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Removal of phenol and zinc by *Candida* isolated from wastewater for integrated biological treatment

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ABSTRACT

The present study aims to evaluate the removal of phenol and zinc by *Candida* strains isolated from sludge and wastewater. It also aims to select candidates for biosorption and bioreduction to improve the traditional treatment process of wastewater and produce reclaimed water for irrigation. Forty-one *Candida* strains (12 *Candida tropicalis*, 13 *Candida lipolytica*, 6 *Candida maltosa*, 10 *Candida parasopilis*) from sludge and wastewater during treatment of sewage water were screened for their Zn biosorption and phenol removing potential. Four *C. tropicalis,* 3 *C. lipolytica*, 2 *C. maltose* and 6 *C. parasopilis* strains demonstrated the highest final population (>7 log CFU/ml) after 3 h of exposure to low pH and temperature. The majority of the tested strains were resistant and exhibited partial phenol degrading up to 700 μg/ml. Scanning electron microscopy of *Candida* indicated that there was neither damage in the cells nor signs of irregular wrinkled outer surface of cells when treated with phenol or Zn compared to the control. These strains are good candidates for further investigation within different sewage treatment plant systems to elucidate their potential biodegradation and bioabsorption of hazard benefits in the system used for treating wastewater.

Keywords: Selection; *Candida*; Phenol; Wastewater; Zinc

1. Introduction

Wastewater poses an environmental hazard as heavy metals, phenolic compounds, organic micropollutants, etc. There are several conventional techniques that have been applied for reclaiming wastewater including membrane separation, chemical precipitation, reverse osmosis, ion exchange, and solvent extraction [1–3]. However, common disadvantages of these methods are secondary pollution, high chemical energy, and cost requirements. So, biosorption and biodegradation are the properties of certain types of inactive, dead, microbial biomass to bind and concentrate heavy metals from even dilute aqueous solutions. Biomaterials including fungal biomass, marine algae, bacteria, yeasts, and waste sludge have been extensively investigated during the last decade for the removal of heavy metals and dyes because of their low cost, high efficiency, reduction in the amount of chemical and biological sludge, regeneration of biosorbent, and the possibility of metal recovery [4].

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Biosorption which is a promising technology for metal pollution control has been investigated for the removal of zinc ions using micro-organisms like *Candida utilis* and *Candida tropicalis* [5]. *Candida* sp. biomass is an effective biosorbents for copper, iron, and zinc and biosorbed about 80% of the metal ions from the effluent [6].

Phenol and chlorophenols are ubiquitous pollutants that contaminate natural water resources from a variety of sources such as wood preservatives, incineration of wastes, pesticides, etc. Besides, they are generated as byproducts during bleaching of pulp with chlorine and during disinfection of water by chlorination [7–9]. Phenols are highly toxic to human beings affecting various biochemical functions. A cute exposure of phenol causes central nervous system disorders leading to collapse, coma, sometimes muscular convulsions, and reduction in body temperature (hypothermia). Inhalation and dermal contact of phenol causes cardiovascular diseases and skin blisters, respectively, while ingestion can cause serious gastrointestinal damage, and oral administration may result in muscle tremors and death [10,11]. Due to these harmful effects, the removal of phenol from industrial wastewater is of great importance. However, the reclamation of polluted areas from organic pollutants such as phenol is difficult because they can't be easily transformed into the non-toxic elements [12]. Therefore, conventional biological treatment methods using activated sludge have been used for the removal of high concentration of phenolic compounds from industrial wastewater [13]. Micro-organisms, e.g. bacteria, fungi, and yeasts, which are able to metabolize phenol can be considered as one of the cheapest and safer alternative approach for the removal of phenol from wastewater [12,14,15]. Enhancing the activity of *Candida* populations and bacterial biodegradation activities is an axial pathway for the removal of phenol from wastewater [16,17]. The purpose of this study is to isolate new strains from *Candida* derived from the sewage sludge and wastewater. Also, to select candidates for biosorption of zinc and bioreduction of phenol to improve the traditional treatment process of wastewater and produce reclaimed water for irrigation.

2. Materials and methods

2.1. Chemicals and strains used

All the chemicals used in this study were of analytical grade while culture media were of biological grade. The stock solution (1,000 mg/l) for zinc ions was prepared from zinc sulfate ($ZnSO_4 \cdot 7H_2O$) using pure distilled water and working solutions of desired concentrations were prepared by the dilution of stock solution. The stock solution (1,500 mg/l) for phenol was prepared from pure phenol (Merck, Germany) using pure distilled water and working solutions of desired concentrations were prepared by the dilution of stock solution. The reference ATCC strains of *Candida albicans* 90028, *C. tropicalis*, *Candida parasopilis* 22017, *Candida krusei* 6258, and *Candida neoformans* 1199 were used in this study.

2.2. Isolation and identification of Candida *spp.*

The *Candida* isolates were obtained from wastewater and sludge samples during a previous research carried out on the microbiological analysis of wastewater and activated sewage sludge from wastewater treatment plant at Zagazig City, Egypt. Briefly, the samples were serially diluted from 10^{-1} to 10^{-5} in sterile saline water, (0.85% NaCl) then 0.1 ml of each dilution samples were spread on *Candida* agar media (Biolife, Milano, Italy), and plates were incubated at 35°C for 48 h. When the cell colonies appeared on *Candida* agar, streaking was repeated on fresh agar to obtain pure cultures. A collection of 41 *Candida* isolates were identified, based on API 20 system (France), as compared with reference strains. Each isolate was identified by API 20 C AUX together with the reference ATCC strains *C. albicans* 90028, *C. tropicalis*, *C. parasopilis* 22017, *C. krusei* 6258, and *C. neoformans* 1199 (bioMérieux S.p.A.), then were verified genotypically according to the method described by Lopandic et al. [18]. Isolation and purification of DNA were performed using the hexadecyl trimethyl (CTAB) method [19]. The purpose of such molecular-based identification was to confirm the fidelity of the phenotypic identification. Two primers ITS3p (5´ GCATCGATGAAGAACGC AGC3´) and NL4 (5´ GGTCCGTGTTTCAAG ACGG3´) were used to amplify a 1 kb fragment of the D1/D2 domain of 26S rRNA according to Lopandic et al. [18]. The PCR program was set as follows: 30 cycles of 98°C/15 s, 59°C/60 s, and 72°C/90 s, with a final extension of 72°C/10 min. A fragment of 600 bp was sequenced using the NL1 (5´GCATATCAATAAGCGG AGGAAAAG3´) and NL4 primers [20]. The sequence was used in a BLAST search in the GenBank, and the identification was based on a minimum identity score of 99%.

2.3. Preparation of inoculums

The yeast cultures were grown in Malt broth (Difco) and the inoculum was prepared at 1% (v/v) from a young growing pure culture at 35°C for 24 h at 150 rpm, when optical density OD_{600} is 1.0, the yeast

cells were then harvested by centrifugation at 10,000 rpm for 10 min at 4˚C. After removing the broth, the cell pellet was rinsed and resuspended in peptone buffer solution (pH 7.4).

2.4. Screening of phenol and zinc tolerance Candida

For selecting phenol and zinc tolerance strains, the *Candida* strains were plated onto yeast nitrogen base agar (YNBA) plates supplemented with phenol (1.0, 2.0, 4.0, 7.0, 10.0, and 15.0 ppm) and glucose (1% w/v) as control and allowed for colony development at 35 ± 1˚C for 48 h. The *Candida* strains were plated onto malt agar supplemented with zinc sulfate concentrations (0.0, 1.0, 3.0, 6.0, 12.0, 24.0, and 30.0 ppm) then allowed for colony development at 35 ± 1˚C for 48 h. Four phenotypically different *Candida* colonies were picked up and purified by repeated plate streak method in the same medium then microscopically examined.

2.5. Effect of phenol on Candida growth

Phenol experiments were conducted to investigate the effect of phenol dosage on *C. tropicalis* or *C. parasopilis* activity. To evaluate the effect of phenol dosage, a contact time of 96 h was selected, and the phenol dosage was changed from 0.0 to 10.0 ppm. The phenol experiments were performed in 100 ml Erlenmeyer flasks with glass stoppers. The flasks were mixed in a constant temperature incubator shaker at 30˚C at 150 rpm. The reported results were the average values of duplicate runs. Samples were periodically (0.0, 6, 12, 18, 24, 48, 72, and 96 h) taken for OD_{600} measured.

2.6. Effect of zinc on Candida growth

Zinc experiments were conducted to investigate the effect of zinc sulfate dosage on *C. tropicalis* or *C. parasopilis* activity. To evaluate the effect of zinc sulfate dosage, a contact time of 96 h was selected, and the zinc sulfate dosage was changed from 0.0 to 1.0, 3.0, and 6.0 ppm. This experiment was performed in 100 ml Erlenmeyer flasks with glass stoppers. The flasks were mixed in a constant temperature shaking incubator at 30˚C at 150 rpm. The reported results were the average values of duplicate runs. Samples were periodically (0.0, 6, 12, 24, 48, 72, and 96 h) taken for counting the viable cells onto malt agar (log CFU/ml).

2.7. Scanning electron microscopy

Scanning electron microscopy (SEM) analysis was conducted for further exploration of the mode of action of the phenol on *Candida* cell morphology. An aliquot of 0.1 ml of *C. tropicalis* culture was inoculated into 10 ml malt broth and cultivated at 30˚C with gentle agitation for 24 h. The cells were collected at 5,000 rpm for 4 min at 4˚C. Cells were washed three times and re-suspended in phosphate buffer (pH 7.2) at the same volume. The phenol (0.0, 1.0, and 7.0 ppm) was added to the cell suspension and incubated at 30˚C with gentle agitation for 6 h. The control sample was prepared similarly but without adding phenol. Yeast cells were recovered by centrifugation at 5,000 rpm at 4˚C, washed with phosphate buffer (pH 7.2), and fixed in 2.5% glutaraldehyde in phosphate buffer. The fixed yeast pellet was then dehydrated in graded alcohol series, dried, and mounted onto stubs using double sided carbon tape, coated with a thin layer of gold. All cell samples were examined by scanning electron microscope (JEOL-SEM, Japan).

2.8. Statistical analysis

The data shown in the corresponding figures were the mean values of the experiments. All analyses were performed in three replicates. Data were statistically analyzed using ANOVA through the general linear models procedure of the statistical analysis system software (SAS version 9.1, SAS Institute, Inc., 2003). Least significant differences were used to separate means at $p < 0.05$.

3. Results and discussion

3.1. Strains identification

The quantitative analysis of *Candida* in wastewater samples showed that the quantity of *Candida* was very different and oscillated in untreated wastewater from 36×10^3 to 67×10^4 CFU/ml, in treated wastewater from 31×10^2 to 55×10^3 CFU/ml, and finally in sewage sludge from 30×10^3 to 43×10^3 CFU/g of dry solids. The pure cultures of *Candida* were identified by API 20 C AUX together with the reference ATCC strains. These purified *Candida* strains were identified as *C. tropicalis*, *C. parasopilis*, *Candida lipolytica*, and *C. maltose*. In primary experiment, a total of 4 *C. tropicalis*, 3 *C. lipolytica*, 2 *C. maltose*, and 6 *C. parasopilis* strains demonstrated the highest final population (>7 log CFU/ml) after 3 h of exposure to low pH and temperature. These *Candida* strains which were found capable of growing at higher phenol concentration were selected as the most potential strain for phenol degradation and zinc biosorption.

The comparative qualitative analysis showed that the most quantitative species in occurrence were

C. tropicalis and *C. parasopilis* occupying about 30% in all *Candida* isolated from untreated wastewater, treated wastewater, and sludge. The other species *C. lipolytica*, *Candida maltosa*, *C. albicans*, *C. krusei*, and *Geotrichum* (*G. candidum*) occupied about 70% in all *Candida* isolated from untreated wastewater, treated wastewater and sludge.

3.2. *Phenol tolerance pattern for* Candida *isolates*

Forty-one *Candida* strains (12 *C. tropicalis*, 10 *C. parasopilis*, 13 *C. lipolytica*, and 6 *C. maltose*), isolated from sludge and wastewater during treatment of sewage water, were screened for their phenol which is used as a source of carbon on YNBA plate supplemented with 1.0, 2.0, 4.0, 7.0, and 10.0 ppm phenol concentration. All *Candida* strains utilized glucose (1% w/v) and phenol (1.0 ppm) as carbon and energy sources showing fast and luxuriant growth onto agar plates (Table 1). But, no growth was observed in all the four *Candida* strains in presence of 15.0 ppm of phenol. Moreover, *C. tropicalis* and *C. parasopilis* strains showed fast and luxuriant growth up to 4.0 ppm phenol concentration (Table 1), whereas *C. lipolytica* and *C. maltose* strains showed very slow growth up to 4.0 ppm phenol concentration. Simultaneously, *C. parasopilis* and *C. tropicalis* also showed very slow growth up to 10.0 ppm phenol concentration. However, *C. lipolytica* and *C. maltose* showed no growth at 7.0 ppm phenol concentration. This might be due to the inhibitory effect of phenol on microbial growth at higher concentrations. So, the *Candida* strains could use either glucose or phenol as carbon and energy sources [16]. These results indicated that *Candida* strains have potential for phenol degradation and hence, these *Candida* strains could be used in further studies for phenol degradation in axenic and mixed culture conditions.

3.3. *Zinc tolerance pattern for* Candida *isolates*

Forty-one *Candida* strains (12 *C. tropicalis*, 10 *C. parasopilis*, 13 *C. lipolytica* and 6 *C. maltose*), isolated from sludge and wastewater during treatment of sewage water, were screened for their growth in presence of different concentrations of zinc sulfate (0.0, 1, 3, 6, 12, 24, and 30 ppm) on malt agar plate. All *Candida* strains grew fast and showed fast and luxuriant growth onto agar plates with concentrations 0.0, 1.0, and 3.0 ppm zinc sulfate (Table 1). But, no growth was observed in all the four *Candida* strains having 30 ppm of zinc sulfate. Moreover, *C. tropicalis* and *C. parasopilis* strains showed very slow growth up to 24 ppm zinc sulfate concentration (Table 1), whereas *C. lipolytica* and *C. maltose* strains showed very slow growth up to 12 ppm zinc sulfate concentration. Simultaneously, *C. parasopilis* and *C. tropicalis* also showed slow growth up to 12 ppm zinc sulfate concentration. However, *C. lipolytica* and *C. maltose* strains showed no growth at 24 ppm zinc sulfate concentration. This might be due to the inhibitory effect of heavy metal on microbial growth at higher concentrations indicating that these strains may be able to tolerate high zinc sulfate concentration. Hence, immobilized biomass of *C. utilis* and *C. tropicalis* strains might be used as biosorbents of heavy metals. *C. utilis* showed better biosorption capacity for zinc ions compared to *C. tropicalis* and it was also better than all other types of microbial biosorbents reported in the literature [5]. These results

Table 1
Phenol and zinc tolerance pattern for *Candida* isolates cultured form wastewater

Strains	Glucose in YNBA 1%	Phenol concentrations onto YNBA (ppm) 1	2	4	7	10	15
Ct ($n = 12$)	+++	+++	+++	++	+	+	–
Cp ($n = 10$)	+++	+++	+++	++	+	+	–
Cl ($n = 13$)	+++	+++	++	+	–	–	–
Cm ($n = 6$)	+++	+++	++	+	–	–	–
	Zinc sulfate concentrations onto malt agar (ppm)						
	0	1	3	6	12	24	30
Ct ($n = 12$)	+++	+++	+++	++	++	+	–
Cp ($n = 10$)	+++	+++	+++	++	++	+	–
Cl ($n = 13$)	+++	+++	+++	++	+	–	–
Cm ($n = 6$)	+++	+++	+++	++	+	–	–

Notes: +, very slow growth; ++, slow growth; +++, fast and luxuriant growth; –, no growth; Ct, *Candida tropicalis*; Cp, *Candida parasopilis;* Cl, *Candida lipolytica;* Cm, *Candida maltose*; YNBA, yeast nitrogen base agar.

indicated that *Candida* strains have potential for biosorption of zinc from aqueous solutions and hence, these *Candida* strains could be used for further studies in zinc biosorption in axenic and mixed culture conditions.

3.4. *Growth of* C. tropicalis *and* C. parasopilis *in phenol*

Fig. 1 shows that the biomass concentration measured as optical density (OD_{600}) continuously increased, indicating the growth of *C. tropicalis* or *C. parasopilis* strain in the liquid medium containing different concentrations of phenol. In these experiments, phenol was added to the YNB medium in a concentration range of 1.0, 2.0, 4.0, 7.0, and 10.0 ppm phenol concentrations. The control experiment was conducted by adding glucose (1% w/v) to the same medium. It could be seen that the existence of low-concentration phenol did not inhibit significantly ($p < 0.05$) the growth of *C. tropicalis* or *C. parasopilis*. Furthermore, by the increase of initial phenol concentration, the strains were able to grow on the liquid medium until it was raised up to 10.0 ppm phenol concentration. It was demonstrated that the cells grown in the sample with 1.0, 2.0, and 4.0 ppm phenol got rid of the lag phase of the cell growth stages rapidly. As seen in Fig. 1, 7.0 and 10.0 ppm phenol in the medium resulted in a slight growth compared with the control and the maximum OD_{600} which was 0.43–0.56 after 96 h, respectively. However, an addition of 1.0, 2.0, and 4.0 ppm phenol to the medium resulted in an increase significantly ($p < 0.05$) of the cell concentration to about 0.8–1.0 OD_{600} after 96 h in case of *C. tropicalis*. Phenol, as a growth substrate, supplied carbon and energy source for *C. tropicalis* and *C. parasopilis* and was easily utilized to synthesize the new cells. *C. tropicalis* could degrade 2,000 mg/l phenol alone within 66 h [21]. On the contrary, the phenol of low concentration from 1.0 to 4.0 ppm supplied a sole carbon and energy source for *C. tropicalis* and *C. parasopilis* in the initial phase of biodegradation without any addition of glucose.

3.5. *Growth of* C. tropicalis *and* C. parasopilis *in zinc solution*

The zinc sulfate concentration effect (0–12.0 ppm) on the growth of the two *Candida* strains (*C. tropicalis* or *C. parasopilis*) as compared with the control (without zinc sulfate) was also evaluated during 96 h of incubation at 35˚C using malt broth (Fig. 2). Concentration-dependent effect can be observed in all cases. It can be seen from Fig. 2 that 6.0 ppm in the medium resulted in a slightly decreased cell concentration of growth compared with controls grown without metal (2.63–3.53 log CFU/ml and 7.95–8.29 log CFU/ml, respectively) after 96 h. However, an addition of 1.0 ppm zinc sulfate to the medium resulted in an increase of the cell concentration compared to the control from 5.94 to 6.07 log CFU/ml and from 7.95 to 8.29 log CFU/ml, respectively after 96 h. Growth of *C. tropicalis* or *C. parasopilis* in a medium with 20.0 ppm zinc sulfate was strongly inhibited. *C. utilis* and *C. tropicalis* were used as biosorbents of heavy metal from aqueous solutions. *C. utilis* showed better biosorption capacity for zinc ions compared with *C. tropicalis*. Furthermore, it was also better than all other types of microbial biosorbents reported in the literature [5]. So, yeasts could be used as a biosorbent of heavy metals. These micro-organisms essentially contain various organic compounds such as acidic polysaccharides, lipids, amino acid, chitins, and other

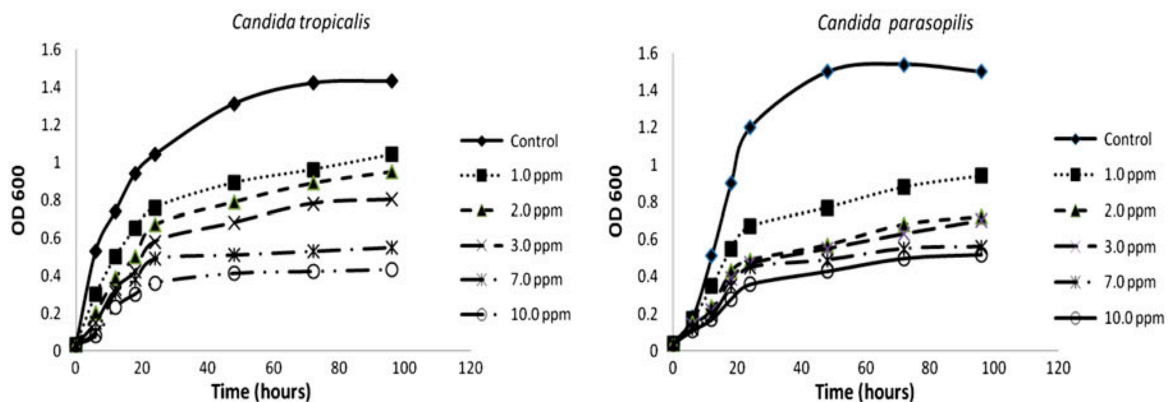

Fig. 1. Effect of different concentration of phenol compared on *C. trpicalis* and *C. parasopilis*. $OD_{600} = 1.0$, corresponding to 0.2 g dw biomass/l.

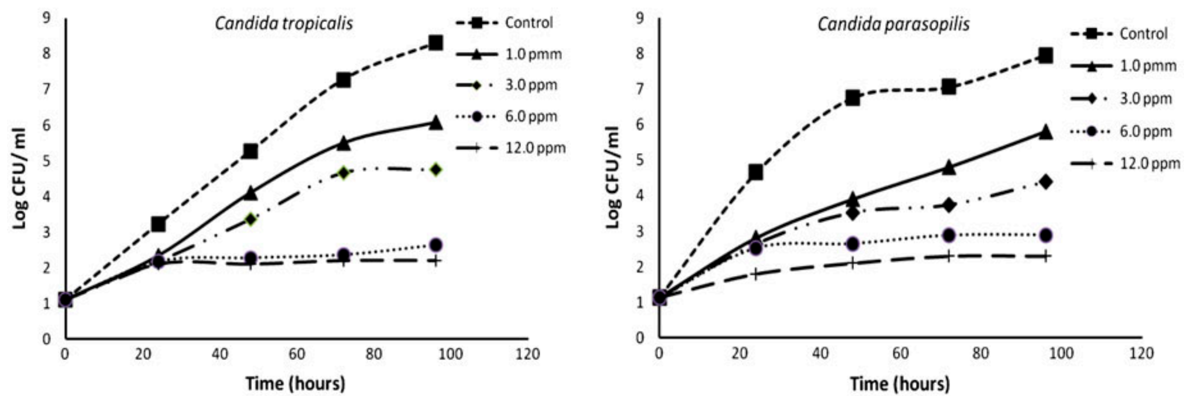

Fig. 2. Effect of zinc (zinc sulfate) on *C. trpicalis* and *C. parasopilis* count (log CFU/ml). Metal was added to the medium in a concentration range of 100–600 μg/ml zinc sulfate.

cellular components. These materials interact with chemicals resulting in a passive uptake of metals from aqueous solution to the microbial cells. The main bio-absorption mechanisms appear to be extracellular interactions, complexion and subsequent accumulation, passive sorption at binding sites on the envelope of cells, and intercellular accumulation [22].

3.6. Scanning electron microscopy

The phenol (0.0, 1.0 and 7.0 ppm) was added to the cell suspension and incubated at 35˚C with gentle agitation for 6 h. SEM at a magnification of 5.000 or 7.500 or 20,000× showed that the *Candida* was morphologically oval or spherical in shape in treated and untreated cells, as demonstrated in Fig. 3, with lengths and diameters reaching 4 μm and 6 μm, respectively, with no cell morphological changes. As it is seen the increasing concentration of phenol (1.0 and 7.0 ppm) does not affect the external shape of the cell and the cell has retained the form which proves that they are still viable. SEM images of *Candida* indicated that there was neither damage in the cells nor signs of irregular wrinkled outer surface of cells when treated with

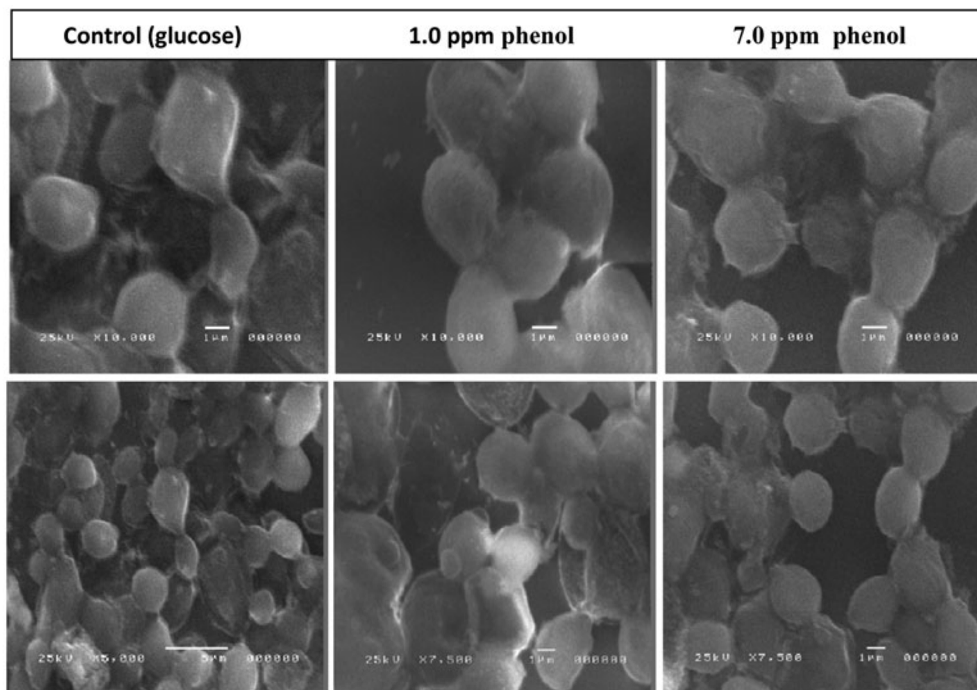

Fig. 3. SEM of *C. tropicalis* treated with 100 or 700 μg/ml of phenol.

phenol up to 7.0 ppm compared with the control. These strains are good candidates for further investigation within different systems of treatment to elucidate their potential biosorption and bioreduction of hazard benefits in the system used for treating wastewater.

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