RESEARCH ARTICLE

Copper Tolerance of Novel *Rhodotorula* sp. Yeast Isolated from Gold Mining Ore in Gia Lai, Vietnam

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ABSTRACT

In this study, twenty-five yeast strains were isolated from soil samples collected in the gold mining ore in Gia Lai, Vietnam. Among them, one isolate named GL1[T] could highly tolerate Cu^{2+} up to 10 mM, and the isolates could also grow in a wide range of pH (3–7), and temperature (10–40 °C). Dried biomass of GL1 was able to remove Cu^{2+} effectively up to 90.49% with a maximal biosorption capacity of 18.1 mg/g at pH 6, temperature 30 °C, and incubation time 60 min. Sequence analysis of rDNA indicated this strain was closely related to *Rhodotorula mucilaginosa* but with 1.53 and 3.46% nucleotide differences in the D1/D2 domain of the 28S rRNA gene and the ITS1-5.8S rRNA gene-ITS2 region sequence, respectively. Based on phylogenetic tree analysis and the biochemical characteristics, the strain appears to be a novel *Rhodotorula* species, and the name *Rhodotorula aurum* sp. nov. is proposed. This study provides us with more information about heavy metal-tolerant yeasts and it may produce a new tool for environmental control and metal recovery operations.

1. Introduction

The rapid development of industry leads to huge amounts of waste, including heavy metals, being disposed of in the environment [1]. Heavy metals are considered among the most toxic and persistent environmental contamination agents. Copper is a critical metal and is extensively used in industries, leading to a hazardous environment of copper contaminant [1]. Copper is an essential trace element that is involved in the function of many cellular enzymes as a cofactor [2]. However, when copper concentration is higher than the tolerance contents of cells, it can be toxic and a threat to the organisms since copper catalyzes the production of reactive oxygen species, causing serious damage to cytoplasmic components through the oxidation of proteins, split DNA and RNA, and lipid peroxidation [3]. Moreover, under anaerobic conditions, copper is also deleterious for microorganisms including yeast [4]. Also, excessive exposure to copper has been linked to pathologies, such as Alzheimer's disease [5] and Wilson's disease

[6] in humans. To overcome the problem, copper ions must be removed from contaminated wastewater.

It is known that heavy metal-tolerant microorganisms such as those that have been isolated from areas contaminated by heavy metals are generally excellent candidates for studying and applying bioremediation, as a sustainable technology for the environmental treatment of heavy metal pollution.

Consequently, several heavy metal tolerant yeasts have been isolated and used for removing heavy metals including copper from industrial wastewater and/or contaminated water, such as *Cryptococcus* sp. [7], *Pichia guilliermondii* [8], *Rhizopus stolonifera* [9], *Rhodotorula mucilaginosa* [10], *Loddermyces elongisporus* [11].

Whilst identifying microorganisms for copper removal, this study, isolated novel copper-tolerant yeast from gold mining ore in the Gia Lai province of Vietnam. Which belongs to the *Rhodotorula* genus, but 9, and 22 nucleotides differ in the D1/D2 region of the ribosomal large subunit, and the ITS (internal transcribed spacer) region, respectively. This paper

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describes it as a new species, *Rhodotorula aurum* sp. nov., and describes its properties in the removal of copper.

2. Materials and methods

2.1. Sample collection and yeast isolation

Five samples were collected from the gold mine area located in Gia Lai province of Vietnam, with approximately 20 g of soil obtained in sterile plastic bags. Samples were then suspended in enrichment media containing yeast extract 0.5%, glucose 0.5%, NaCl 0.05%, and 0.01% of each $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4$, and $CaCl_2$. After shaking incubation for 72 h at 30 °C, the suspension was plated on YM agar (1% malt extract, 0.5% yeast extract, 0.3% peptone, 2% glucose, and 1.5% agar) supplemented with 50 mg/L copper, plates were then incubated for 3 days at 30 °C.

The morphological, biochemical, and physiological characterizations of isolated yeasts were described according to standard methods reported previously [12]. Images of colony morphology were taken using a Canon camera (model EOS 5D Mark IV; Canon, Tokyo, Japan). Cell shape and morphology were photographed by light microscopy (CX33, Olympus, Tokyo, Japan). The ability of yeast cells to assimilate carbon and nitrogen sources was evaluated according to a previous study [13].

2.2. Phylogenetic analysis

For yeast identification, one colony of each isolate was picked and inoculated overnight in a YD medium (1% yeast extract, 1% peptone, and 2% glucose). Yeast cells were collected by centrifugation and used for genomic DNA extraction using the Favorgen DNA extraction kit (Favorgen, Ping Tung, Taiwan) according to the manufacturer's instructions. Subsequently, the internal transcribed spacer (ITS) 1 and 2 regions of the ribosomal DNA, and D1/D2 domains of the large subunit RNA gene were amplified using primers as previously described [14]. PCR was carried out in an MJ mini thermal cycler (Biorad, Hercules, California) using OneTaq® 2X master mix with standard Buffer (New England Biolabs, Ipswich, Massachusetts) in a final volume of 25 μL containing 1 μL of each primer (10 pM), 1 μL of genomic DNA, and 9.5 μl of nuclease-free water. The PCR program was an initial denaturation for 5 min at 95 °C; 35 cycles of denaturation at 95 °C for 30 s, annealing at 54 °C for 30 s, and 72 °C for 30 s, and final extension at 72 °C for 5 min. The PCR products were further verified by being subjected to 1.5% agarose gel electrophoresis and stained with SYBR^{TM} Green I (Invitrogen,

Waltham, Massachusetts) for visualization under the MUPID® One LED Illuminator (Nippon genetics Europe, Duren, Germany). Amplicons were then purified using the AccuPrep® PCR/Gel Purification (Bioneer, Daejeon, Korea) and sequenced by Macrogen Inc. (Seoul, South Korea). Sequences were then aligned and compared with known sequences in the Genbank using the Basic Logarithmic Alignment Search Tool (BLAST) algorithm in the National Center for Biotechnology Information (NCBI) database. Multiple sequence alignment was carried out using the MUSCLE algorithm. Phylogenetic tree based on the D1/D2 domains of the large subunit RNA gene using the maximum likelihood (ML) method was constructed by MEGA11 [15] with bootstrap values based on 1000 random resampling to determine the exact taxonomic positions of isolated yeast. Our sequence and the references of sequences used for phylogenetic analysis in this study are shown in Table S1.

2.3. Determination of copper tolerance by yeast isolates

The copper tolerance of yeasts was examined by allowing yeast growth in flasks containing 100 mL a YNB glucose medium (YNB 0.7%, glucose 0.5%) supplemented with different concentrations of Cu^{2+} ($CuSO_4 \cdot 5H_2O$). Overnight cultured yeast cells were added to flasks (to a final concentration of 10^5 cells/ mL) and incubated at 30 °C for 72 h in a shaking incubator at 150 rpm. The kinetic growth of yeast was checked by measuring optical density at 600 nm on a spectrophotometer (Optizen POP UV-VIS Spectrophotometer, K LAB, Daejeon, Korea). OD_{600} was also used to check the kinetic growth of yeast in a YNB glucose medium with different pH ranges of 3–8 at 30 °C, and in a range of different temperatures (10–40 °C). All experiments were carried out in triplicate.

2.4. Biosorption assay

The effects of initial pH (2, 3, 4, 5, 6, 7) and temperature (20, 30, 40, 50 °C) on the Cu^{2+} removal efficiency were investigated according to a previous study [16]. In brief, yeast isolate was cultured in a YM medium for 3 days at 30 °C, the cells were then collected by centrifugation and washed thoroughly with deionized water before being dried at 80 °C for 48 h. Dried biomass was subsequently ground and used as adsorbents. Biosorption was performed under the following conditions: 1 g of biomass was exposed to 200 mL of solution containing 100 mg/L of $CuSO_4 \cdot 5H_2O$ in flasks at optimal pH and temperature, and shaken at 150 rpm. After different

interval times (5, 10, 20, 40, 60, 90, and 120 min) of incubation, 0.5 mL of the mixture was collected and centrifuged at 10,000 rpm for 10 min. The residual Cu^{2+} content was analyzed by atomic adsorption spectroscopy (ContrAA 800D, Analytik Jena, Germany). The Cu^{2+} binding capacity to biomass was calculated as follows: $q = (C_o - C_t)^*V/m$, where C_o is the initial Cu^{2+} concentration (100 mg/L); C_t is the final metal ion concentration (mg/L); V is the volume of the reaction solution (in mL); m is yeast biomass dry weight (g); and q is mg of Cu^{2+} adsorbed per g of biomass.

3. Results and discussion

3.1. Yeast isolation

After 4 days of enrichment culture, 25 yeasts were isolated from 5 samples. One isolate named GL1^T was selected according to its ability to tolerate high Cu^{2+} concentration. Sequence analysis of the D1/D2 domain of the 28S rRNA gene showed that the GL1^T strain was closely related to *Rhodotorula mucilaginosa* but with 1.53% nucleotide differences (8 substitutions and 1 gap out of 586 nucleotides). In the same way, the ITS1-5.8S rRNA gene-ITS2 region sequence of the strain GL1^T differed from *R. mucilaginosa* by 22 nucleotides (19 substitutions and 3 gaps out of 636 nucleotides). These conserved sequences were submitted to Genbank with accession numbers OP214778, and OQ073533, respectively.

Kurtzman et al. (1998) [14] indicated that yeast strains exhibiting nucleotide substitutions >1% of the D1/D2 domain usually are considered different species. Results from the phylogenetic tree on the basis of the D1/D2 sequence indicated that GL1 belongs to the *Rhodotorula* species and that is a new sister species of *R. mucilaginosa* (Figure 1). In addition, it can assimilate several hydrocarbon sources including glucose, fructose, maltose, trehalose, glycerol and soluble starch. But in contrast to *R.*
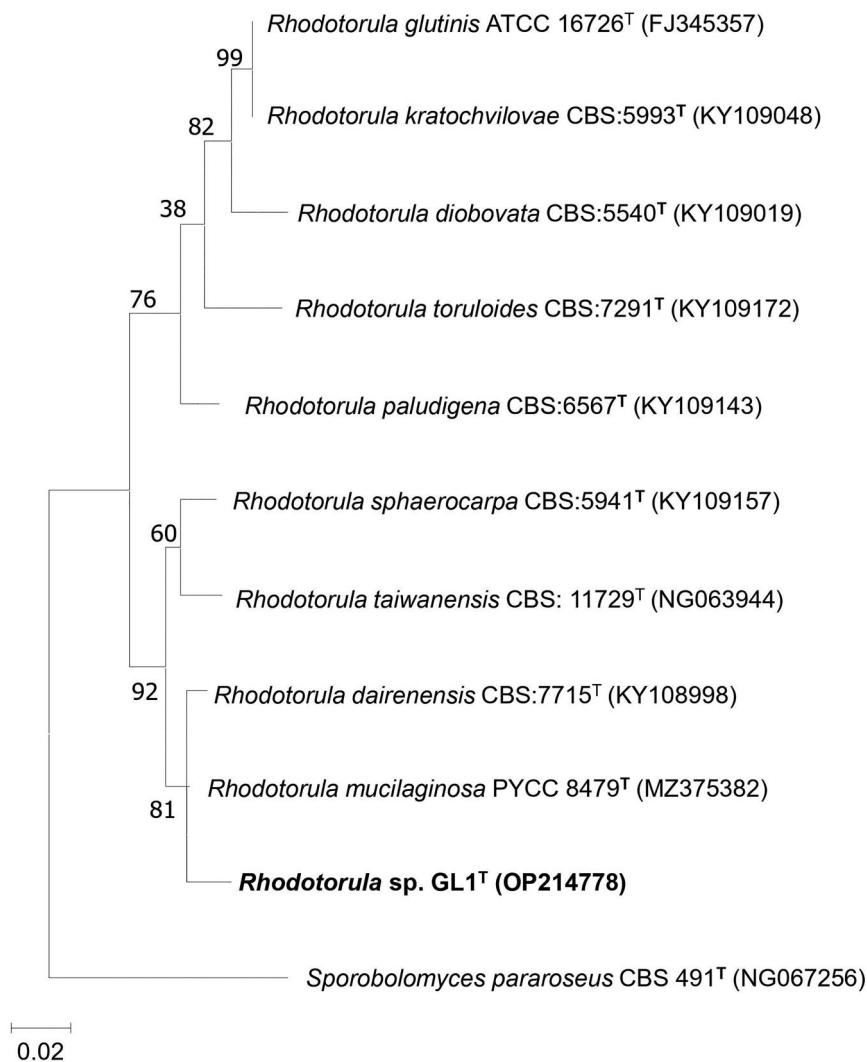

Figure 1. Phylogenetic tree showing the position of *Rhodotorula* sp. GL1 (*Rhodotorula aurum* sp. nov.) and their related species constructed by the maximum-likelihood method based on the D1/D2 nucleotide sequences. The numbers given at the branching points indicate levels of bootstrap support (%) with which a given branch appeared in 1000 bootstrap replications. Only values >50 are shown. *Sporidiobolus pararoseus* CBS 491 (NG 067256) was used as an outgroup. Bar, 0.02 nucleotide substitution rate. The GenBank accession numbers are reported in brackets.

mucilaginosa, *Rhodotorula* GL1 was able to assimilate ethanol and inositol, but it could not assimilate citrate, arabinose, and xylose (Table 1).

According to the findings presented here, we propose that *Rhodotorula* sp. GL1^T is a novel species in the *Rhodotorula* genus and is named *Rhodotorula aurum* sp. nov. (aurum in Latin meaning gold).

3.2. Phenotypic and physiological characteristics

Yeast colonies *Rhodotorula aurum* sp. nov. strain were glistening, butyrous to mucoid, smooth with entire margins, and red-pigmented white at 3 days old on PDA agar. Yeast cells are ovoid to elliptical in shape with size in the range of 4–7 μm, and budding cell is commonly unipolar (Figure 2). Moreover, yeast cell didn't produce spores, hyphae, and pseudohyphae after 21 days at 25 °C in yeast sporulation medium (Formedium, Norfolk, UK) or in corn meal agar (Himeidia, Mumbai, India). *Rhodotorula aurum* sp. nov. could grow at temperatures ranging from 10 to 40 °C (Figure S1A). The optimal temperature was observed at 30 °C since growth performance reached the maximum, whereas at 45 °C no growth was observed (data not show), and poor growth was found at 10 and 40 °C. Similar results were also observed in the study of Mundra

et al. (2011) [18], who reported that *Rhodotorula* sp. PS4 could grow at temperatures in the range of 5–40 °C. While the ability to grow at different temperatures in *R. calyptogenae* and *R. minuta* was varied, some strains could grow at temperatures above 35 °C, others were not able to grow at this temperature [19].pH is a critical factor for yeast growth, most of yeasts grow well at a pH of between 4.5 and 6.5, but some species can grow in a more acidic or alkaline medium [20]. In agreement with this, *Rhodotorula aurum* sp. nov. grew well in the pH range of 3–7, and the optimal pH for yeast growth was determined at pH 5-6 (Figure S1B). The strain was able to grow at pH 8, but the growth kinetic was significantly decreased compared to that in the pH range of 3–7. Similar results were also observed in previous studies, in which pH 5.5 was determined as the optimal pH for *Rhodotorula* sp. RY1801 [21], *R. acheniorum* [22], and *R. glutinis* [23]. A higher pH (7) for *Rhodotorula* sp. PS4 growing and dissolving phosphate was determined [18].

The results of biochemical tests indicate the similarity of *Rhodotorula aurum* sp. nov. and other strains, in that *Rhodotorula aurum* sp. nov. can assimilate many different sugars, but could not utilize xylose, arabinose, and citrate (Table 1).

Table 1. Biochemical characteristics of *Rhodotorula* sp. GL1 compared to other *Rhodotorula* species.

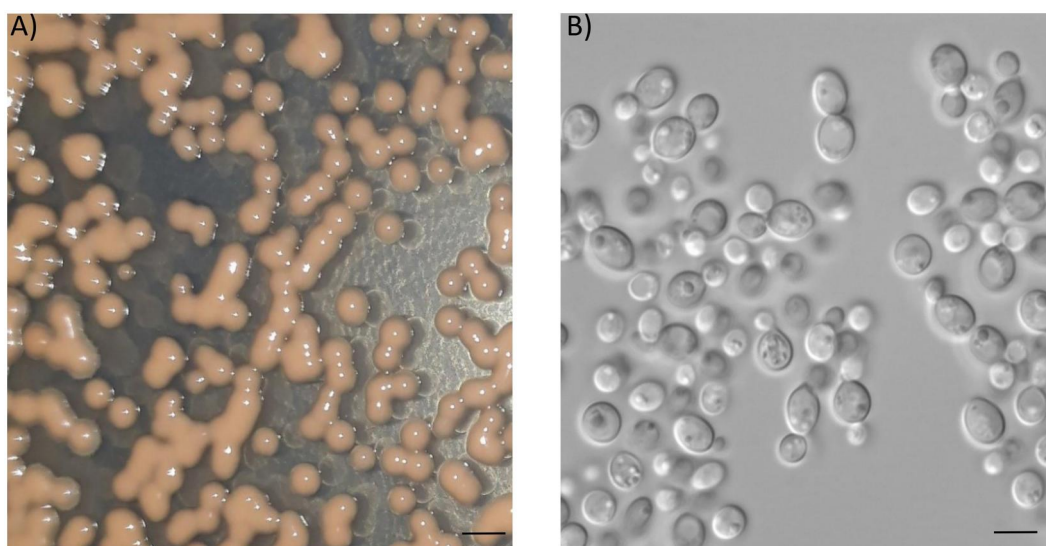| Assimilation reactions	*Rhodotorula* sp. GL1	*R. mucilaginosa* [17]	*Rhodotorula* sp. RY1801 [20]
Raffinose	+	−	+
Xylose	−	+	+
Inositol	+	−	−
Ethanol	+	−	−
Cellobiose	+	−	+
Arabinose	−	+	+
Citrate	−	+	+
Urea	+	+	+

Figure 2. Morphology of *Rhodotorula aurum* sp. nov. (A) Colonies grown on YM plate for 2 days. Bar 1 mM. (B) Cells under microscopy (magnification of 100×). Bar 10 μM.

3.3. Effect of copper on kinetic growth of Rhodotorula aurum sp. nov.

The growth of *Rhodotorula aurum* sp. nov. in YNB glucose containing different copper concentrations varied, at low concentrations (0.5–2 mM) Cu^{2+} *Rhodotorula aurum* sp. nov. could grow well as a control (without copper). However, the growth ability of yeast cells was severely affected when copper was above these concentrations by extending the lag phase and reducing the maximal OD_{600} it could reach (Figure 3(A)). These results are in agreement with previous studies, which suggested that high concentrations of heavy metals cause stress for yeast cells, prolonging lag phage extension or decreasing specific growth rate [24,25]. As Figure 3 indicates *Rhodotorula aurum* sp. nov. could grow in a medium containing 10 mM copper. This finding is consistent with that of Villegas et al. (2005) [26], who found *R. mucilaginosa* RCL-11 could grow in a medium supplemented with 10 mM copper. In contrast, 8 mM of copper concentration is the maximum level of copper tolerated by *R. mucilaginosa* LM9 [27].

The effect of copper on cell morphology was also investigated as yeast cells were cultured in a medium without Cu^{2+} and in medium containing different concentrations of Cu^{2+}. As shown in Figure 3(B), cells exposed to copper exhibit altered morphology with some strangely rough dots inside, but cells were severely affected and damaged the cell wall when Cu^{2+} reached 7 mM or more (10 mM) which could explain the dynamic growth being decreased. A detailed understanding of the mechanism by which copper alters yeast cell morphology may need further study, but the dots (dark bodies) of yeast cells in medium containing Cu^{2+} (≤ 4 mM)

may represent resistance mechanisms of yeasts through sequestration and extrusion of cytoplasmic copper ions rather than avoidance or compartmentalization [28].

3.4. Biosorption assays

3.4.1. Effect of initial pH on the removal efficiency of Cu^{2+}

It is well known that pH has significant effects on the biosorption capacity of heavy metals because it is actively engaged in the competition between protons and heavy metal ions for the surface biosorption sites [29]. Figure 4(A) shows that, in a strong acidic medium (pH 2), the adsorption of Cu^{2+} on the biosorbent surfaces was slow as only 6.75% of Cu^{2+} was removed. This is because at very low pH a high concentration of H^+ ions competes with Cu^{2+} to fill the active sites on the surface of the biosorbent. The removal efficiency was dramatically increased from 29.6 to 81.67% as pH increased from 3 to 5, and reached a maximum of 90.28% at pH 6 since increasing pH leads to a decrease in H^+ ions in the solution which brings about an increase in the number of active sites for Cu^{2+} adsorption. However, when pH reached 7, OH^- competed with functional groups of biosorbent surfaces, and also reacted with Cu^{2+}, then a precipitate of $Cu(OH)_2$ may appear that interferes with the adsorption process, causing a reduction in the adsorption efficiency to 72.52%. These results are consistent with those of Say et al. (2001) [30] who used *Phanerochaete chrysosporium* for biosorption of Cu^{2+}, and with those of Chen et al. (2021) who also found that 6 is the optimal pH for biosorption of Cu^{2+} by yeast *Pichia pastoris* [31]. Meanwhile, pH 5 was determined as the
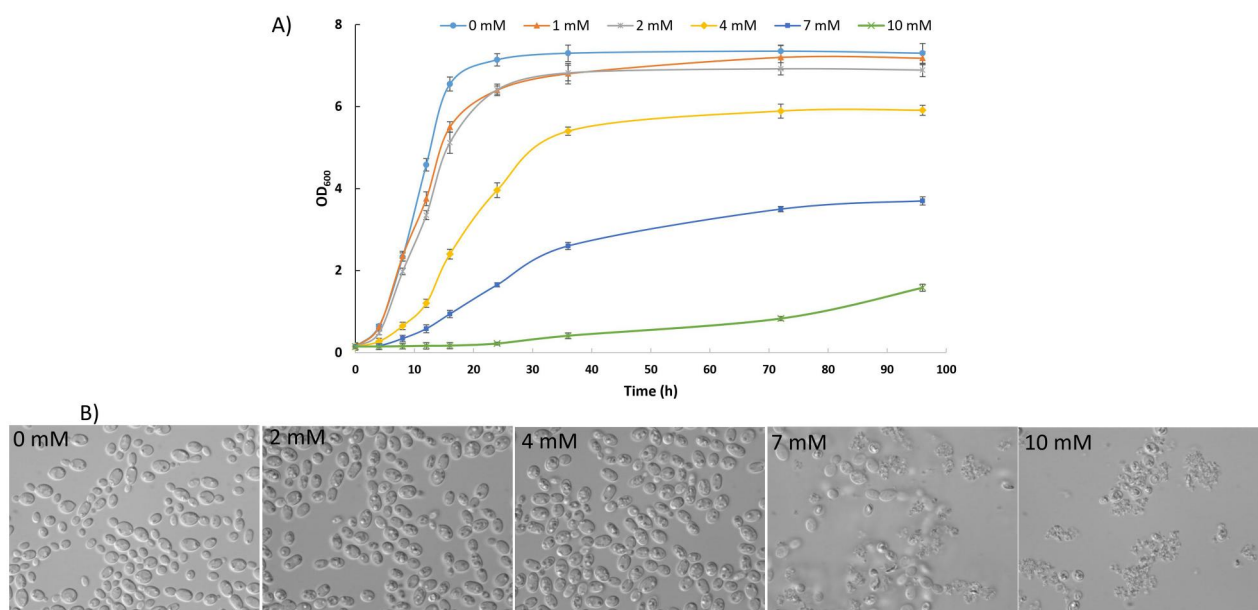

Figure 3. Effect of Cu^{2+} on the growth (A) and cell morphology (B) of *Rhodotorula aurum* sp. nov. Bar 10 μM.
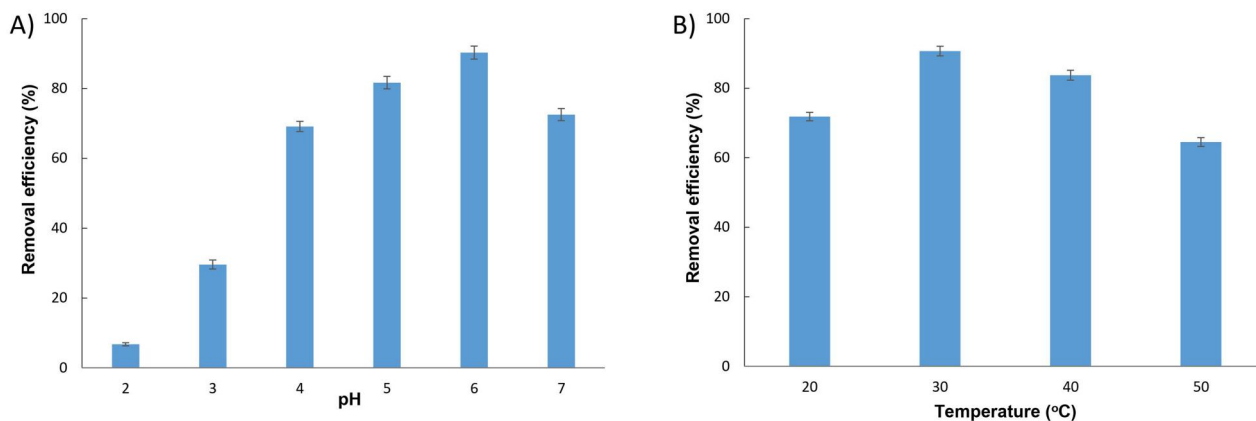
Figure 4. Effect of initial pH (a) and temperature (b) on Cu^{2+} adsorption by *Rhodotorula aurum* sp. nov.
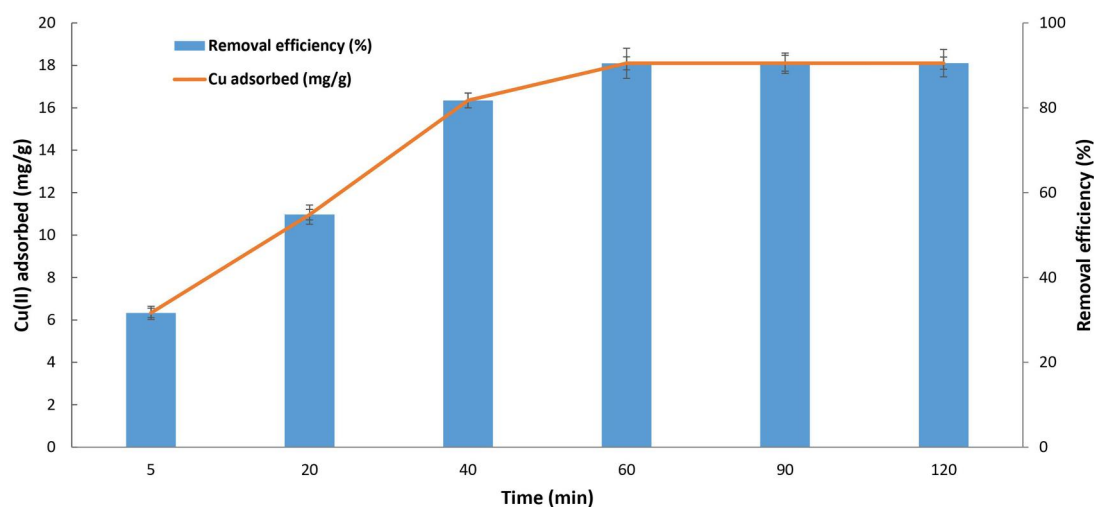

Figure 5. Biosorption of Cu^{2+} by *Rhodotorula aurum* sp. nov., initial copper concentration 100 mg/L at pH 6, temperature 30 °C, using 1 g dried biomass.

optimal pH value for Cu^{2+} adsorption by *Saccharomyces cerevisiae* [32], by *Papiliotrema huenov* [16]. pH in the range of 3.5–4 was the most favorable for dried yeast *S. cerevisiae* in adsorption of Cu^{2+} [33], a higher pH (7) was optimum for copper ion adsorption by *Spirulina platensis* [34]. Thus, the optimal pH for copper ion adsorption may be dependent on the biological material used. The pH 6 was therefore chosen as the optimal pH for subsequent experiments.

3.4.2. Effect of temperature on biosorption of Cu^{2+}

Temperature is among the most important factors that influence the kinetics of biosorption [35]. The Cu^{2+} adsorbed by the *Rhodotorula aurum* sp. nov. was evaluated under four different temperatures: 20, 30, 40, and 50 °C. As the results shown in Figure 4(B), at 20 °C the removal efficiency of copper was 71.84%, and it was significantly increased to 90.66% when the temperature increased to 30 °C. However, the removal efficiency gradually decreased to 83.75% at 40 °C, and then dramatically reduced to 64.52% when the

temperature reached 50 °C. This reduction in adsorption when the temperature increased was possibly due to the weakening of adsorptive forces between Cu^{2+} and the active sites of surface biosorbents [36] or to a change in the functional groups on the surface of yeast [37]. Our results demonstrate that biosorption of Cu^{2+} by *Rhodotorula aurum* sp. nov. is endothermic, an increase in temperature led to expedited ion diffusion. This finding broadly supports the work of other studies about the effect of temperature on biosorption [16,38,39].

3.4.3. Biosorption of Cu^{2+} by Rhodotorula aurum sp. nov. biomass under optimal conditions

The results from Figure 5 indicate that the adsorption of Cu^{2+} to yeast biomass reached equilibrium within 60 min. The biosorption capacity was increased from 6.32 to 18.1 mg/g when the contact time increased from 5 to 60 min, and the Cu^{2+} removal efficiency in the solution increased from 31.66 to 90.49%, respectively. Thus, at pH 6, temperature 30 °C, and incubation time 60 min the maximum Cu^{2+} adsorption to the yeast biomass

was around 18.1 mg/g, which is significantly higher compared to those of previous studies in which the maximal Cu^{2+} binding capacity ranged from 2.59 to 15.85 mg/g using different sorbents such as *Saccharomyces cerevisiae* [32,33], *Candida* spp. [26] and *Pichia stipitis* [39]. However, the findings of the current study are far below those observed by Salvadori et al. (2014) [36] who found the biosorption capacity of copper was 26.2 mg/g when using the dead biomass of *R. mucilaginosa* isolated from a wastewater mine in Brazil. It has been reported that the pretreatment of yeast biomass by phosphate [40], by NaOH, or by Na_2CO_3 [41] dramatically improves biosorption capacity. Thus to improve the Cu^{2+} biosorption capacity using biomass *Rhodotorula aurum* sp. nov. as a biosorbent, it is necessary to develop an effective pretreatment method, which is presently underway and the results will be reported upon completion.

4. Conclusion

In this study, a highly copper-resistant strain named *Rhodotorula aurum* sp. nov. was isolated from a gold mining area. It is affiliated with *R. mucilaginosa* but exhibited some differences in the nucleotide sequence of the SLU gene (>1%) and in biochemical characteristics. The dried biomass of *Rhodotorula aurum* sp. nov. was able to absorb 18.1 mg Cu^{2+}/g biomass that makes it potentially useful in the bioremediation of heavy metals.

5. Taxonomy

Rhodotorula aurum sp. nov., A, B, & C.

MycoBank: MB 848923

Etymology: *Rhodotorula aurum* sp. nov. in reference to gold mining located in Chư'pah, Gia Lai Province, and identified by KCT Nguyen, PH Truong, TC Ho, CT Le, KD Tran, TL Nguyen, MT Nguyen, and PV Nguyen.

Strain GL1[T] exhibits red-pigmented white and smooth colonies that are glistening, butyrous to mucoid, with entire margins in 3 days old on PDA agar. Microscopic morphology shows ovoid and elliptical budding yeast-like cells which are unipolar, and have a size of 4–7 μm. Spores, hyphae, and pseudohyphae were not observed after 21 days at 25 °C of cultivation. The maximum growth of *Rhodotorula aurum* sp. nov. was observed at 30 °C, but the yeast cells exhibit poor growth at 10 and 40 °C. Strain GL1[T] was able to grow in medium containing 10 mM of Cu^{2+} and in pH ranged 3–7. Strain GL1[T] could assimilate ethanol and inositol, but neither use citrate, arabinose or xylose.

The type strain GL1[T] was isolated from Gia Lai province, Vietnam (GPS: 14°21'47.7"N 108°12'53.9"E), and deposited in the Institute of Biotechnology, Hue University Vietnam with accession number 062022.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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