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Heavy Metal Resistance and Biosorption of Acid-Tolerant Yeasts Isolated from Tea Soil

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Abstract: The heavy metal resistance and biosorption of two acid-tolerant yeast strains isolated from tea soils in Kagoshima Experimental Station (Japan) were investigated. *Cryptococcus* sp. AH-13 was more resistant to Cd, Cu, Zn, Co, Hg, Ag, Fe, Mn, Ni (except Pb) on the YG solid medium than *Candida palmioleophila* KB-6. The resistance to heavy metals in the YG solid medium were higher than those in the liquid medium. When being cultivated in YG liquid medium (pH 3.0) containing various concentrations of heavy metals, the growth of *Candida palmioleophila* KB-6 was considerably inhibited at 0.05 mM Cd, 0.3 mM Cu and 0.5 mM Zn whilst the growth of *Cryptococcus* sp. AH-13 in was inhibited at 0.5 mM Cd, 1.5 mM Cu and 1.5 mM Zn. Both types of living and dead cells of *Candida palmioleophila* KB-6 and *Cryptococcus* sp. AH-13 could remove heavy metals from their salt solutions. The amount of heavy metals accumulated in above types of yeast cells increases along with the concentration of heavy metals, but seems to be constant at a certain saturable concentration of heavy metals. Heavy metal biosorption by *Cryptococcus* sp. AH-13 appeared to be higher than that by *Candida palmioleophila* KB-6.

Keywords: Tea soil, acid-tolerant yeast, heavy metal resistant yeasts, *Cryptococcus* sp. AH-13, *Candida palmioleophila* KB-6.

1. Introduction

The application of nitrogenous fertilizers, especially ammonium sulfate fertilizer, to naturally acidic tea soils at rates in excess of tea plant need and leaching of nitrate nitrogen has speeded up the process of acidification. Here there is a doubt that heavy metals in tea soils might become more soluble, posing a significant threat to the activities of soil microorganisms as well as the health of tea consumers. The point thus was made that the microbial ecology of this extreme acidic environment should be realized to possibly suggest an appropriate solution to surmount the increase in the content of soluble heavy metals. Among microorganisms, yeasts are capable of tolerating high levels of acidity [1-5]. Moreover, they possess the potential to accumulate a range of metal cations. Up to 90% of the yeast cell wall is polysaccharide complexed with proteins, lipids and other substances. Biosorption may be primarily a function of the binding of heavy metal cations

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to chemical functional groups on the yeast cell wall via ionic and coordination bonds [6]. However, the potential function of yeasts in soil is not well understood, largely because yeasts are thought to make up an insignificant proportion of the soil's microbial population. Moreover, little is known about resistance to heavy metals of yeasts in acidic soils, especially in tea garden soils. Therefore, the aim of the present study was to investigate the heavy metal resistance and biosorption of two indigenous yeast strains, *Cryptococcus* sp. AH-13 and *Candida palmioleophila* KB-6, isolated in acidified tea soils in Kagoshima (Japan). These initial results may facilitate studies which offer the potential application for improving the acidified tea garden soils.

2. Materials and Methods

2.1. Soil samples

Samples of Kuroboku (high-humic Andosol) and Akahoya (light-colored Andosol) soils used for isolation of yeast strains were collected from tea gardens at a depth of 0-20 cm at the Kagoshima Tea Experimental Station. All the fresh soil samples were passed through a 2 mm mesh sieve (JIS standard), dried for 24 hours, passed through a 0.5 mm mesh sieve (JIS standard) and kept in closed glass bottles for storage at 5^{o}C.

2.2. Quantification of water soluble heavy metal content of the soil samples

The soluble heavy metals in soils were extracted with pure water (1:20), followed by shaking for 2 hours [7], diluted with 1% nitric acid and then quantified by using Inductively coupled plasma-mass spectrometry (ICP-MS).

2.3. Heavy metal resistance of yeasts on YG media

Solutions of $CdCl_2.2.5H_2O$, $CuSO_4.5H_2O$, $ZnCl_2$, $NiCl_2.6H_2O$, $CoCl_2.6H_2O$, $MnCl_2.4H_2O$, $PbCl_2$, $HgCl_2$, $AgNO_3$ and $FeCl_3.6H_2O$ (pH 3.0) were filter- sterilized, and added to YG solid (yeast extract 1.0 g, glucose 1.0 g, KH_2PO_4 0.2 g, $MgSO_4.7H_2O$ 0.2 g, agar 15 g, water up to 1000 ml, pH 3.0) and liquid (without agar) media autoclaved at 121^{o}C for 15 min to final desired concentrations of heavy metals. The media were inoculated with yeasts and subsequently incubated at 30^{o}C for 3-5 days. The resistance to heavy metals of yeasts was determined by assessing minimal inhibitory concentration of heavy metals to their growth. The appearance of colonies on plates or turbidity in each of the tubes containing a certain concentration of heavy metal after incubation would affirm their growth at given condition.

2.4. Yeast growth in the presence of heavy metals

Each culture of 50 ml of YG liquid medium with a certain concentration of Cd, Cu, Ni or Zn was inoculated with 1 ml of yeast suspension and incubated by shaking at 150 rpm, 30^{o}C for 5 days. A culture grown in the absence of heavy metals served as the control. Samples of cultures (5 ml) were collected daily from each of the cultures. The growth was monitored as absorbance at 660 nm using a spectrophotometer. The pH of the medium was measured using pH meter with a glass electrode.

2.5. Heavy metal biosorption analysis

The types of yeast cells used for the analysis were prepared as follow: (i) Living-cells: From early-stationary cultures incubated

in YG medium by shaking at 30°C, 150 rpm, the cells were harvested by centrifugation at 2500g for 30 minutes and washed in quarter strength Ringer's solution before re-centrifugation and final washing with de-ionized water; (ii) Dead-cells: The cells at early-stationary phase were killed by autoclaving, and then the same procedure as above was followed. The analysis was carried out according to the method of Scott (1990) [8] with some modifications. The cells (30 mg) were placed in a 100 ml solution of each heavy metal with certain concentration (pH 3.0) for 10 minutes. This solution was then filtered with a sterilized filter (0.50 μm pore size) to separate the cells from the filtrate. Next, the cells were washed with distilled water. The yeast cells retained on the filter papers were then weighed after drying to a constant weight at 80°C for 24 hours, decomposed by concentrated nitric acid and subjected to ICP-MS analysis to determine the amount of heavy metal bound to the cells.

2.6. Statistical analysis

All the values represented the means of three independent experiments and were plotted along with their respective standard deviations. Differences of means were tested with the Turkey-Kramer's method.

3. Results and Discussion

3.1. Water soluble heavy metal content of soil samples

The water soluble heavy metal content of soil samples were determined and presented in Table 1. The Cu and Fe contents in Akahoya sample were higher in Kuroboku sample. Besides, the other heavy metal contents were lower and not almost different in both of samples.

Table 1.Water soluble heavy metal content (mg Kg^{-1}) in tea soils

Soil sample	Zn	Cu	Cd	Co	Fe	Mn	Ni	Pb
Kuroboku	1.6	0.4	0.0	0.1	7.8	27.3	0.4	0.4
Akahoya	1.9	0.6	0.01	0.0	14.8	14.9	0.4	0.4

3.2. Heavy metal resistance of yeasts in YG media

The heavy metal minimal inhibitory concentrations to the growth of *Candida palmioleophila* KB-6 and *Cryptococcus* sp. AH-13 in YG media were determined and described in Tables 2 and 3.

Table 2. Minimal inhibitory concentrations of metals (mM) of yeasts on YG solid medium

	Cd	Cu	Zn	Co	Pb	Hg	Ag	Fe	Mn	Ni
Cryptococcus sp. AH-13	5.0	10.0	10.0	5.0	2.0	0.1	5.0	15.0	250	10.0
Candida palmioleophila KB-6	2.0	5.0	5.0	5.0	5.0	0.05	0.1	10.0	20	5.0

Table 3. Minimal inhibitory concentrations of metals (mM) of yeasts on YG liquid medium

	Cd	Cu	Zn	Co	Pb	Hg	Ag	Fe	Mn	Ni
Cryptococcus sp. AH-13	1.0	2.0	2.0	0.5	0.001	0.01	1.0	1.0	100	1.0
Candida palmioleophila KB-6	0.1	0.5	0.5	0.5	1.0	0.001	0.001	1.0	0.1	0.5

The yeasts demonstrated resistance to substantial concentrations of heavy metals. The order of toxicity of the heavy metals on the YG solid medium plates to *Candida palmioleophila* KB-6 and *Cryptococcus* sp. AH-13 was Hg (0.05 mM) > Ag (0.1 mM) > Cd (2.0 mM) > Co = Cu = Zn = Ni = Pb (5.0 mM) > Fe (10.0 mM) > Mn (20 mM) and Hg (0.1 mM) > Pb (2.0 mM) > Cd = Co = Ag (5.0 mM) > Cu = Zn = Ni (10.0 mM) > Fe (15.0 mM) > Mn (250 mM), respectively (Table 2). Whereas, the order of toxicity of the heavy metals in YG liquid medium was Hg = Ag (0.001 mM) > Cd = Mn (0.1 mM) > Cu = Zn = Ni = Co (0.5 mM) > Fe = Pb (1.0 mM) and Pb (0.001 mM) > Hg (0.01 mM) > Co (0.5 mM) > Cd = Ag = Fe = Ni (1.0 mM) > Cu = Zn (2.0 mM) > Mn (100 mM), respectively (Table 3).

Cryptococcus sp. AH-13 appeared to be more resistant to heavy metals (except Pb) than *Candida palmioleophila* KB-6. The minimal inhibitory concentrations of heavy metals in YG solid medium were higher than those in liquid medium. This may be ascribed to the fact that conditions for diffusion, complexation and the availability of heavy metals in solid media differs from liquid media. As a matter of fact, Hg appears to be the highest toxic one among tested metals. It is reasonable because the affinity of Hg^{2+} to thiol groups is very strong.

Obviously, the tolerance to acidity and resistance to heavy metals of yeast strains depended on type of yeast, medium (solid or liquid), and heavy metal and its concentration presented in the culture medium. Besides, it appears that there is a relation between tolerance to acidity, resistance to heavy metals of a yeast strain and the properties of the soil from which it was isolated.

Abdullah (1998) [9] reported that *Candida tropicalis*, *Geotrichum capitatum*, *Rhodotorula minuta* and *Williopsis californica* isolated from Saudi Arabian soil were able to survive and grow in Czapek-Dox liquid medium (pH 6.0) containing up to 400 µg/ml of Cd and Cu. However, it is difficult to make comparisons of the heavy metal resistance levels of yeasts from different studies because of the various culture media and incubation conditions employed. Regarding to mechanisms, detoxification by phyto-chelatins or metallothionens and reduced accumulation by active efflux have been known as two major mechanisms of metal resistance. After the complete genome sequence of *Saccharomyces cerevisiae* was determined, the mechanism in yeast seemed to be detoxification. In this case, the pH of the spent YG liquid medium containing heavy metals of yeasts increased slightly from 3.0 to 3.5 after 5 days of incubation. However, elucidation of the precise mechanisms requires further study.

3.3. Yeast growth in the presence of heavy metals

The growth of yeasts in the presence of different heavy metal concentrations in YG liquid medium (pH 3.0) are determined and expressed in Figures 1a-d and 2a-d.

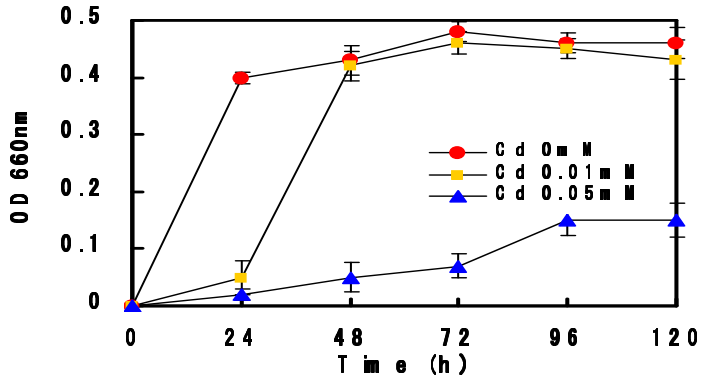

Fig. 1a. The growth of strain *Candida palmioleophila* KB-6 in YG liquid medium (pH 3.0) with various concentrations of Cd, 0 mM (●), 0.01 mM (■), and 0.05 mM (▲).

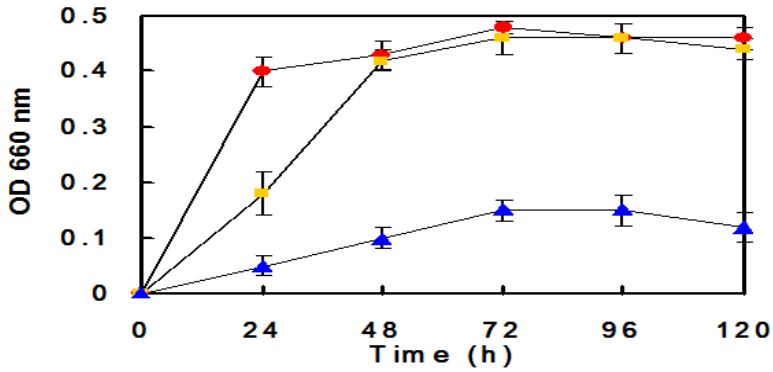

Fig. 1b. The growth of strain *Candida palmioleophila* KB-6 in YG liquid medium (pH 3.0) with various concentrations of Cu, 0 mM (●), 0.1 mM (■), and 0.3 mM (▲).

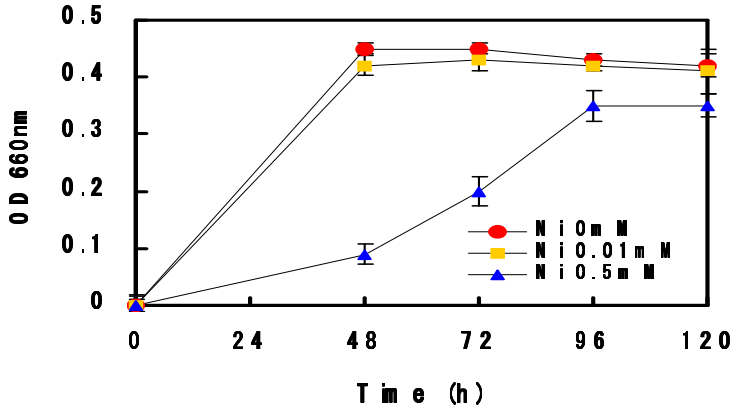

Fig. 1c. The growth of strain *Candida palmioleophila* KB-6 in YG liquid medium (pH 3.0) with various concentrations of Ni, 0 mM (●), 0.01 mM (■), and 0.05 mM (▲).

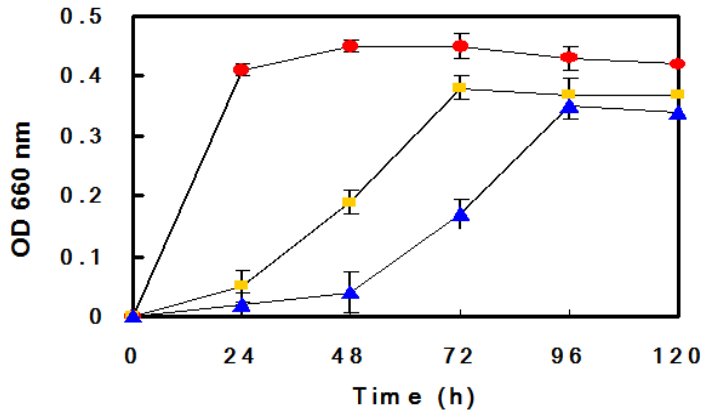

Fig. 1d. The growth of strain *Candida palmioleophila* KB-6 in YG liquid medium (pH 3.0) with various concentrations of Zn, 0 mM (●), 0.5 mM (■), and 1.5 mM (▲).

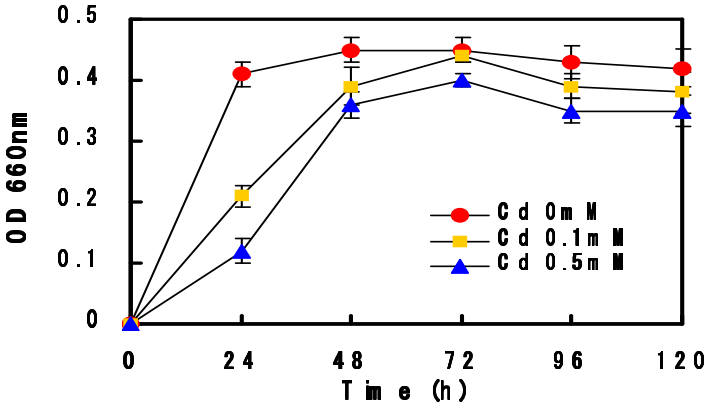

Fig. 2a. The growth of strain *Cryptococcus* sp. AH-13 in YG liquid medium (pH 3.0) with various concentrations of Cd, 0 mM (●), 0.1 mM (■), and 0.5 mM (▲).

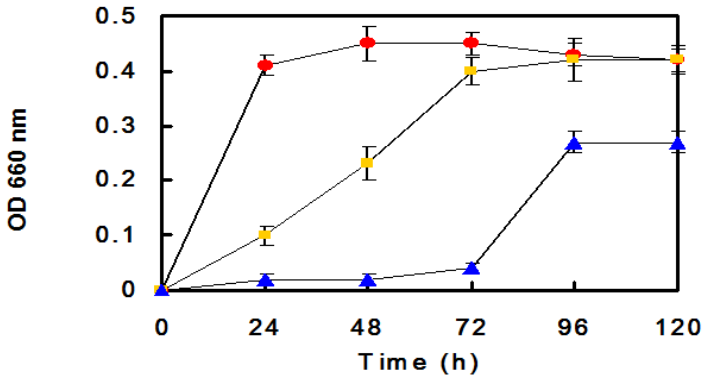

Fig. 2b. The growth of strain *Cryptococcus* sp. AH-13 in YG liquid medium (pH 3.0) with various concentrations of Cu, 0 mM (●), 0.5 mM (■), and 1.5 mM (▲).

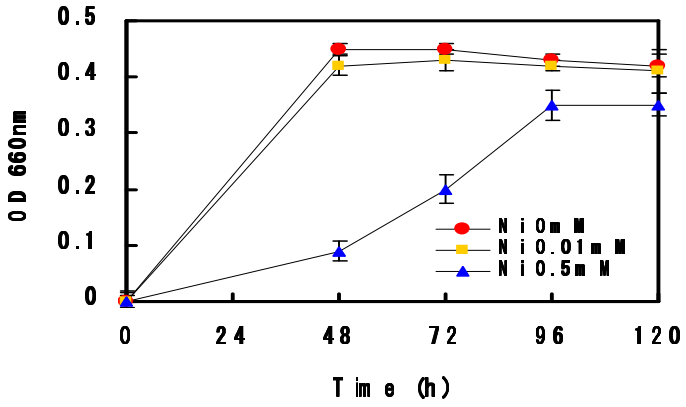

Fig. 2c. The growth of strain *Cryptococcus* sp. AH-13 in YG liquid medium (pH 3.0) with various concentrations of Ni, 0 mM (●), 0.01 mM (■), and 0.5 mM (▲).

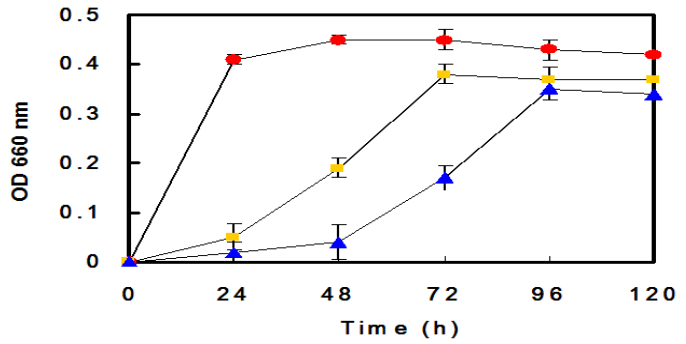

Fig. 2d. The growth of strain *Cryptococcus* sp. AH-13 in YG liquid medium (pH 3.0) with various concentrations of Zn, 0 mM (●), 0.5 mM (■), and 1.5 mM (▲).

Generally, increasing heavy metal concentrations in the culture medium inhibited their growth, especially 0.05 mM Cd, 0.3 mM Cu, Ni and 0.5 mM Zn, for *Candida palmioleophila* KB-6 and 0.5 mM Ni, 1.5 mM Cu and Zn, for *Cryptococcus* sp. AH-13. Compared to the rate in the absence of heavy metals in the culture medium, the optimal growth rate of *Candida palmioleophila* KB-6 in the presence of 0.05 mM Cd, 0.3 mM Cu, 0.3 mM Ni and 0.5 mM Zn was only 30%, 65%, 30% and 40%, respectively. Whereas, that of *Cryptococcus* sp. AH-13 strain in the presence of 0.5 mM Cd, 0.5 mM Ni, 1.5 mM Cu and 1.5 mM Zn was about 90%, 75%, 60% and 75%, respectively. The pH of the spent YG liquid medium containing heavy metals of yeasts increased slightly from pH 3.0 to 3.5 after 5 days of incubation.

Although the survival and growth of strains *Candida palmioleophila* KB-6 and *Cryptococcus* sp. AH-13 were observed in the presence of heavy metals, it appeared to be inhibited at increasing concentrations of these heavy metals. The precise above-conducted experiments indicated to what extent the presence of high levels of heavy metals influenced the growth of the respective yeast strains. The inhibition may be accounted for by the toxicity of heavy metals at high

concentrations. Toxic effects may include the blocking of functional groups of biologically important molecules, substitution of essential metal ions from their native binding sites in biomolecules [10], alterations in the conformational structure of nucleic acids and proteins, interference with oxidative phosphorylation and osmotic balance, denaturation and inactivation of enzymes, and disruption of cellular and organelle integrity [11]. As a consequence of these effects, microbial growth will be restricted.

3.4. Biosorption of Cu and Zn by yeast cells

Biosorption of Cu, Zn and Ni by the yeasts is investigated and shown in Figures 3, 4 and 5.

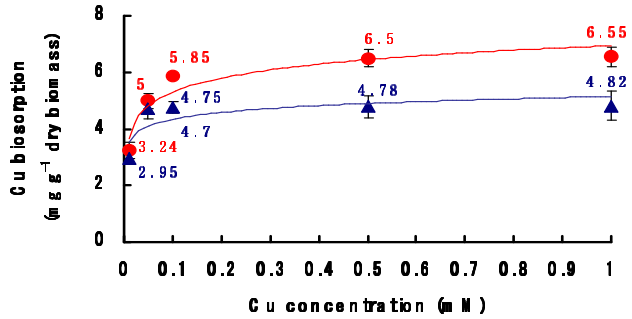

Fig. 3. Biosorption of Cu by *Candida palmioleophila* KB-6 (▲) and *Cryptococcus* sp. AH-13 (●).

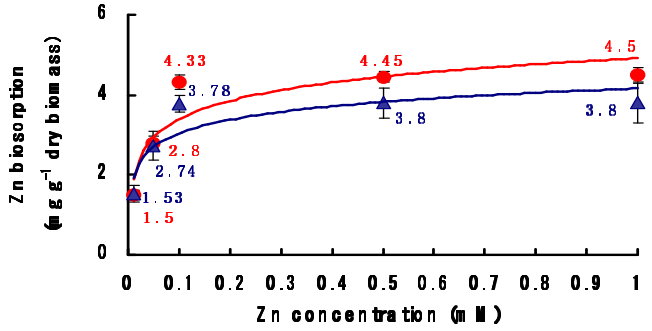

Fig. 4. Biosorption of Zn by*Candida palmioleophila* KB-6 (▲) and *Cryptococcus* sp. AH-13 (●).

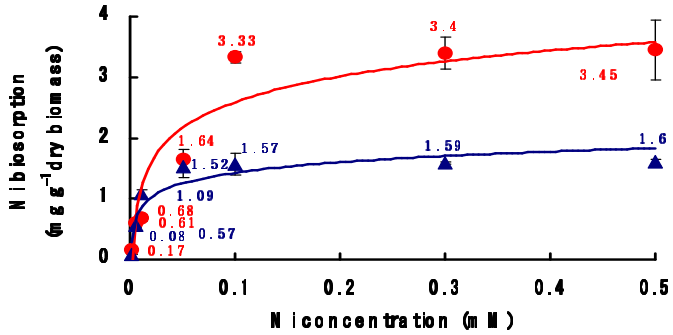

Fig. 5. Biosorption of Ni by *Candida palmioleophila* KB-6 (▲) and *Cryptococcus* sp. AH-13 (●).

The results have affirmed the removal of heavy metals from their solutions by binding to the yeast cells. The amount of heavy metals bound to cells increases along with the concentration of heavy metals, but appeared to be constant at a certain concentration of heavy metals. The highest percentages of heavy metals removed from solutions at concentrations tested by *Candida palmioleophila* KB-6 were about 29% (Cu 0.01 mM), 14% (Zn 0.01 mM) and 11.5% (Ni 0.005mM) and those of *Cryptococcus* sp. AH-13 were approximately 30% (Cu 0.01 mM), 17% (Ni 0.001 mM) and 14% (Zn 0.1 mM).

3.5. Effect of type of yeast cell on cadmium biosorption

The capacity for biosorption of cadmium by two types of yeast cells (living and dead) was determined and described in Figures 6 and 7. The ability of *Candida palmioleophila* KB-6 to remove cadmium was significantly greater ($P \leq 0.05$) in living-cells than in dead- cells for all cases tested. Meanwhile, the ability of *Cryptococcus* sp. AH-13 to remove cadmium at concentrations of 0.05 and 0.1 mM was not significantly different ($P > 0.05$) between living- and dead-cells.

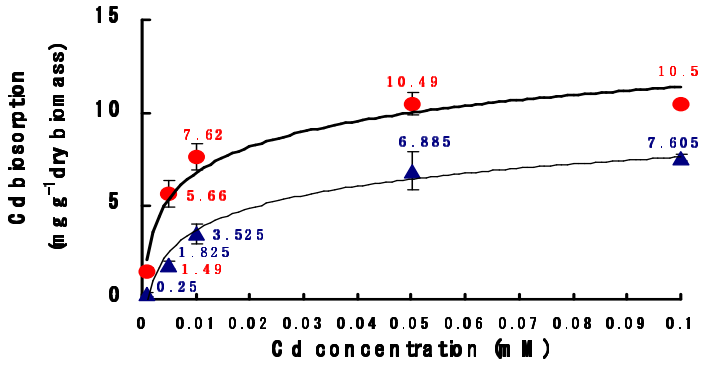

Fig. 6. Biosorption of Cd by *Candida palmioleophila* KB-6 in living-cells (●) and dead-cells (▲)

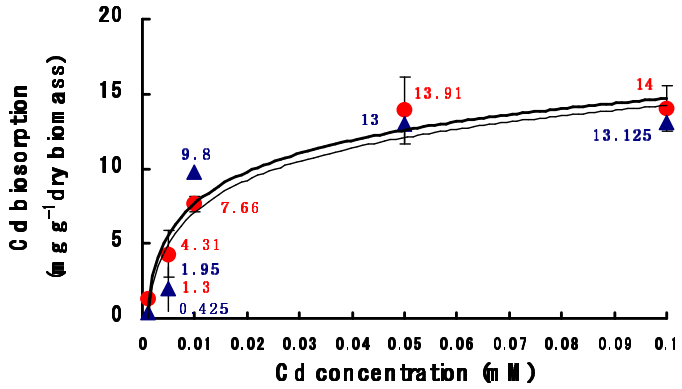

Fig. 7. Biosorption of Cd by *Cryptococcus* sp. AH-13 in living-cells (●) and dead-cells (▲)

The highest percentage of cadmium removed from the initial solution by yeasts was obtained at a concentration of 0.001 mM in the living-cells. At this concentration, the percentage was about 80% in living- cells but only 13.5% in dead-cells of *Candida palmioleophila* KB-6 and about 70% in living-cells but only 23% in dead-cells of *Cryptococcus* sp. AH-13.

Yeasts possess an acknowledged potential for multi-metal accumulation from the environment. In general, biosorption seems to be a universal and inherent characteristic of yeasts. Both living- and dead- cells were able to take up heavy metals via physico-chemical mechanisms such as adsorption or ion-exchange. When living cells are used, metabolic uptake mechanisms may also contribute to the process [10], including metal precipitation as sulphides, complexation by siderophores and other metabolites, sequestration by metal-binding proteins and peptides such as methalothioneins and phytochelatins, transport and intracellular compartmentation, and metal transformation resulting in oxidation, reduction or methylation [12]. Intracellularly accumulated metals are most readily associated with the cell wall and vacuole but may also be bound by other cellular organelles and biomolecules [13].

Even though there is ambiguity concerning whether living or dead cells are the better metal biosorbent, some research results have suggested that living cells seems to be more effective in the biosorption of heavy metals than dead cells [14]. In other words, pretreatment prior to use for metal biosorption affects the uptake capacity of the cells. In the present study, for cell types of strain *Candida palmioleophila* KB-6, the result is likely to agree with the above suggestion. The amount of heavy metals bound to the living cells was significantly larger than that of the dead cells. This is possibly due to the destruction of metal binding sites by heating which affects the cell wall character and in turn affects the nature of the uptake. On the other hand,there was no significant difference in the biosorption of heavy metals between living and dead cells of strain *Cryptococcus* sp. AH-13. The discrepancy in the result may be ascribed to the fact that, for a variety of reasons, the capacity for heavy metal biosorption of living cells may be greater, equivalent to or less than that of dead cells derived from the same microbial strain.

4. Conclusion

Two yeast strains isolated from tea soils in Kagoshima (Japan) and identified as *Cryptococcus* sp. AH-13 and *Candida palmioleophila* KB-6, have been shown to tolerate acidity, resist heavy metal toxicity and remove them from culture medium. Therefore, these yeasts may be a potential indigenous microbial resource for the improvement of acidified tea soils.

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Sự kháng và hấp thụ kim loại nặng của các chủng nấm men chịu axit được phân lập từ đất trồng chè

Ngô Thị Tường Châu

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Tóm tắt: Khả năng kháng và hấp thụ kim loại nặng của hai chủng nấm men chịu acid phân lập từ các mẫu đất trồng chè ở Kagoshima (Nhật Bản) đã được khảo sát. Chủng *Cryptococcus sp.* AH-13 có khả năng kháng với Cd, Cu, Zn, Co, Hg, Ag, Fe, Mn, Ni (ngoại trừ Pb) trong môi trường YG dạng rắn cao hơn so với chủng *Candida palmioleophila* KB-6. Khả năng kháng kim loại nặng của các chủng nấm men trong môi trường YG dạng rắn là cao hơn so với dạng dịch thể. Khi được nuôi cấy trong môi trường YG dạng lỏng (pH 3.0) chứa các nồng độ khác nhau của các kim loại nặng, sự phát triển của *Candida palmioleophila* KB-6 đã bị ức chế đáng kể tại các nồng độ 0.05 mM Cd, 0.3 mM Cu và 0.5 mM Zn, trong khi đó sự phát triển của *Cryptococcus sp.* AH-13 bị ức chế tại các nồng độ 0.5 mM Cd, 1.5 mM Cu và 1.5 mM Zn. Bên cạnh đó, cả hai dạng tế bào sống và chết của các chủng *Candida palmioleophila* KB-6 và *Cryptococcus sp.* AH-13 cũng đã có thể loại bỏ các kim loại nặng ra khỏi dung dịch muối của chúng. Lượng các kim loại nặng được tích lũy trong các dạng tế bào nấm men nói trên đã tăng lên cùng với sự tăng của các nồng độ kim loại nặng tuy nhiên sẽ đạt đến một giá trị ổn định tại nồng độ cực đại cho sự tích lũy kim loại. Khả năng hấp thụ kim loại của *Cryptococcus sp.* AH-13 dường như là cao hơn so với *Candida palmioleophila* KB-6.

Từ khóa: Đất trồng chè, nấm men chịu axit, nấm men kháng kim loại nặng, *Cryptococcus* sp. AH-13, *Candida palmioleophila* KB-6.