Copper Tolerance and Copper Accumulation of Culturable Endophytic Yeasts of *Phragmites Australis* Cav. (Trin) ex Steud. From the Mine Tailings Pond in Mankayan, Benguet, Philippines

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ABSTRACT— ***Three endophytic yeasts of* Phragmites australis *Cav. (Trin) ex Steud. from the tailings pond 5 of Lepanto Consolidated Mining Co. in Mankayan, Benguet, Philippines were investigated for their capacity to tolerate and accumulate copper. Two of the isolates were successfully identified through sequence homology of their ITS genes. All isolates were able to tolerate 500ppm Cu concentrations. The isolate that sequestered the most copper was D. hansenii Yph 4 at 9.38 µg/mg. The isolate that had the best bioconcentration factor was Candida parapsilosis Yph5 at 74.09%. Ascorbate peroxidase and catalase assays of yeasts grown on 50 ppm copper were done. The highest producer of APX was C. parapsilosis Yph5. On the other hand, the best catalase producer was the unidentified yeast Yph3.***

Keywords— Endophytic yeasts, copper tolerance, *Phragmites australis*, bioconcentration factor, ascorbate peroxidase, catalase

1. INTRODUCTION

Remediation efforts that use physical and chemical approaches are both expensive and non-sustainable [1]. This is the reason why phytoremediation is gaining popularity as a remediation option. Phytoremediation is considered as an environmentally friendly, gentle management option for polluted soil as it uses solar-driven biological processes to manage pollutants [2], including heavy metals (HM). Phytoremediation consists of four different plant-based technologies each having a different mechanism of action for the remediation of metal-polluted soil, sediment, or water. These include: rhizofiltration, phytostabilization, phytovolatilization, and phytoextraction [3]. Phytoremediation however suffers from several limitations which include the extent of metal availability, metal uptake and phytotoxicity for the plant [4]. A promising approach to address this limitation is the optimization of the synergistic effect of plants and microorganisms [5]. This research was done to this end. Endophytic yeasts were tested *in-vitro* for their possible in-vivo contribution toward successful phytoremediation of Cu contaminated landscapes.

All organisms when exposed to environmental stress react appropriately to allay the experienced stress. Accordingly, when organisms are exposed to increased concentrations of HM, they express adaptive responses to alleviate the effect of the stressful condition; and fungal endophytes are not an exception.

Fungi in general are known to tolerate and detoxify metals by several mechanisms [6]. These include HM compartmentation, chelation and intra and extracellular complexation mechanisms [7]. Various compounds with high affinity with HM provide this function for the plant. Such compounds include metallothioneins (MT) and phytochelatins (PC). MTs are cystein-rich polypeptides while PCs consist of only three amino acids, glutamine (Glu), cystine (Cys), and glycine (Gly) and are enzymatically synthesized from glutathione (Yang, Feng, He, & Stoffella, 2005). The metal-chelate complex is then sequestered into the vacuole or into other sub-cellular compartments [8].

Another mechanism of HM tolerance is the more immediate and useful response of producing antioxidant enzymes. The toxicity of HMs to organisms is due, in part, to oxidative stress due to the production of reactive oxygen species (ROS) [9]. Ascorbate peroxidase and catalase are two enzymes that are said to be the primary line of defense in destroying free radicals [10].

In this study, the yeast isolates endophytic to *Phragmites australis* Cav. (Trin) ex Steud. from the tailings pond 5A of the Lepanto Consolidated Mining Co. in Mankayan, Benguet were investigated for their capacity to tolerate elevated

levels of copper *in-vitro*. Possible evidences and reasons for their tolerance to increased Cu levels were investigated. All of these were in view of the possible role of the endophytic yeasts to the survival of their host *P. australis* in the Cu contaminated tailings pond 5A of the Lepanto Consolidated Mining Corporation (LCMC). Specifically, the research investigated the minimum inhibitory concentrations of copper for each of the yeast isolates; the quantity of copper accumulated by each of the yeast isolates, and the effect of increased Cu concentration on the activity of the antioxidant enzymes ascorbate peroxidase and catalase.The entire manuscript, including mathematical equations, tables, and figures must be prepared in electronic form and submitted as Word for Windows files. Use only fonts that come with Windows software. For the text use Times New Roman size 10. For all special characters (e.g., Greek characters) use the font **Symbol**. Line spacing is single; spacing after paragraphs is 6 pt; first line is indented **.2** inches; text alignment is justified. Use carriage returns only to end headings and paragraphs, not to break lines of text. Verify the correct spelling for the final version with the Spelling and Grammar function of Word.

2. MATERIALS AND METHODS

2.1 Isolation and Identification of Endophytic Yeasts

Endophytic yeasts were isolated from fifteen (15) healthy looking *Phragmites* sp. plants that were observed to be the dominant plant species occurring at the tailings dam of LCMC. The plants were then transported to the University of the Philippines Baguio for the surface sterilization of the roots and lower stems found to be submerged in the substratum following the protocols suggested by Stone et al. [11]. Representative segments of the roots and lower stems were plated on potato dextrose agar (PDA) amended with chloramphenicol and rose bengal. The plates were incubated in an incubator oven for seven days. Emerging hyphae were isolated into PDA slants for pure cultures.

Pure cultures of the yeast isolates were grown in potato dextrose broth (PDB) to grow mycelia for DNA extraction. Small aliquots (1.5 ml) of broth cultures of the isolates were centrifuged to pellet the cells. The cells were then suspended in 50 ul of 33.5 mM KH2PO4, pH 7.5. Lyticase was added (20ul of 2.5 Units/ul) and incubated at 37 °C for 1 h. 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.

The endophytic yeast isolates were identified by sequencing the internal transcribed region (ITS) of the 18S rDNA, using universal primers ITS-1 and ITS-4. The PCR mixture (36 µl) contained 12.5 µl of the Vivantis PCR Master Mix, 0.75 µl $MgCl_2$, 1µl each of the forward and reverse primers, 18.75 µl of PCR water, and 2 µl of the isolated DNA. The reaction cycle consisted of a pre-denaturation phase of 5mins at 95°C, followed by 35 cycles of 30s at 95°C, 1min at 60°C and 1min at 72°C. Final extension phase of 6 mins 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 1st Base Sequencing Facility in Singapore for cleaning and subsequent sequencing using ITS 1 as the sequencing primer.

The Basic Local Alignment Sequence Tool using nucleotides (BLASTn) search program was used to look for nucleotide sequence homology for the 18s ITS (1/4) region for the fungal isolates for their identification. Isolates whose sequences had a similarity greater than 97% was considered to belong to the same species. This 3% difference used to define species boundaries appears to correlate well with differences among known endophytic species [12]. In instances where the similarity is 95-97%, only the genus was accepted. Sequence homology below 95% was treated as an unidentified taxon [13]. Morphological examination was used to clarify ambiguities and to confirm results of sequence similarity searches.

2.2 Comparative tolerances of the yeast isolates to varying concentrations of copper

Isolates were grown in 2500, 1500, 750, 500, 250 and 100 ppm Cu using $CuSO_4$ in potato dextrose (PD) broth. Optical density at 600nm (OD_{600}) was used to determine if yeast cells propagated at these concentrations of Cu. OD_{600} was measured after 8 days incubation [14] using a UV-VIS Spectrophotometer (Shimadzu UV mini 1240 UV-VIS) and an increase in absorbance was considered evidence for growth.

2.3 Accumulation of copper of the yeast isolates

The strains were also tested for their capacity to accumulate copper by growing them in PD broth with 50 ppm copper for 7 days 30 °C in a shaker incubator. Afterwhich, 1ml aliquots of the broth were centrifuged at 3,000 rpm for 5 min to separate the cells from the broth. The supernatant was sent to the Natural Science Research Unit of Saint Louis University to test its copper content to determine whether the inoculated yeast isolate was able to remove copper from the PD broth. The pelleted biomass was dried at 50^0C in a drying oven until measured weight was constant to determine the dry weight of the yeast cells per ml of the broth. The dry weight was used to compute for the efficiency of copper accumulation per unit dry mass of the yeast isolate. The bioconcentration factor (BCF) was also calculated following Ait Ali *et al.*, [15].

$$BCF = A/B$$

Where: BCF = Bioconcetration Factor
A = metal (ppm) accumulated
B = metal (ppm) of solution

2.4 Enzyme antioxidants: ascorbate peroxidase activity (APX)

This experiment used supernatants of PDB cultures incubated for 7 days in 50 ppm Cu PD broth centrifuged at 5,000 rpm for 3 min. APX levels for each isolate were determined according to Jiang and Zhang [16]. One reaction mixture contained 1.5ml 50mM potassium phosphate buffer (pH 7.0), 85 µl 0.5mM ascorbic acid, 75 µl 0.1 mM H_2O_2 and 125 µl of the culture filtrate was prepared. The reaction was monitored by following the decrease in A_{290} (extinction coefficient 2.8 mM^{-1} cm^{-1}) for three minutes as ascorbate was oxidized. APX activity was quantified as enzyme units following the formula of Wilson [17] below, modified so as to measure enzyme activity per mg dry wt of the yeast isolates.

$$Enzyme\ units\ (katals\ per\ mg\ dry\ wt\ of\ yeast\ isolate) = \left(\frac{\Delta A290}{\epsilon}\right) x \left(\frac{a}{wt}\right) x \left(\frac{1000}{x}\right)$$

Where: ϵ = extinction coefficient of APX (2.8 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

2.5 Enzyme antioxidants: catalase activity (CAT)

This experiment used supernatants of PD broth cultures incubated for 7 days in 50 ppm Cu PD broth centrifuged at 5,000 rpm for 3 min. Catalase activity was measured using the methods of Posmyk [18] and Odjegba [19]. The enzyme assay contained 3.125 mM H_2O_2 in 50mM phosphate buffer (pH 7.0) and 200 µl of the culture filtrate in a total volume of 3ml. The yeast culture filtrates that were used in this experiment were from the yeast cultures previously grown for 7 days in 50 ppm Cu potato dextrose broth that were centrifuged at 5,000 rpm for 3 min to get the supernatant. The activity was determined by the decrease of 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 [17] below, modified so as to measure enzyme activity per mg dry wt of the yeast isolates.

$$Enzyme\ units\ (katals\ per\ mg\ dry\ wt\ of\ yeast\ isolate) = \left(\frac{\Delta A290}{\epsilon}\right) x \left(\frac{a}{wt}\right) x \left(\frac{1000}{x}\right)$$

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 µl

2.5 Statistical analysis

All the copper treatments were set up in triplicates. The values were subjected to Analysis of Variance to determine if there are significant differences between the measured plant lengths using SPSS® version 17.0. The Duncan post-hoc test algorithm of the same software was used to show the different homogenous subsets that are significantly different from each other.

3. RESULTS

Three endophytic yeasts were successfully isolated into pure cultures from the roots and lower stems of *P. australis*. Of the three isolates, only two isolates were successfully identified through sequence similarity of their ITS sequences with the entries in the GenBank database. These were isolates Yph4 and Yph5 identified as *Debaryomyces hansenii* and *Candida parapsilosis* respectively. Table 1 summarizes the GenBank query details using the Basic Local Alignment Sequence Tool using nucleotides (BLASTn) algorithm. The isolate Yph3 was unsuccessfully identified due to the unsuccessful PCR protocol employed for this isolate. The sequences for *D. hansenii* Yph4 and *C. parapsilosis* Yph5 were submitted to GenBank and were given the accession numbers KJ908845 and KJ908846 respectively.

Table 1: 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%

3.1. Comparative tolerances of the yeast isolates to varying concentrations of copper

Yeast growth in PDB measured as absorbance at 600 nm showed that yeast growth for all the isolates were significantly higher at 100 ppm, 250 ppm, and 500 ppm (Fig. 1). Looking at the three yeast isolates, *C. parapsilosis* Yph5 consistently had a higher absorbance as compared to the other two in all copper concentrations of copper. It is only in the highest concentration at 2500 ppm where the absorbances measured from each isolate were not statistically different from each other.

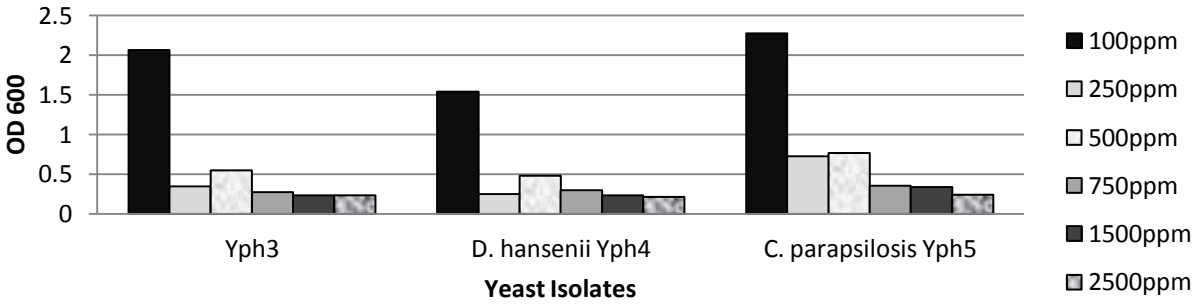

Figure 1. Absorbance at 600 nm indicating growth of endophytic yeasts at specific concentrations of Cu.

3.2. Accumulation of Copper of the Yeast Isolates

Figure 2 shows both the remaining copper in solution after incubation and the computed bioconcentration factors for each of the yeast isolates. The unidentified yeast Yph3 absorbed a small amount of copper from the solution at 5.7%. The highest computed bioconcentration factor was for *C. parapsilosis* Yph5 at 74.1%. *D. hansenii* Yph4 displayed intermediate capacity to accumulate copper at 44.3%. The amount of copper per dry weight of yeast however showed that the isolate that accumulated the most was also *D. hansenii* Yph4 at 9.37 µg Cu/mg (Fig. 3). *C. parapsilosis* Yph5 accumulated 8.57 µg Cu/mg. The amount accumulated by Yph3 was just around 10% of the other two at 0.89 µg Cu/mg yeast dry wt.

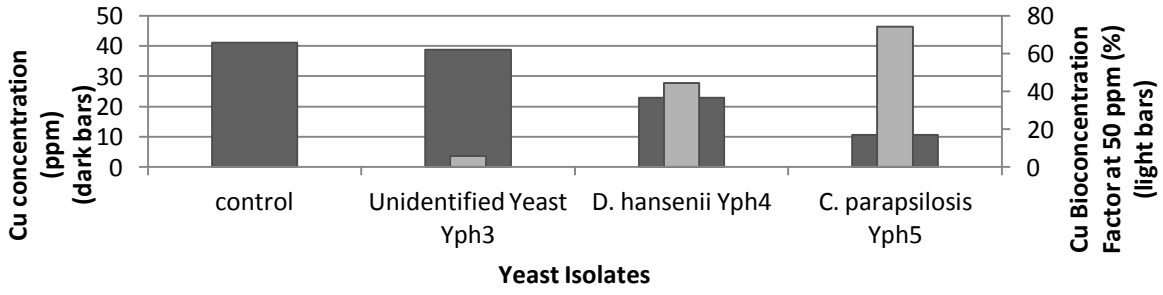

Figure 2. Resultant copper concentration (ppm) remaining in solution and computed bioconcentration factor after 8 days of incubation with the diffferent endophytic yeast isolates.

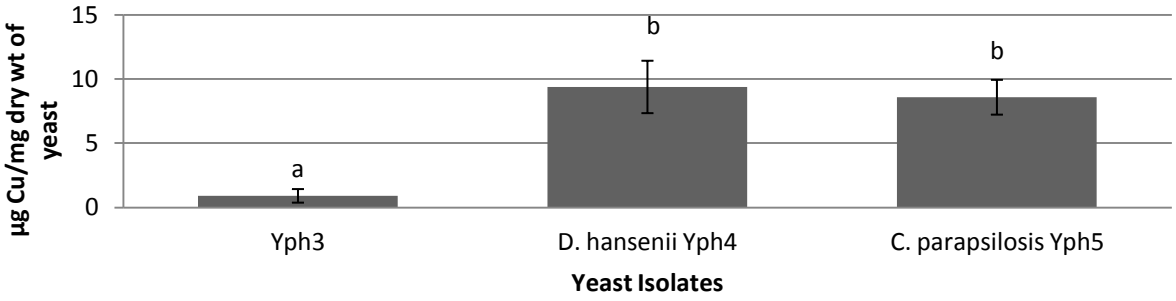

Figure 3. Accumulated copper of the three endophytic yeasts. Error bars are standard deviations. Letters denote homogeneous groups at p=0.05.

3.3. Enzyme Antioxidants: Ascorbate Peroxidase Activity (APX)

Upon exposure to 50 ppm Cu, isolate *C. parapsilosis* Yph5 stood out as a significant producer of APX (Fig. 4). This isolate produced 31.83 U of APX. This was more than 4 times than the next producer which was isolate Yph3. *C. parapsilosis* Yph5 was also more than 44x better than the average of the three lowest producer which *D. hansenii* Yph4. *D. hansenii* Yph4 produced only 0.75 U of APX.

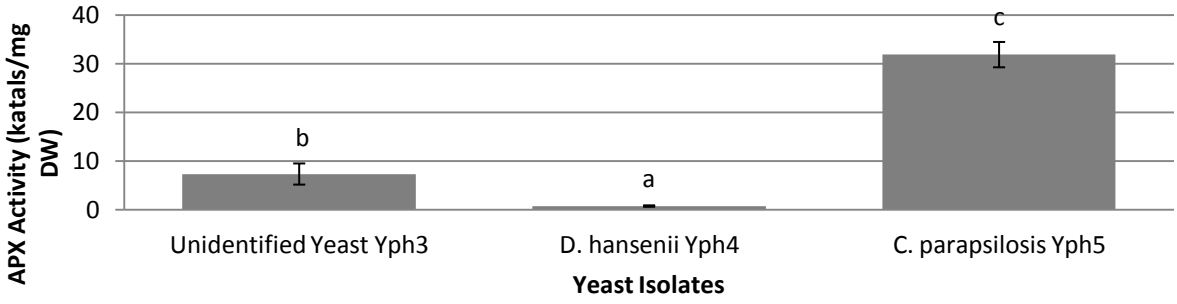

Figure 4. APX activity of the different yeast isolates measured as enzyme units per mg dry wt of the yeasts. Error bars are standard deviations. Letters denote homogeneous groups at p=0.05.

In Fig. 5, the ascorbate peroxidase activity under normal conditions and under 50 ppm Cu concentration for each yeast isolate was compared for the three yeast isolates *D. hansenii* showed a reduction in APX activity under exposure to 50 ppm Cu. On the other hand, the other two isolates Yph3 and *C. parapsilosis* Yph5, expressed an increase of APX activity. The increase in APX activity in *C. parapsilosis* Yph5 was remarkable. The observed activity of APX under 50 ppm copper treatment was 10x compared to APX production without the copper. The measured basal APX activity was 3.02 U and the measured APX activity under 50ppm copper was 31.83 U. Isolate Yph3 increased by only 0.73x.

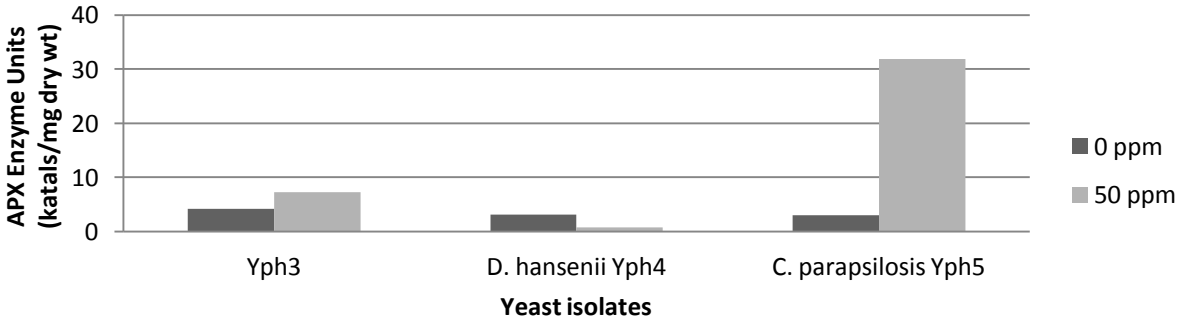

Figure 5. Comparative APX activities of the different endophytic yeast isolates under normal conditions and under 50 ppm Cu concentrations. Error bars are standard deviations. Letters denote homogeneous groups at p=0.05.

3.4. Catalase Activity (CAT)

The yeast isolates were also measured of their catalase activity after exposure to 50 ppm Cu (Fig. 6). It is evident that Yph1 was the best producer of catalase at elevated concentrations of copper; producing 0.34 units of catalase. *D. hansenii* Yph4 and *C. parapsilosis* Yph5 only produced 0.09 and 0.06 units of catalase respectively. Figure 7 shows the catalase activities under normal and under 50 ppm Cu. Only Yph3 showed an increase in catalase activity upon exposure to 50 ppm copper while the other two were observed to have a decrease in this enzyme's activity. The observed increase in Yph3 at 50 ppm copper was about 2.5x the basal catalase activity.

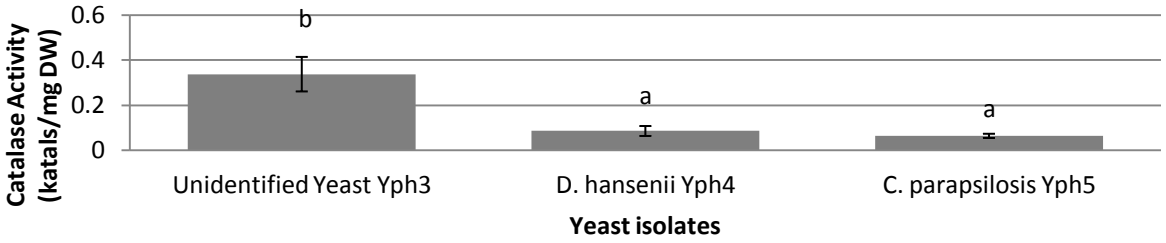

Figure 6. Catalase activity of the different yeast isolates measured as enzyme units per mg dry wt of the yeasts. Error bars are standard deviations. Letters denote homogeneous groups at p=0.05.

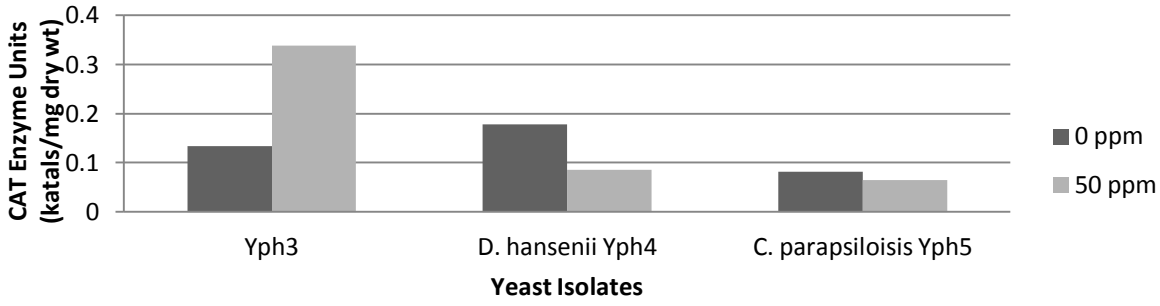

Figure 7. Comparative CAT activities of the different endophytic yeast isolates under normal conditions and under 50 ppm Cu concentrations. Error bars are standard deviations. Letters denote homogeneous groups at p=0.05.

4. DISCUSSION

The results of this research show that yeast endophytes of *P. australis* plants from the Cu stressed tailings pond 5A of LCMC in Mankayan are themselves Cu tolerant. All of them evidently tolerated 500ppm copper concentration. The observed limit of tolerance was about 2x more than the measured Cu concentration in the roots of *P. australis* (at 295 ppm). These results is similar to that of Rajapaksha [20] where he found that a large fraction of culturable fungi was able to tolerate 300 mg Cu/L (~5 mM) in agar media, which were several folds higher than the highest total Cu found in Cu and Zn polluted agricultural soils of Sri Lanka. The highest concentration that a yeast can tolerate is 2300 µg/mL observed in *Candida tropicalis* isolated from the wastewaters of Lahore, Sri Lanka [21]. Another yeast species that was observed to be copper tolerant is *Candida argentea* from an abandoned mine in Cwmystwyth in Wales, UK [22].

The capacity of fungi to tolerate or even thrive in heavy metal polluted soils may be gleaned from their capacity to accumulate these elements within their biomass and as measured with the Bioconcentration Factor. This mechanism is referred to as sequestration [23]. Except for isolate Yph3, all isolates accumulated significant amounts of Cu in their biomass. Of the three yeasts, the best Cu accumulator was *D. hansenii* Yph 4 at 9.38 µg/mg. This value is almost half to the accumulating capacity of *S. cerevisiae* cited by Wang and Chen [23] at 20mg/g yeast dry wt. The Cu sequestering activity of the different yeast isolates in this research can also be appreciated in reference to the accumulating capacity of other fungi as reviewed by Fujii & Fukunaga [1] : *Cryptococcus* sp. up to 35 mg Cu/ mg dry wt yeast; 11.5 mg/g in *Candida* sp.; 10.8 mg/g for *A. flavus* and 12.3 mg/g for *S. cerevisiae*. However, compared with the excellent biosorbent of fungi *Rhizopus* sp. for lead, cadmium, copper, zinc, and uranium (160 to 460 µmol/g), the common yeast *S. cerevisiae*, and possibly the other groups of yeasts, are regarded as 'mediocre' metal biosorbents [23].

As reviewed by Wang and Chen [24] , there may be several ways by which yeasts bioaccumulate copper using *S. cerevisiae* as the example. One mode is by extracellular accumulation/precipitation of Cu. This is where the yeast cells produce or excrete exracellular polymeric substances (EPS), such as polysaccharides, glucoprotein, lipopolysaccharide, soluble peptide etc. These substances possess a substantial quantity of anion functional groups which can adsorb metal ions. Another is cell surface sorption/precipitation. Since the cell wall tends to be the first cellular structure to come in contact with metal ions, HM can attach to the various functional groups (phosphate, carboxyl, amine as well as phosphodiester) the cell wall possesses. The third possible mechanism of metal accumulation is cellular sequestration. This is where the yeasts produce metalloproteins and other metal chelating compounds. These metal-chelate complexes are retained in the cytoplasm as inert inclusion bodies or are transported to storage organelles such as the vacuole. Examples of metal chelating compounds containing thiol (-SH) functional groups which have high affinity for metal ions include glutathione, metallothioneins and phytochelatins [25, 26] . Phytochelatins are involved in the accumulation, detoxification, and metabolism of metal ions such as cadmium, zinc, copper, lead, and mercury [27]. There is evidence that phytochelatin-metal complexes are pumped into the vacuole in fission yeast (*Schizosaccharomyces pombe*) [28]. Other molecules that are involved in HM sequestration are siderophores and organic acids. Most yeasts are capable of producing organic acids such as α-ketoglutaric, pyruvic, isocitric, citric, malic, succinic and acetic acid. Siderophores and organic acids are important in the precipitation of an excess of this metal due to their chelating ability [29].

The primary effect of increased Cu concentrations within the biomass is the generation of reactive oxygen species (ROS) through direct transfer of electrons in single electron reactions [25,30]. This leads to severe damage of cytoplasmic constituents through the oxidation of proteins, cleavage of DNA and RNA, and lipid peroxidation. Copper also binds with high affinity to histidine, cysteine and methionine, resulting to the inactivation of proteins [21]. The extent of injury suffered by cells depends on the rate of ROS generation and the rate at which repair and detoxification is [30].

Defense mechanisms against ROS injury utilize both enzymatic and non-enzymatic systems. In this research, ascorbate peroxidase (APX) and catalase (CAT) activity were assayed in response to 50 ppm Cu stress. Of the three isolates, *C. parapsilosis* Yph5 was the highest producer of APX. The increase in APX in this isolate was10 times more compared to the levels of the enzymes without Cu stress. The observed increase was a response by the yeasts toward increased ROS that may have been produced by the Cu in excess of the normal concentration. Similar response was observed in *Rhodotorula mucilaginosa*. With regard to catalase activity, exposure to 50ppm Cu induced the doubling of activity in the unidentified yeast Yph3. Irazusta *et al*. [31] observed that exposure to elevated Cu concentrations do increase catalase activity. Superoxide dismutase and catalase were also seen to have increased in *Candida fukuyamaensis* and *Rhodotorula mucilaginosa* with exposure to high Cu concentrations [32].

5. ACKNOWLEDGEMENT

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