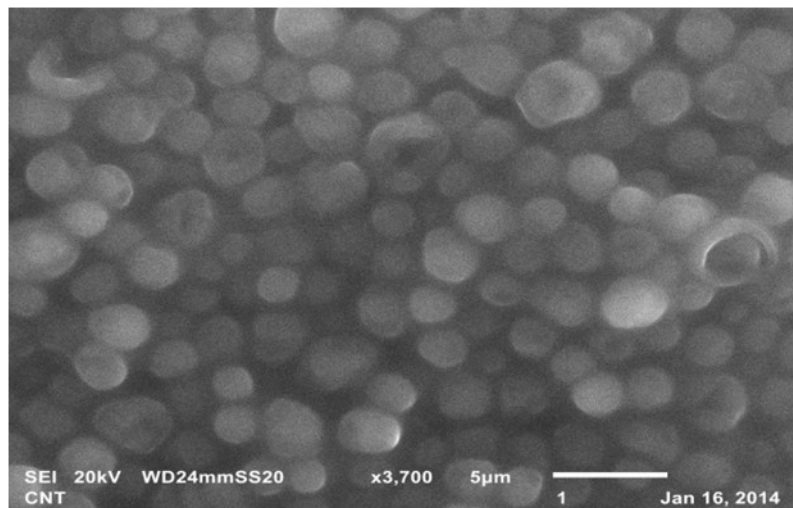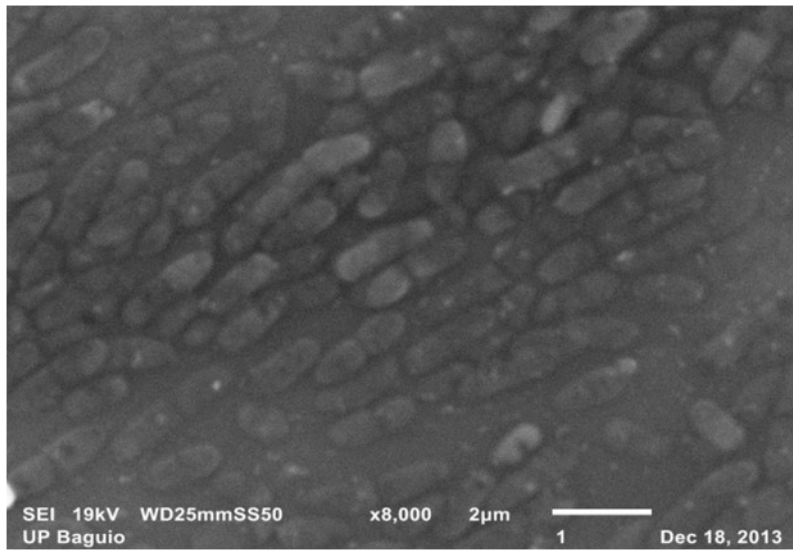
Figure 3. Scanning electron micrographs of the yeast isolates. (Top) Yph3; (Middle) *D. hansenii* Yph4; (Bottom) *C. parapsilosis* Yph5.

Enzyme Antioxidants: Ascorbate Peroxidase Activity (APX)

APX levels for each isolate were determined according to Jiang and Zhang (2002). One reaction mixture contained 1.5 ml of 50 mM potassium phosphate buffer (pH 7.0), 85 µl of 0.5 mM ascorbic acid, 75 µl of 0.1 mM H_2O_2 and 125 µl of the culture. The reaction was monitored by following t-he decrease in A_{290} (extinction coefficient 2.8 mM^{-1} cm^{-1}) for three minutes as ascorbate is oxidized. APX activity was quantified as enzyme units following the formula of Wilson (2010) below, modified so as to measure enzyme activity per mg DW of the yeast isolates.

$$Enzyme\ units = \left(\frac{\Delta A290}{\epsilon}\right) \times \left(\frac{a}{wt}\right) \times \left(\frac{1000}{x}\right)$$

(katals per mg dry wt of yeast isolate)

Where: ϵ = extinction coefficient of Catalase (39.4 mM^{-1} cm^{-1})
a = total volume of the reaction mixture in µl
wt = dry wt of the yeast isolates in mg
x = the volume of test solution added to the reaction mixture in µl

Enzyme Antioxidants: Catalase Activity (CAT)

Catalase activity necessary for the dissipation of H_2O_2 was measured using the methods of Posmyk et al. (2009) and Odjegba and Fasidi (2006). The enzyme assay mixture contained 3.125 mM H_2O_2 in 50 mM phosphate buffer (pH 7.0) and 200 µl of the culture filtrate in a total volume of 3 ml. The activity was determined by the decrease in absorbance at 240 nm due to H_2O_2 consumption, using a UV-VIS spectrophotometer. APX activity was quantified as enzyme units following the formula of Wilson (2010) below, modified so as to measure enzyme activity per mg DW of the yeast isolates.

$$Enzyme\ units = \left(\frac{\Delta A240}{\epsilon}\right) \times \left(\frac{a}{wt}\right) \times \left(\frac{1000}{x}\right)$$

(katals per mg dry wt of yeast isolate)

Where: ϵ = extinction coefficient of Catalase (39.4 mM^{-1} cm^{-1})
a = total volume of the reaction mixture in µl
wt = dry wt of the yeast isolates in mg
x = the volume of test solution added to the reaction mixture in µl

RESULTS AND DISCUSSION

The growth characteristics of the three endophytic yeasts were documented. The observed characteristics are enumerated in Table 1. Of the three isolates, only two were successfully identified by comparing their ITS sequences with those in the GenBank database. These were isolates Yph4 and Yph5, identified as *Debaryomyces hansenii* and *Candida parapsilosis*, respectively. Table 2 summarizes the GenBank query details using the BLASTn algorithm. Yph3 was unsuccessfully queried into the GenBank database possibly due to an unsuccessful PCR protocol employed for this isolate.

Total Antioxidant Capacity Assays

All isolates exhibited notable antioxidant potentials as they all displayed significantly lower absorbances than the negative control (sterile potato dextrose broth) in both the DPPH and ABTS assays (Fig. 4). All isolates showed similar radical-scavenging activity with the ABTS assay (Fig. 5). The computed average for all the three isolates was 88%. The DPPH assay showed varying radical-scavenging activities for the three isolates; the unidentified yeast Yph3 showing the highest activity with a decrease in absorbance of 41.4%. Using the results of the ABTS assay, the ascorbic acid (AA) equivalent (µg) per mg DW of the yeasts was computed. Figure 6 shows that the isolate with the highest AA equivalent per mg DW was *C. parapsilosis* Yph5 at 8.4 µg/mg.

Non- Enzyme Antioxidants: Soluble Phenols Assay

All isolates produced significant amounts of phenolic compounds measured as gallic acid equivalents per ml of broth culture (Fig. 7). Together, all isolates produced 4.4x more phenolic compounds than what was in the control. The two best isolates were *D. hansenii* Yph4 and *C. parapsilosis* Yph5, on the average producing 4.8x more phenolics than the control. These two isolates also produced phenolic compounds in amounts significantly higher than the other isolate - the unidentified yeast Yph3. When measured as µg gallic acid per mg DW of the yeast isolates, the best performer was isolate *C. parapsilosis* Yph5 at 46.92 µg gallic acid equivalent/mg DW (Fig. 8).

Table 1. Morphological characteristics of the three yeast isolates from the roots and lower stems of *P. australis*.

ISOLATE	COLOR	TEXTURE	MARGIN	SURFACE	ELEVATION	CELL SHAPE	CELL SIZE
Yph3	Yellow	Mucoid	Entire	Glistening Smooth	Dome-like	Cylindrical	1-2 µm
Yph4	White	Viscous	Entire	Glistening Smooth	Raised	Spherical	2-4 µm
Yph5	White	Butyrous	Entire	Dull Smooth	Dome-like	Ellipsoid	2-5 µm

Table 2. Molecular identification of the yeast isolates using the BLASTn algorithm of NCBI.

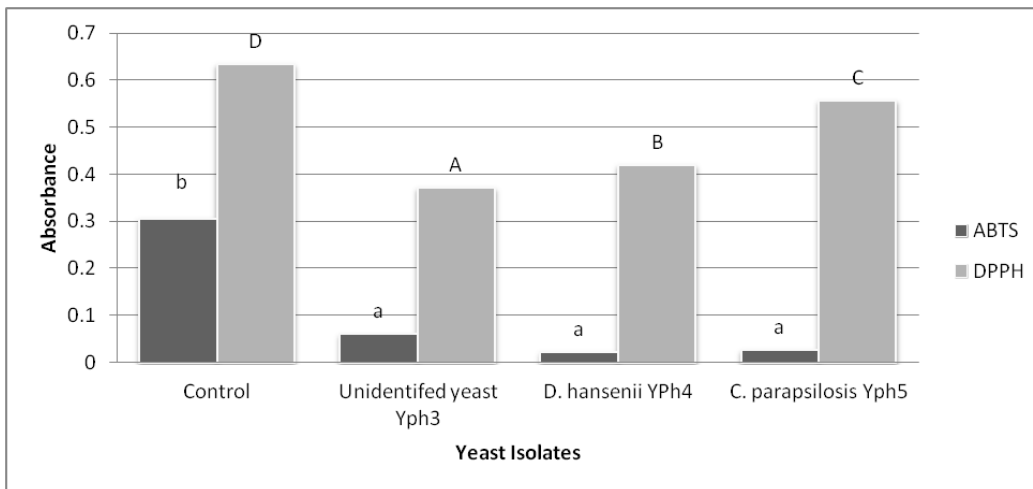| YEAST ISOLATES	CLOSEST MATCH	GENBANK ACCESSION #	TOTAL SCORE	QUERY COVER	E-VALUE	SIMILARITY
Yph3		*Unidentified*				
Yph4	*Debaryomyces hansenii* (JN851059.1)		1070	98%	0	99%
Yph5	*Candida parapsilosis* (KC462059)		1745	82%	0	99%

Figure 4. Spectrophotometric absorbances of the different yeast isolates measured in the ABTS and DPPH assays. Letters denote homogenous groups using ANOVA at p=0.05; n=3.
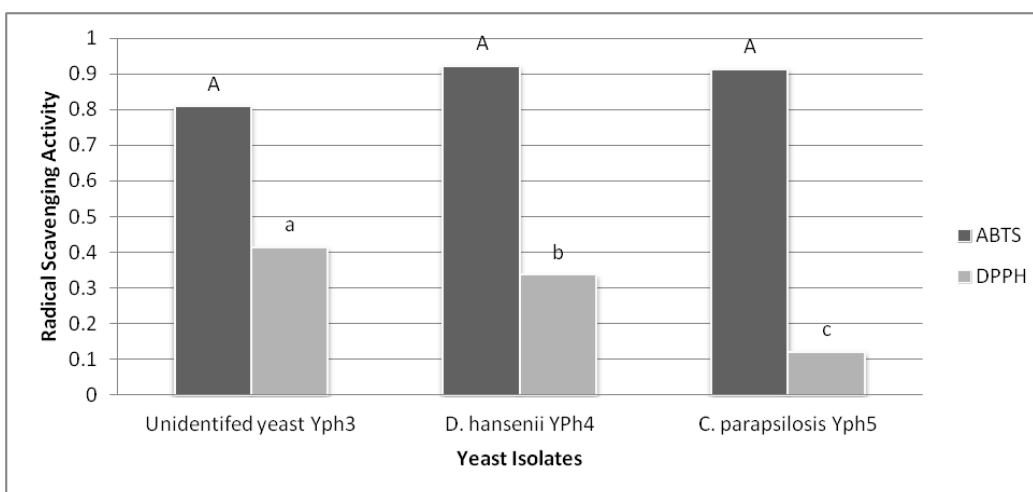

Figure 5. Radical scavenging activities of the different yeast isolates using ABTS and DPPH radical quenching assays. Letters denote homogenous groups using ANOVA at p=0.05; n=3.
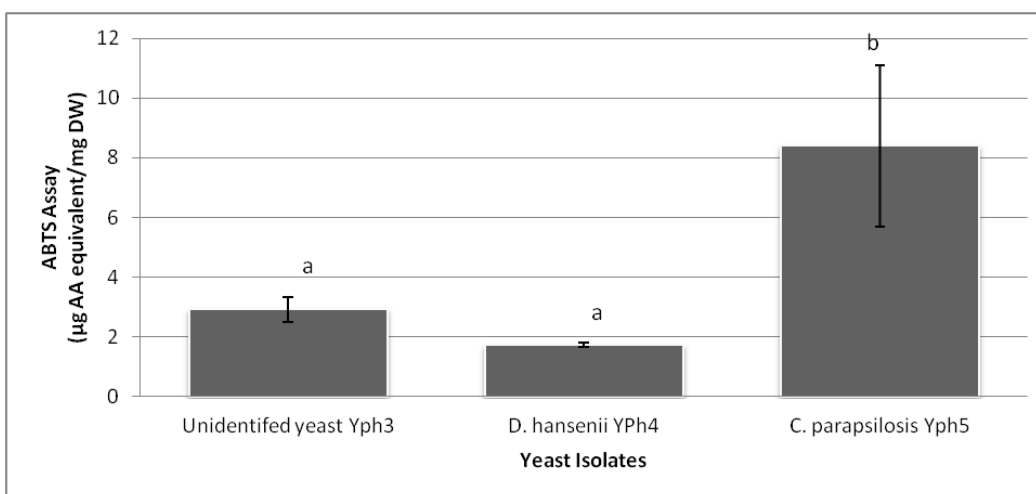

Figure 6. Ascorbic acid equivalent (µg/mg DW) determined from the different yeast isolates. Letters denote homogenous groups using ANOVA at p=0.05; n=3.

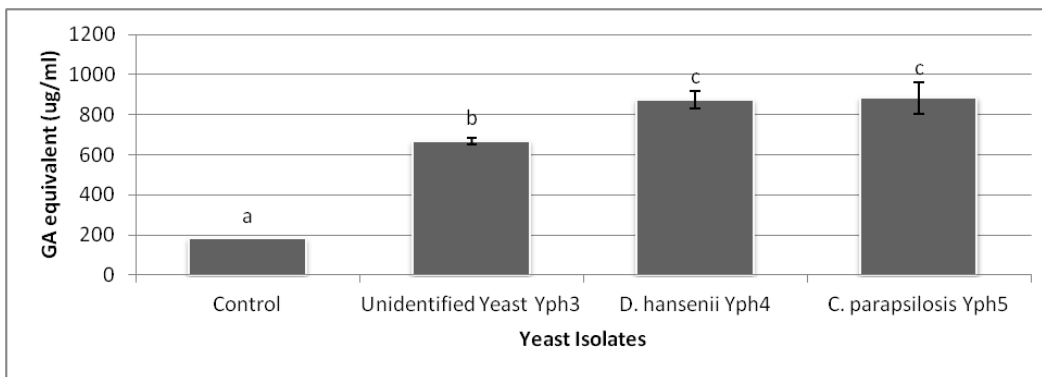**Figure 7.** Production of phenolic compounds of yeast isolates measured as gallic acid equivalent (µg/ml). Letters denote homogenous groups using ANOVA at p=0.05; n=3.

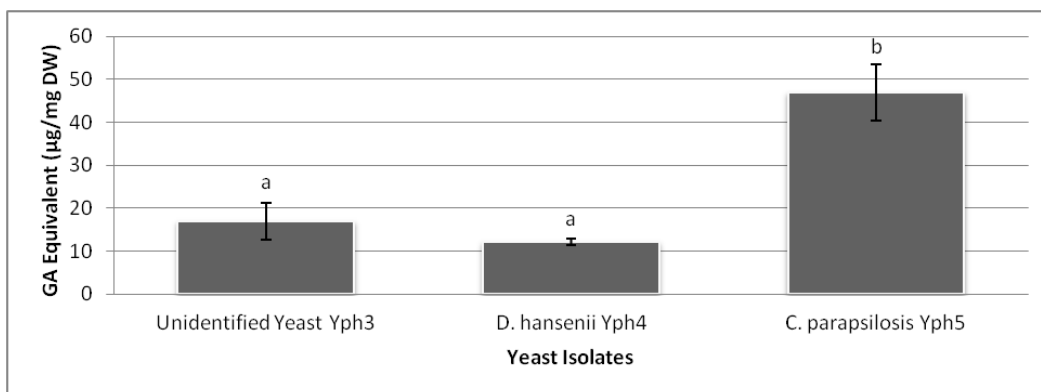**Figure 8.** Production of phenolic compounds of yeast isolates measured as gallic acid equivalent (µg/ mg DW). Letters denote homogenous groups using ANOVA at p=0.05; n=3.
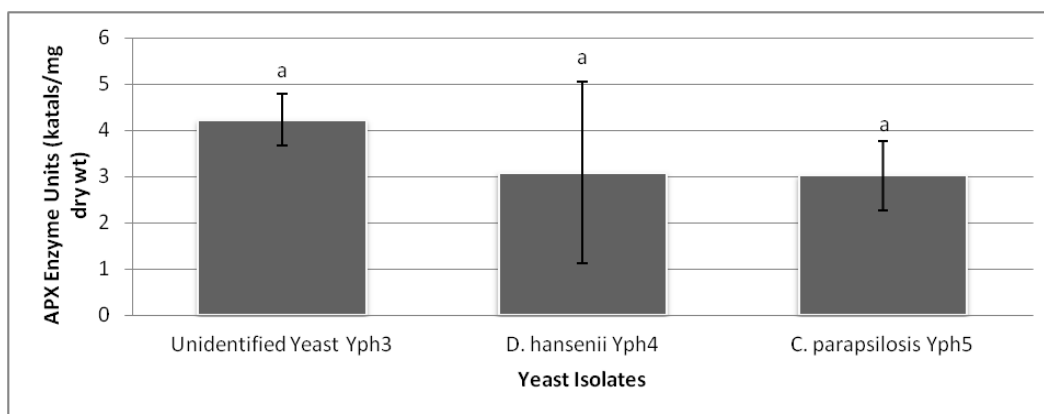

Figure 9. APX units of activity (katals/mg DW) computed for each of the yeast isolates. Letters denote homogenous groups using ANOVA at p=0.05; n=3.
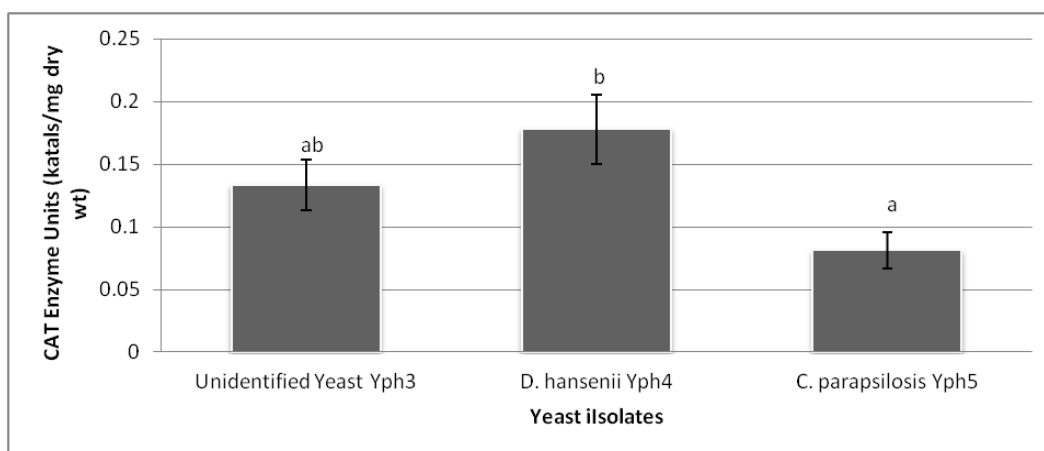
Figure 10. CAT units of activity (katals/mg DW) computed for each of the yeast isolates. Letters denote homogenous groups using ANOVA at p=0.05; n=3.

Enzyme Antioxidants

The highest activity of APX was in the unidentified yeast isolate Yph3 (Fig. 9) at 4.2 U. *D. hansenii* Yph4 and *C. parapsilosis* Yph5 had similar APX activities at 3.08 and 3.02 U, respectively. Yph3 showed 28% more APX activity than the other two yeast isolates. However, the statistical difference between them was insignificant. With regard to catalase activity, isolate *D. hansenii* Yph4 was observed to have the highest at 0.18 U (Fig. 10). Catalase activity in this isolate was 1.64 times higher than the isolate with the lowest CAT activity, which was *C. parapsilosis* Yph5.

Recent researches have been focusing on fungal endophytes as novel sources of secondary metabolites for various applications (Smith et al. 2008, Strobel 2003, Strobel and Daisy 2003) including antioxidants (Hamilton and Bauerle 2012). Antioxidant systems control oxidant levels of ROS that, if left unchecked, may lead to the unspecific oxidation of proteins and membrane lipids, which could result in nutrient leakage from plant cells and may even cause DNA damage (Ravindran et al. 2012). Fungal endophytes have been found to contribute to the enhancement of overall oxidative stress tolerance in host plants (White and Torres 2010). Studies have been done to investigate the possible contribution of fungal endophytes to plant response to various environmental stresses such as temperature, salinity and even biotic stresses caused by fungal pathogens (Ravindran et al. 2012). However, there have been no reports on the contribution of yeast endophytes to the HM tolerance of *P. australis*.

In this study, three endophytic yeast isolates were tested for their antioxidant potential. Using different assays, results show that all isolates displayed antioxidant activities although at varying degrees. This probably means that the yeast isolates exert their antioxidant properties by different mechanisms. *C. parapsilosis* Yph5 was found to produce the highest amounts of phenolic compounds. Phenolics and their derivatives include phenolic acids, isobenzofuranones and isobenzofurans (Aly et al. 2011).

Phenolic compounds are good antioxidants because their hydroxyl groups confer scavenging ability (Ravindran et al. 2012).

Ascorbate peroxidase and catalase are the two antioxidant enzymes that were assayed in the study. These two enzymes are said to be the primary line of defense against free radicals (Ravindran and Naveenan 2011). Other detoxifying antioxidant enzymes include glutathione reductase and superoxide dismutase (Hamilton and Bauerle 2012). It was found that the three isolates had similar APX activities. On the other hand, *D. hansenii* Yph4 was observed to have the highest catalase activity. It is notable that isolate Yph3, which had the best antioxidant capacity as measured with DPPH, did not produce as much phenolics, APX or catalase. It may be that this isolate has other antioxidant systems different from those that were assayed in this experiment and which conferred its relatively high antioxidant potential.

The successful assays for phenolics and for the antioxidant enzymes, APX and catalase, are just a few of the many *in vitro* assays for antioxidant activity. Already, these assays have shown that the isolated yeast endophytes have promising antioxidant activities *in vivo*. Together with the innate capacity of *P. australis*, the endophytic yeasts provide various mechanisms of adaptation to the adverse conditions created by the high concentrations of Cu and other heavy metals in constructed wetlands such as the tailings pond of LCMC.

ACKNOWLEDGEMENTS

CONFLICTS OF INTEREST

None

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