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Copper Tolerant Yeast, *Candida tropicalis*, Isolated from Industrial Effluents: its Potential use in Wastewater Treatment

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Abstract.- The present study is aimed at assessing the ability of Cu^{2+} resistant yeast, *Candida tropicalis*, to uptake metal from the medium. The minimum inhibitory concentration of Cu^{2+} against *Candida tropicalis* ranged between 2300-2300 µg/mL. The yeast could also tolerate Pb^{2+} (1200 µg/mL), Cr^{6+} (2000 µg/mL), Cd^{2+} (2800 µg/mL), Zn^{2+} (3100 µg/mL), Ni^{2+} (3000 µg/mL) and Hg^{2+} (2400 µg/mL). The yeast isolate showed typical growth curves but lag and log phases extended in the presence of copper. Yeast isolate showed optimum growth at 30ºC and pH 4. Metal processing ability of the isolate was determined in a medium containing 100 µg/mL of Cu^{2+}. *Candida tropicalis* could reduce 74% of copper from the medium after 96 hours and was also capable to remove Cu^{2+} 16%, 20%, 29%, 43%, 46%, 55, and 68% from the medium after 6, 12, 18, 24, 30, 48 and 72 hours, respectively. *C. tropicalis* could also efficiently uptake 82% copper from the medium after 96 hours and was also able to remove Cu^{2+} 64% and 74% from the wastewater after 4 and 8 days, respectively. The metal uptake ability suggests possibility of using this yeast strain for removal of copper from Cu^{2+} contaminated wastewater.

Keywords: Copper, wastewater, *Candida tropicalis*, bioremediation

INTRODUCTION

Heavy metals in the environment have been prioritized as major inorganic contaminants due to their recalcitrance and consequent persistence (Atkinson *et al.*, 1998). The main sources for heavy metal contamination are mining activities and industrial wastewaters, discharging a variety of toxic metals such as Cd, Cu, Ni, Cr, Hg, Zn and Pb into the environment (Soares *et al.,* 2003; Malik, 2004).

Trace amounts of copper are essential for life, copper also catalyses the synthesis of reactive oxygen species, leading to severe damage of cytoplasmic constituents through the oxidation of proteins, cleavage of DNA and RNA, and lipid peroxidation (Halliwell and Gutteridge, 1984; Garcia *et al*., 2002). Copper also binds with high affinity to histidine, cysteine and methionine, resulting in the inactivation of proteins (Camakaris *et al*., 1999). Cu^{2+} is known to be the most commonly used heavy metal and is some of the more widespread heavy metal contaminants of the environment (Dönmez and Aksu, 1999).

Traditional technologies for heavy metals removal such as chemical precipitation, ion exchange, or reverse osmosis processes are very expensive and have several disadvantages, such as unpredictable metal ion removal, high reagent requirements and generation of toxic sludge, which are often difficult to dewater and require extreme caution when disposing of them (Siloniz *et al.*, 2002a). New technologies, like biosorption, are required to reduce heavy metal concentrations to acceptable environmental levels at low costs.

Microorganisms may be used to remediate wastewaters or soils contaminated with heavy metals. The metal processing capacity of microorganisms can be used to concentrate, remove and recover metals from aqueous streams and enhance the efficiency of wastewater treatment processes (Amoroso *et al*., 1998). They have proven capability to take up heavy metals from aqueous solutions, especially when the metal concentrations in the effluent range from less than 1 to about 20 mg/L (Brierley, 1990).

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In a biotechnological context, yeasts may be useful in the metal-containing effluents treatment (Blackwell *et al*., 1995). Metal accumulation bioprocesses generally fall into one of the two categories, biosorptive uptake by non-living or non-growing biomass and bioaccumulation by living cells (Akzu and Donmez, 2001).

Active uptake systems can take up both essential and non-essential metal ions and thus are of interest in bioremoval. The essential characteristics of a living biomass used in a metal ion removal process are tolerance and uptake capacities (Macaskie and Dean, 1989; Aksu, 1998; Suh, 1998). One of the most ubiquitous biomass types available for bioremediation of heavy metals at low pH is yeast. Yeast biomass is an inexpensive, readily available source of biomass. Furthermore, yeast cells retain their ability to accumulate a broad range of heavy metals to varying degrees under a wide range of external conditions.

The aim of this work was to isolate copper resistant yeasts from polluted areas, and to investigate the ability of yeast isolates to remove copper from culture medium and wastewater.

MATERIALS AND METHODS

Sample collection

Wastewater samples were collected in screw capped sterilized bottles from industrial area of Sialkot and Kot lakhpat. Some physicochemical parameters of wastewater *viz.*, temperature (ºC), pH and copper (µg/mL) were measured (APH, 1992).

Isolation of copper resistant yeast

For isolation of copper tolerant yeasts, 100µL of the wastewater sample was spread on YEPD agar plates containing 50µg of Cu^{2+}/mL of the medium. YEPD agar plates were prepared by dissolving 1 g of yeast extract, 0.5 g peptone and 0.2 g glucose in 100 mL distilled water, pH adjusted at 7.2 to 7.5 and then 1.5 g agar was added in the 250 mL flasks. The medium was autoclaved at 121ºC and 15 Lb pressure for 15 minutes. The growth of the yeast colonies was observed after 48 hours of incubation at 30ºC. Isolated colonies were picked up with sterilized wire loop and streaked on YEPD agar medium plate containing 100µg Hg^{2+}/mL. It was again incubated at 30ºC for 48 hours. This process was repeated with successively higher concentrations of Cu^{2+} (150, 200, 250------- 2300µg Cu^{2+}/mL) until the minimum inhibitory concentration (MIC) of each isolate was obtained. The MIC is defined as the lowest concentration of Cu^{2+} at which a single colony-derived streak could not grow.

Physical, biochemical and molecular characterization of the yeast isolate

For biochemical characterization the yeast isolate was tested for colony morphology, spore staining, starch hydrolysis, ester production, nitrate reduction, yeast-malt agar test, citrate utilization, acid production from glucose, ammonia from urea, fermentation of carbohydrates, and tolerance of 1% acetic acid. For physical and biochemical characterization of yeast isolate the criteria adopted by Benson (1994) was followed. For further identification, genomic DNA was isolated and the 18S rRNA gene was amplified by PCR using two general yeast 18S rRNA primers (ITS-5; 5′-GGAAGTAATAACAACG-3′, ITS-4; 5′-TCCTCCGCTTATTGATATGC-3′). The PCR product of 0.58 kb was removed from the gel and cloned in pTZ57R/T vector. The amplified 18S rRNA gene was purified with a Fermentas purification kit and the amplified products were electrophoresed on 1% agarose gel. Sequencing was carried out by Genetic analysis system model CEQ-800 (Beckman) Coulter Inc. Fullerton, CA, USA. The 18S rRNA gene sequences were compared with known sequences in the GenBank database to identify the most similar sequence alignment.

Determination of optimum growth conditions

For optimum growth of the yeast isolate, two parameters *i.e.* temperature and pH were considered. For determination of optimum temperature, 5 mL YEPD broth was added in 5 sets, each of three test tubes, autoclaved and inoculated with 20 µL of freshly prepared culture of yeast isolate. The five sets of tubes were incubated at 20ºC 25ºC, 30ºC, 35ºC and 40ºC. After an incubation of 16 hours, the absorbance was taken at 600 nm.

For determination of optimum pH, test tubes having 5 mL YEPD broth were prepared in 7 sets,

each containing 3 test tubes, and pH was adjusted at 4, 5, 6, 7, 8, 9 and10 then autoclaved. These tubes were inoculated with 20 µL freshly prepared culture of the yeast isolate. After incubation period of 16 hours, the absorbance was taken at 600 nm.

Growth curves of yeast isolate

Growth curves of yeast isolates were determined with (100µg Cu^{2+}/mL) and without copper. For yeast isolate 100 mL YEPD broth was taken in two sets consisting of 3 flasks, autoclaved and then one set (3 flasks, treated) inoculated with 100 µL of the freshly prepared inoculum. The other three flasks (control) were maintained at a concentration of 100µg Cu^{2+}/mL. These flasks were incubated at 30ºC in a shaker at 60-80 rpm. An aliquot of culture was taken out in an oven sterilized tube, at regular intervals of 0, 4, 8, 12, 16, 20, 24, 28, 32, 36 and 48 hours. Absorbance was taken at 600 nm. Growth was plotted graphically.

Cross metal resistance

The cross heavy metal resistance of yeast isolate was determined by using stock solutions of 10 mg/mL of different metal salts such as, lead nitrate, cadmium chloride, copper sulphate, potassium dichromate, mercuric chloride, zinc sulphate and nickel chloride. The cross metal resistance was determined by increasing the concentration of respective metal in a stepwise manner with 100 µg/mL of metal checked resistance. Streaked plates containing metal ions, incubated at 30°C for 48 hours and growth was observed for four days.

Estimation of Cu^{2+} processing ability of the yeast isolate

The metal processing capability of yeast isolate was checked by adding Cu^{2+} at a concentration of 100 µg/mL in the culture medium. The control culture medium was also run for copper containing the same concentration as in treated one *i.e.* 100 µg/mL but was without the yeast isolate. The cultures were incubated for 96 hours and from each medium (control and treated) 5 mL culture was taken out under sterilized conditions after 0, 6, 12, 18, 24, 30, 48, 72 and 96 hours, respectively. The cultures were spun down at 3000 rpm for 5 minutes and the supernatants were used for the estimation of Cu^{2+} by atomic absorption spectrophotometer (Varian, U.S.A) at wavelength 324.7nm. The amount of metal in the supernatants was determined by using standard curve. The percentage reduction in the amount of Cu^{2+} in the medium was calculated.

Bioaccumulation of copper by yeast isolate

The uptake of copper by yeast isolate in YEPD medium was carried out by acid digestion. Copper (100 µg/mL) was added in the culture medium and cells were harvested by centrifugation at 4000 rpm after 96 hours of incubation at 30ºC, washed three times in saline solution. After that, 100 µL of concentrated nitric acid was added to the pellet and mixture was boiled until the cells were completely dissolved (Abe *et al*., 2001). Samples volume was adjusted to 5 mL with distilled water. Metal content of the digest was measured by atomic absorption spectrophotometer (AAS) at 253.7nm. Amount of copper uptake by yeast cells was calculated in µg/mL by using standard curve.

Uptake of copper at large scale

To check the efficacy of yeast cells to remove copper from wastewater a large-scale experiment was set up. Three plastic containers were taken. In first container 10L of tap water was taken along with 1.5L of yeast isolate grown to log phase. In second container 10L of industrial effluent was taken along with 1.5L of 48 hours grown yeast culture. In the third container only 10L of industrial effluent was taken and 100µg/ml of Cu stress was maintained in each container. Experiment was carried out at room temperature (25±2ºC). After four and eight days of incubation samples were taken out, centrifuged to separate the cells, and supernatants used to estimate the amount of Cu^{2+} in wastewater and the quantity removed by the yeast cells.

Statistical analysis

Observations were made and all the experiments run in triplicate. At least three separate

flasks were usually maintained for one treatment. Each time three readings were taken; their mean and standard error of the mean were calculated.

RESULTS

Physicochemical characteristics of industrial wastewater

Some physicochemical characteristics of industrial wastewater were ascertained, from where chromium tolerant bacterium was isolated. The temperature of different samples ranged between 22ºC to 36ºC, pH ranged between 7.5 and 9.0, dissolved oxygen between 0.38±0.03 and 1.35±0.01 mg/L and Cu^{2+} ranging between 1.20±0.04 and 1.80±0.03 µg/mL.

Identification of yeast isolates

The morphological and biochemical characteristics of yeast isolates have been shown in Table I. The partially amplified (580bp) and sequenced 18S rRNA gene from local isolate (CBLYCu2) was blast to check the maximum homology of this gene to confirm the species of the locally isolated yeast. The blast query revealed that this gene is 95% homologous to already reported gene of *Candida tropicalis* (CBLYCu2). The nucleotide sequences coding for 18S rRNA gene of *Candida tropicalis* have been submitted to the GenBank database under accession number EUO17507.

Table I.- Morphological and biochemical characteristics of bacterial isolates.

Characters	*C. tropicalis*
Shape	Round
Size	0.1-0.4 mm
Colour	Off-white
Texture	Dull
Margin	Round
Elevation	Round
Type	Budding
Starch hydrolysis	Negative
Ester production	Positive
Citrate utilization	Negative
Tolerance of 1% acetic acid	Negative
Acid production from glucose	Negative
Production of ammonia from urea	Positive
Nitrate reduction	Positive
Glucose	Positive
Sucrose	Negative
Maltose	Positive

Optimum growth conditions

The most suitable temperature for the copper resistant yeast isolate was found to be 30ºC. Maximum growth for *Candida tropicalis* was observed at pH 4. The growth curve pattern was studied by growing the *C. tropicalis* in the presence of Cu^{2+} (100µg/mL) and comparing with the control culture in which no metal ions were added. Although the growth pattern of the isolate was not significantly different from that of control but the growth of yeast isolate was inhibited in the presence of Cu^{2+}. It is interesting to note that the lag phase of the yeast isolate was extended from 16 hours to 32 hours in cu-treated culture medium. The growth pattern has been shown in Figure 1.

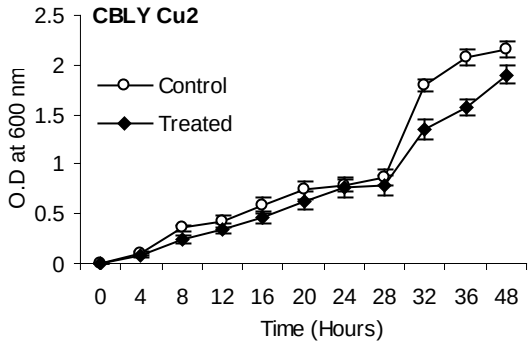

Fig. 1. Growth curves of copper resistant *Candida tropicalis* in YEPD medium containing 100µg Cu^{2+}/mL after incubation at 30°C.

Heavy metal resistance

C. tropicalis was found to be resistant to copper at a concentration of 2300 µg/mL. The yeast isolate was also checked for its resistance to various other heavy metals, *viz.*, chromium, cadmium, mercury, lead, zinc and nickel (Table II). *C. tropicalis* showed maximum resistance against Zn^{2+} at a concentration of 3000 µg/mL and the order of resistance regarding the metal concentration was $Zn^{2+} > Ni^{2+} > Cd^{2+} > Hg^{2+} > Cr^{6+} > Pb^{2+}$.

Metal processing ability

Copper processing capability the yeast isolate was checked by adding Cu^{2+} at 100µg/mL in the culture medium (Fig. 2). *C. tropicalis* could remove74% of copper from the medium after 96 hours of incubation. The yeast was also capable to decrease Cu^{2+} ions by 16%, 20%, 29%, 43%, 46%, 55% and 68% from the medium after 6, 12, 18, 24, 30, 48 and 72 hours, respectively.

Table II.- Cross resistance of copper resistant yeast isolate from industrial wastewater against other heavy metals.

Heavy metals (µg/mL)	*C. tropicalis*
Cr^{6+}	2000
Cd^{2+}	2800
Hg^{2+}	2400
Pb^{2+}	1200
Zn^{2+}	3100
Ni^{2+}	3000

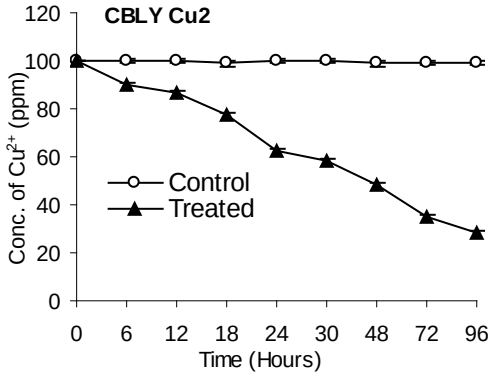

Fig. 2. Biosorption of copper by *Candida tropicalis* in YEPD medium incubated at 30°C.

Copper uptake by yeast isolate

The biosorption term has been used in the present study to indicate that one or more of these processes removed the metal. Metal accumulation bioprocesses generally fall into one of two categories, biosorptive uptake by non-living or non-growing biomass and bioaccumulation by living cells. The amount of copper estimated after acid digestion was 82µg/mL for *C. tropicalis* and the concentration of copper left in the medium was 18 µg/mL (Fig. 3). The percent bioaccumulation was 82 for *C. tropicalis*.

Removal of copper from aqueous solutions at large scale

In order to assess the ability of yeast *C. tropicalis* to remove Cu^{2+} ions in contaminated industrial effluents a large-scale experiment was performed. *C. tropicalis* was observed to remove 64% copper from the wastewater after 4 days and was also able to remove 74 % from the wastewater after 8 days. The yeast took up 50 and 75 µg/mL of the metal from 10Lof water containing 100 µg/mL copper within 4 and 8 days, respectively. The percent removal of $Cu^{\square 2+}$ from water and industrial wastewater by *C. tropicalis*, was 74% and 75%, respectively (Table III).

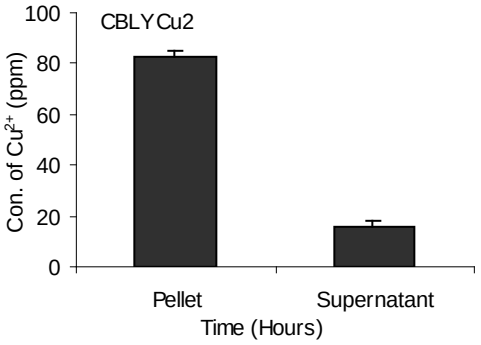

Fig. 3. Amount of copper accumulated by *Candida tropicalis* (pellet) and in the medium (supernatant) after 96 hours of incubation.

Table III.- Removal of Cu^{2+} by yeast isolate with initial concentration of 100 µg/mL of Cu^{2+} in the industrial wastewater at room temperature.

Time	Removal of Cu^{2+} by *C. tropicalis* (µg/mL)		
	Distilled water + H_2O+ Metal	Effluent + Strain	Effluent + Metal
4 days	50	64	30
8 days	75	74	50

DISCUSSION

Isolation of copper tolerant yeast from

polluted areas was performed in order to investigate the ability of the strain to remove copper from the culture medium. In the present study growth rate of yeast isolate in the presence of Cu^{2+} was slightly slower as compared with that of non-treated (control) yeast culture. This happened because of higher concentration of metals that probably poisoned essential biochemical reactions (Perego and Howell, 1997). Growth period was delayed when concentration of heavy metal was increased (Brady *et al.*, 1994). Abe *et al*. (2001) isolated yeasts from deep-sea sediments and studied the mechanisms of the cells for growing in the presence of different concentrations of $CuSO_4$. They also concluded that the decrease of cell viability presumably was due to the considerable increase of copper in the cells.

Removal of heavy metals from wastewater is normally achieved by advance technologies such as ion exchange, chemical precipitation, ultra filtration, or electrochemical deposition do not seem to be economically feasible for such industries because of their relatively high costs. Therefore, there is a need to look into alternatives to investigate a low-cost method, which is effective and economic, and can be used by such industries. More practical methods are being explored. One of these methods is to isolate heavy metal resistant microorganisms as these have evolved strategies to cope up with stressed conditions (Stadler *et al.,* 2004). Bioremediation of heavy metals using microorganisms has received a great attention in recent years for its potential application in industry, as it is nondestructive, cheap and economical (Rise-Roberts, 1998; Rehman *et al.,* 2007).

Copper tolerance and bioaccumulation has been studied in bacteria (Remonsellez *et al.,* 2006; Shakoori and Muneer, 2002), algae (Feng and Aldrich, 2004; Chojnacka *et al*., 2004), fungi (Yan and Viraraghavan, 2003; Dönmez and Aksu, 2001). Different types of plant species successfully used to remove copper ions are reported by many workers (Keskinkan *et al*., 2004; Gardea-Torresdey *et al*., 2004).

In the present investigation budding yeast, *C. tropicalis*, was found to be resistant to copper at a concentration of 2300 µg/mL. *C. tropicalis* showed maximum resistance against Zn^{2+} at a concentration of 3000 µg/mL. The isolate was also found to tolerate Ni^{2+} Cr^{6+}, Cd^{2+}, Hg^{2+} and Pb^{2+} at a concentration of 3000 µg/mL, 2000 µg/mL, 2800 µg/mL, 2400 µg/mL and 1200 µg/mL respectively. The order of resistance regarding the metal concentration was, therefore, Zn^{2+}> Ni^{2+}> Cd^{2+} >Hg^{2+} Cr^{6+}>Pb^{2+}. Abe *et al.* (2001) isolated highly copper-tolerant *Cryptococcus* sp. yeast, with the ability to grow in YEPD broth containing 1 and 10mM Cu^{2+}.

In the present study, *C. tropicalis,* showed fairly high capability to uptake metal from the environment. The percent bioaccumulation of copper was 74%. The strain was also capable to decrease Cu^{2+} ions (100 µg/mL) by 16%, 20%, 29%, 43%, 46%, 55% and 68% from the medium after 6, 12, 18, 24, 30, 48 and 72 hours, respectively. In the same context, Shakoori *et al.* (2005) reported the potential of CMBLY Pb2 to remove 62% of Pb^{2+} within 96 hours in a culture medium containing 100 µg Pb^{2+}/mL. Maximum uptake of copper (0.14 mM) was achieved by *Candida* sp. after 30 hours of cultivation (Villegas *et al*., 2005).

In order to assess the ability of yeast isolates to decrease Cu^{2+} in contaminated industrial effluents a mini large-scale experiment was done. Industrial wastewaters harbor a variety of microorganisms including bacteria, fungi, algae and ciliates. *C. tropicalis* was able to remove 64% copper from the wastewater after 4 days and was also capable to remove 74% from the wastewater after 8 days of incubation at room temperature. Siloniz *et al*. (2002b) described the ability of yeast, isolated from sewage sludge, to take up copper in response to increasing concentrations of this metal in the culture medium. Moreover Balsalobre *et al.* (2003) indicated that both the tolerance to metals and the capacity of the uptake are dependent on ionic metal and yeast species.

During the present study *C. tropicalis* showed good biosorption ability to uptake copper from the medium *i.e.* 74%. Further work is needed to know that what mechanism this yeast employs after up taking the copper from the environment. The copper resistant yeast isolated during the present study showed high level of metal resistance and accumulated substantial amount of Cu^{2+} from the

medium and wastewater and therefore may be applicable for the treatment of heavy metals containing wastewater.

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