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Identification and characterization of heavy metal-resistant unicellular alga isolated from soil and its potential for phytoremediation

Naoto Yoshida *, Ryuichiro Ikeda, Tomoko Okuno

Department of Biochemistry and Applied Biosciences, Faculty of Agriculture, University of Miyazaki, 1-1 Gakuen Kibanadai-Nishi, Miyazaki 889-2192, Japan

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Abstract

A unicellular alga displaying a high growth rate under heterotrophic growth conditions was isolated from soil and identified as *Chlorella sorokiniana*. The optimal temperature for growth was 35 °C and the optimal pH was 6.0–7.0. Glucose, sucrose, galactose, maltose, and soluble starch served as carbon sources supporting growth under dark conditions. The cell yield was 50 g/l (wet weight) in a heterotrophic medium containing 3% glucose. Isolated unicellular algae were highly resistant to heavy metals such as Cd^{2+}, of which the minimal inhibitory concentration was 4 mM. Algae were capable of taking up the heavy metal ions Cd^{2+}, Zn^{2+} and Cu^{2+} at 43.0, 42.0 and 46.4 μg/mg dry weight, respectively. Growth inhibition of *Oryza sative* shoots by 5 ppm Cd^{2+} in hydroponic medium was completely prevented by the addition of 0.25 mg of wet *Chlorella* cells. These results indicated that this isolate was potentially useful for phytoremediation by preventing environmental dispersion of heavy metals.

Keywords: Bioremediation; Cadmium; *Chlorella sorokiniana*; Green algae; Heterotrophic growth; Phytoremediation

1. Introduction

Many metal ions are essential as trace elements, but at higher concentrations, they become toxic. Heavy metals are difficult to remove from the environment and are ultimately indestructible, unlike many other pollutants that can be chemically or biologically degraded (Ozaki et al., 2003). Today, heavy metals constitute a global environmental hazard. For example, environmental pollution by Cd^{2+}, arising mainly from mining, smelting, dispersal of sewage sludge (Hutton, 1983), and the use of phosphate fertilizers (Crisanto Herrero and Lorenzo Martin, 1993) is increasing. Microorganisms could be used to clean up metal contamination by removing metal from contaminated water and waste streams, sequestering metals from soil and sediments, or solubilizing metals to facilitate their extraction. Bacteria and higher microorganisms have developed resistance to toxic metals and are able to make them innocuous. Microorganisms respond to heavy metals using various defense systems, such as exclusion (Ortiz et al., 1992), compartmentalization (Valls et al., 2000), complex formation (Wang et al., 1997), and synthesis of binding proteins, such as metallothioneins (Adamis et al., 2004). Microorganisms with unique abilities such as metal absorption, accumulation or resistance can be identified among naturally occurring organisms. Alternatively, these systems can be utilized in engineering bacteria for remediation of polluted waters and soils. Thus, the use of microorganisms for decontaminating heavy metals has attracted growing attention because there are several problems associated with pollutant removal using conventional methods. Bioremediation strategies have been proposed as an attractive alternative owing to their low cost and high efficiency (Mejare and Bülow, 2001).

Heavy-metal-resistant microorganisms show us possible methods to prevent environmental contamination. The newly discovered metal sequestering properties of certain

* Corresponding author. Tel./fax: +81 985 58 7218.
E-mail address: a04109u@cc.miyazaki-u.ac.jp (N. Yoshida).

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types of fungi (Svoboda and Kalac, 2003) and algae (Chaisuksant, 2003) hold considerable promise. Heavy metals can be eliminated from polluted environments by utilizing their natural heavy metal disposing abilities. The objective of this study was thus to isolate and characterize a cadmium-tolerant eukaryotic microorganism from soil and to determine its potential for reducing toxic heavy metals. Cadmium reduction by microorganisms has suggested the importance of microorganisms in bioremediation and environmental cleanup operations (Niu et al., 1993; Chen and Wilson, 1997; Morris et al., 1999). The tolerance of isolated cells to elevated Cd^{2+} in the environment and the biochemical basis of this tolerance, as well as the metal adsorption capacity of these cells, were examined.

2. Methods

2.1. Isolation of heavy-metal-resistant microorganisms

YPG media (peptone, 10 g; yeast extract, 5 g; glucose, 10 g) was sterilized by autoclaving at 120 °C for 15 min. A sterilized solution of 0.5 M $CdCl_2$ through a Millipore filter was aseptically added to the medium at a final concentration of 2 mM. Soil obtained from random areas was dissolved in 10–20 volumes of sterilized physiological saline and was spread onto a YPG plate containing 2 mM of Cd^{2+} and 50 μg/ml of chloramphenicol. After incubation at 30 °C for 3 days, colonies formed were removed and streaked onto another plate, and this was used as the experimental strain.

2.2. Determination of chlorophyll

The growth at 30 °C in YPG broth (pH 7.0) containing glucose was determined by measuring packed cell volume per liter of culture using a hematocrit and cell weight (dry weight of cells, g/l). Chlorophyll *a* and *b* levels were determined by measuring the optical density at 660 nm and 642.5 nm in ethyl ether cell extract, and were calculated according to Jeffrey's equation (Jeffrey, 1976). Cells were then observed by fluorescence microscopy (Exciter: 520–550 nm, Dichroic mirror: 565 nm, Emitter: 580 nm) (Olympus, Tokyo).

2.3. Saccharide utilization

The pre-culture was maintained in 3 ml of yeast extract peptone dextrose (YPG) broth (pH 7.0) at 30 °C for 2 days in the dark. Assimilation of various carbon sources was investigated using MBM medium [KNO_3, 25 mg; $MgSO_4 \cdot 7H_2O$, 7.5 mg; K_2HPO_4, 7.5 mg; KH_2PO_4, 17.5 mg; NaCl, 2.5 mg; $CaCl_2 \cdot 2H_2O$, 1 mg; Fe-mixture ($FeSO_4 \cdot 7H_2O$, 1 g; distilled water, 500 ml), 0.1 ml; A5-metal mixture (H_3BO_3, 286 mg; $MnSO_4 \cdot 7H_2O$, 250 mg; $ZnSO_4 \cdot 7H_2O$, 22.2 mg; $CuSO_4 \cdot 5H_2O$, 7.9 mg; Na_2MoO_4, 2.1 mg; distilled water 100 ml), 0.1 ml; distilled water 99.8 ml; pH 6.0] with or without 0.5% (w/v) of each saccharide. A total of 1 ml of pre-cultured cells was inoculated into 100 ml of MBA broth. MBA broth was then shaken at 150 rpm at 30 °C for 5 days. Growth was compared by measuring optical density at 550 nm.

2.4. Optimum pH and temperature for growth

The pre-culture was maintained in 3 ml of YPG broth (pH 7.0) at 30 °C for 2 days in the dark. The heterotrophic culture was performed using 100 ml of YPG broth (pH 7.0) in each 300 ml cotton plugged Erlenmeyer flask by adding 1 ml of the pre-culture broth, on a rotary shaker (100 rpm) at 20–40 °C for 4 days in the dark. The growth was compared by measuring the optical density at 550 nm. The optimum pH for growth was determined using YPG broth that was adjusted to a desired pH value with 0.1 N HCl or 0.1 N NaOH.

2.5. Amplification and sequencing of 18S rDNA

Isolated microorganisms were grown in 3 ml of YPG broth at 30 °C for 3 days by agitation. DNA was extracted from 1.5 ml of culture broth (approximately 0.04 g cells) using a DNA extraction kit (Isoplant II; Nippon Gene, Toyama) according to the manufacturer's instructions. Sense and antisense primers were designed (5′-ACGGAGG ATTAGGGTTCGATTCCG-3′ and 5′-GCTTCCATTG GCTAGTCGCCAATA-3′, respectively). Both primers were used in PCR with chromosomal DNA acting as a template. The reaction mixture contained 5 μg of chromosomal DNA, 400 pmol of each oligonucleotide primer, and 0.1 U of Taq DNA polymerase in a volume of 50 μl. Thirty thermal cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min, were carried out. PCR products were purified by agarose gel electrophoresis. The resulting DNA fragment (1800 bp) was ligated into a pCRII vector (Invitrogen, CA, USA). The PCR-amplified fragment was sequenced using the dideoxy chain termination method (Sanger et al., 1977). Searches for similar sequences were carried out using the BLAST program (Altschul Gapped BLAST 2001). Multiple alignments were run using GENETIX ver. 10. Distance matrix trees were constructed using Kimura's two-parameter model (Kimura, 1980).

2.6. Metal resistance and minimum inhibitory concentrations

Resistance of the isolated strain to cadmium chloride, cupric sulfate, zinc chloride, nickel chloride and aluminum chloride was determined by the dilution method (Nieto et al., 1989). Metal ions were added separately to YPG broth at concentrations of 1–10 mM. YPG broth without the metal ions was also inoculated with the isolated strain for use as controls. Each broth was cultured at 30 °C for 4 days by agitation at 100 rpm. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of metal that completely inhibited growth.

2.7. Uptake of heavy metals by isolated microorganism

The isolated microorganism was cultured in YPG broth at 30 °C for 4 days and was then harvested by centrifugation at 2000*g* for 15 min. Next, 100 mg (dry weight) of harvested cells were suspended in 100 ml of 10 mM Tris–HCl buffer (pH 7.0) containing 1–5 mg of either Cd^{2+}, Zn^{2+} or Cu^{2+}, or 2.5 mg each of $Cd^{2+} + Zn^{2+}$, $Cu^{2+} + Zn^{2+}$, and $Cd^{2+} + Cu^{2+}$. These suspended solutions were shaken at 100 rpm at 30 °C. Residual heavy metals in the upper phase following centrifugation at 2000*g* for 15 min were quantified by atomic absorption spectrophotometry (Shimadzu, Japan).

2.8. Possibility of bioremediation

Rice seeds (*Oryza sative* L. cv. Koshihikari) were surface sterilized in 70% (v/v) aqueous ethanol for 15 min, rinsed 5 times with distilled water and allowed to germinate on a sheet of moist filter paper at 25 °C with a 12-h photoperiod in a growth chamber. Light was provided from above with a white fluorescent tube. After 3 days, uniform seedlings ($n = 30$) were transferred onto a sheet of plastic mesh (3×3 cm) that was floated on distilled water (100 ml) in the presence or absence of 5 mg/l of Cd^{2+} in plastic containers ($5 \times 5 \times 8$ cm). Next, 0.03–0.50 g (wet weight) of the isolated microorganism was added to the media. Seedlings were grown at 25 °C with a 12-h photoperiod. The water in the plastic container was kept at the same level by adding distilled water at 24-h intervals and only the roots of the seedlings were immersed in the water during the incubation. After 3 weeks, the shoot length was measured.

3. Results

3.1. Isolation of heavy-metal-resistant microorganisms

Soil samples were collected from various districts in Japan and more than a 100 strains were found to be Cd^{2+} and chloramphenicol resistant. A yeast-like strain that formed large green colonies was selected and its physiological profile was subsequently examined. This strain, designated ANA9, was then used as the experimental organism. The cells of the isolated microorganism were always spherical and were 5.35–8.56 μm in diameter. The cells were grown on YPG and were always green, regardless of lighting conditions.

3.2. Determination of chlorophyll

Strain ANA9 was observed as bright red cells on fluorescence microscopy, thus indicating that it possessed chloroplasts. Chloroplasts were excited at 520/550 nm and emission signals were collected at wavelengths above 580 nm. The heterotrophic cells contained chlorophylls *a* and *b* (96 and 34 μg/mg dry weight, respectively) and carotenoids (2.2 μg/mg dry weight). Chlorophylls were constituents of chloroplasts, but were synthesized even under dark conditions when cells grew by metabolizing an organic carbon source.

3.3. Saccharide utilization

Utilization of organic carbon sources was investigated on MBM medium containing 0.5% of each sample in the dark. Only a few were found to support growth as the sole carbon source: glucose, sucrose, maltose, galactose and soluble starch. Cellobiose, sorbose, raffinose, xylose and lactose were unable to support growth under the experimental conditions used. Most algal strains showed higher growth rates under autotrophic conditions than under heterotrophic conditions. In contrast to these strains, heterotrophic growth rate of strain ANA9 was rather higher than the autotrophic rate. *Chlorella ellipsoidea* (Yamada and Shimaji, 1986) was used as a control and was unable to grow under heterotrophic or dark conditions.

3.4. Optimum pH and temperature for growth

The optimal temperature and pH for growth under heterotrophic conditions without light were found to be 35 °C and pH 6.0–7.0, respectively (Fig. 1). Isolated strain ANA9 did not exhibit substantial growth at pH > 8.0 or <4.0. The growth at 40 °C was suppressed, thus suggesting that this was not a thermophilic microorganism.

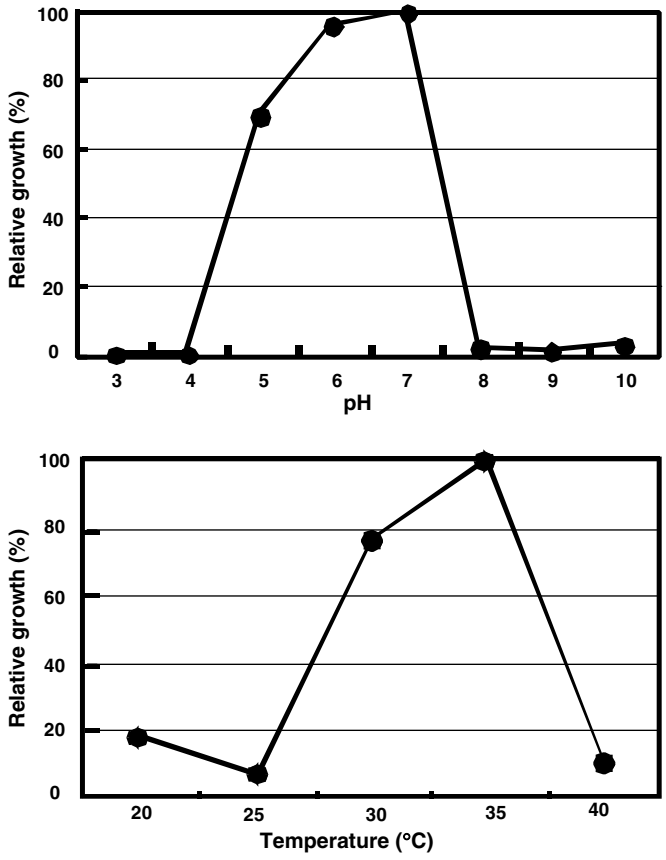

Fig. 1. Effects of pH and temperature on growth of isolated strain ANA99 in YPG broth.

3.5. Heterotrophic growth

The cells were cultured on YPG medium in which glucose concentration was adjusted 0–4% (w/v). As shown in Fig. 2, the optimum concentration of glucose for growth was 3%, where the cell yield was 50 g/l after culturing at 30 °C for 120 h. The growth rate and yield of a number of microalgae have been reported (Endo et al., 1974). Compared with these strains, isolated strain ANA9 can be recognized to have a much higher growth under heterotrophic conditions.

3.6. Amplification and sequencing of 18S rDNA

The small subunit ribosomal RNA gene of strain ANA9 was amplified from bulk genomic DNA by PCR. The isolated small subunit rRNA sequence was 1870 nucleotides long and showed similarities with other known sequences from green algae, ranging in homology from 98% with *Chlorella sorokiniana* (Huss et al., 1999) to 95% with *Chlorella saccharophila* (Krienitz et al., 1996). Strain ANA9 was thus identified as belonging to the genus *Chlorella*. Strain ANA9 was subsequently identified as belonging to the species *C. sorokiniana* and was therefore designated *C. sorokiniana* ANA9.

3.7. Metal resistance and minimum inhibitory concentrations

The MICs of 5 metal ions tested against strain ANA9 are shown. For the purpose of defining metal resistance, strains that were not inhibited by 1 mM of heavy metal ions were regarded as resistant (Nieto et al., 1989). The isolate was resistant to multiple metals. The isolated microorganism was particularly resistant to the five metal ions, the highest MIC was seen for Cd^{2+} (4 mM). Strain ANA9 was able to grow in YPG broth containing Cd^{2+} at concentrations of 3 mM. This strain had a higher resistance to Cu^{2+} and Al^{3+} (MICs of 8 mM and 7 mM, respectively) than to Cd^{2+}, Zn^{2+} and Ni^{2+} (MICs of 4 mM). In the present study, toxicity of metal ions was ranked as follows: $Cd^{2+} = Zn^{2+} = Ni^{2+} > Al^{3+} > Cu^{2+}$.

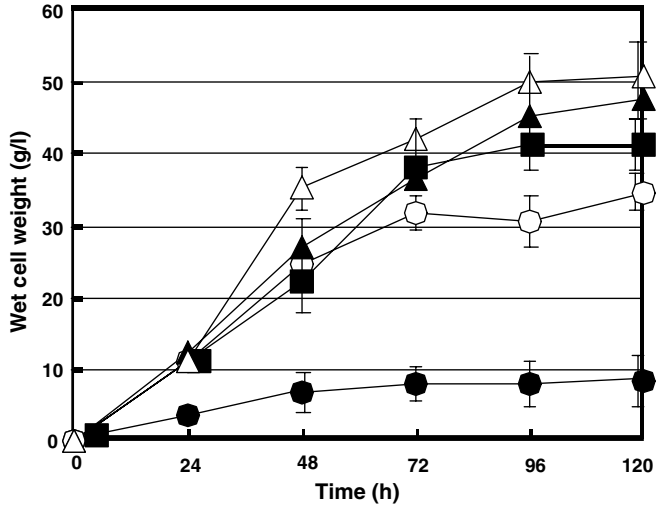

Fig. 2. Typical growth curve of isolated strain ANA9 in YPG broth at glucose concentrations of 0% (●), 1% (○), 2% (▲), 3% (△), and 4% (■). Mean ± SD from three independent experiments with three cultures for each determination is shown.

3.8. Uptake of heavy metals by the isolated microorganism

Fig. 3 shows the time course of heavy metal uptake by the isolated microorganism. The amount of Cd^{2+}, Zn^{2+} and Cu^{2+} taken up by the cells increased rapidly during the first 30 min after the application of cells, and then steadily increased with time. When the initial amount of Cd^{2+} was 5 mg, 55% was taken up from the Tris–HCl buffer after 24 h

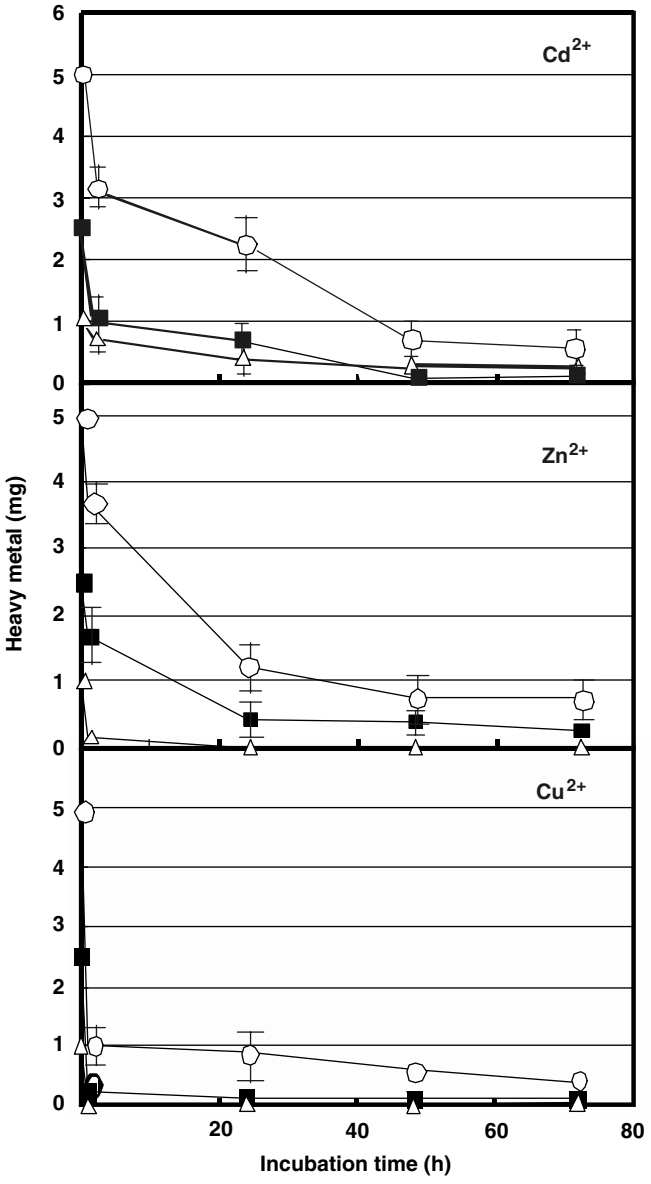

Fig. 3. Time course of Cd^{2+}, Zn^{2+}, or Cu^{2+} elimination in 100 ml of 10 mM Tris–HCl buffer (pH 7.0) by isolated strain ANA9 (dry weight; 100 mg). Initial amount of 5 mg (○), 2.5 mg (■), and 1 mg (△) of each heavy metal were added to the buffer. Values indicate the means ± SD of three independent experiment.

and 90% was taken up after 48 h. When the initial amount of Cd^{2+} was 2.5 and 1 mg, 90% of the Cd^{2+} was also taken up after 48 h. When the initial amount of Zn^{2+} was 5 mg, the reduction in Zn^{2+} concentration was 75% after 24 h and 85% after 48 h. The adsorption of Cu^{2+} by the isolated strain was first very rapid within the first 30 min, resulting in more than 80% being taken up. The maximum amounts of Cd^{2+}, Zn^{2+}, and Cu^{2+} taken up by the cells were 43.0, 42.0, and 46.4 μg, respectively, per milligram of dry weight.

The effects of the presence of co-ions in the solution on the heavy metal uptake capacity of the isolated organism were also examined. As seen in Fig. 4, Zn^{2+} had no effect on Cd^{2+} uptake, despite the 2.5 mg co-ion concentration. The Cd^{2+} and Zn^{2+} uptake capacity of the isolated strain was progressively and negatively affected by Cu^{2+}, indicating that the isolated strain had a higher affinity for Cu^{2+} than for Cd^{2+} and Zn^{2+}. After 48 h of incubation, 50% and 80% of the Zn^{2+} and Cu^{2+}, respectively, was removed from the solution. After 48 h incubation, 60% and 80% of the removed Cd^{2+} and Cu^{2+}, respectively, was accumulated intracellularly or adsorbed on the cell surface. The isolate exhibited a higher selectivity for Cu^{2+} than for other the metal ions and the preference was as follows: $Cu^{2+} > Cd^{2+} > Zn^{2+}$.

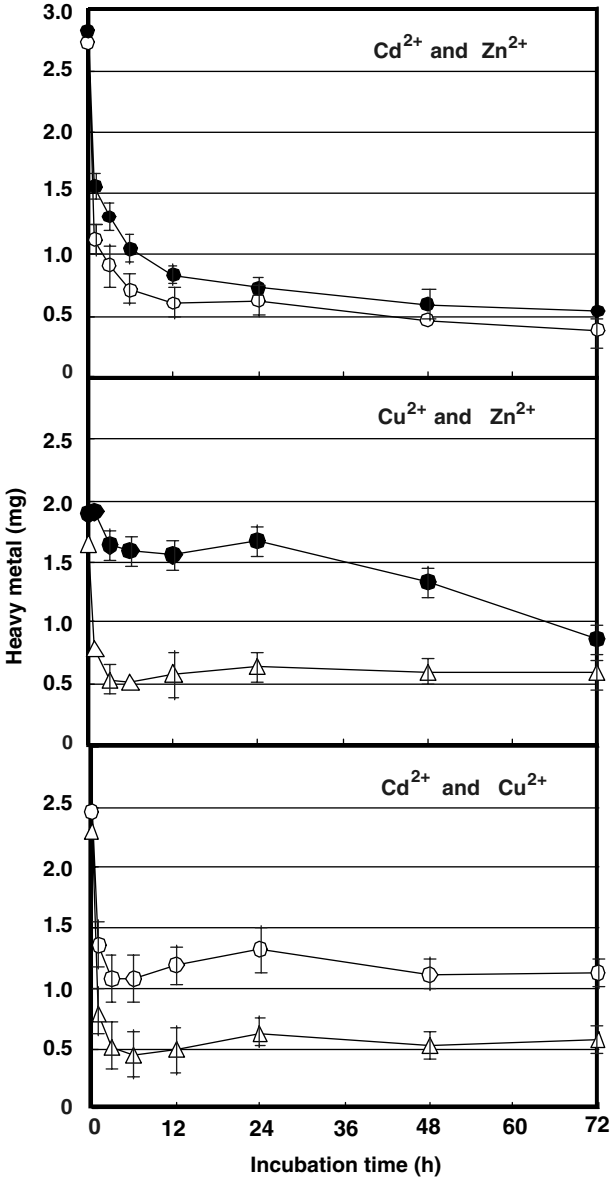

Fig. 4. Time course of co-elimination of heavy metals in 10 mM Tris–HCl buffer (pH 7.0) by isolated strain ANA9. Combinations of Cd^{2+} (○) and Zn^{2+} (●), Cu^{2+} (△) and Zn^{2+} (●), and Cd^{2+} (○) and Cu^{2+} (△) were added to the buffer. Initial amount of each heavy metal were 2.5 mg. Values indicate the means ± SD of three independent experiment.

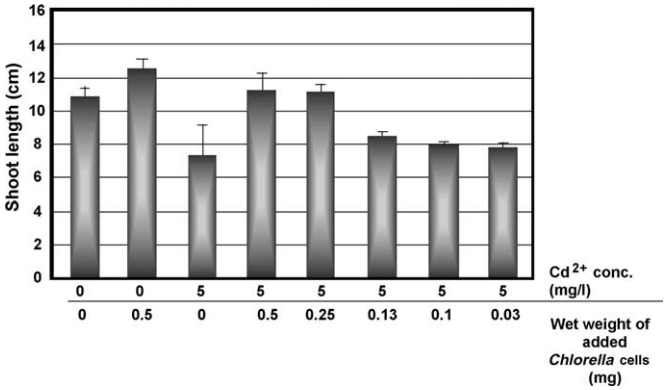

Fig. 5. Effects of Cd^{2+} on rice shoots grown under hydroponic conditions with or without isolated strain ANA9. Vertical bars represent SE ($n = 30$).

The distribution and binding states of Cd^{2+} in the isolated microorganism were examined by washing with 10 mM EDTA solution. Isolated microorganism cells were suspended in 10 mM EDTA solution (pH 5.0) by agitation at room temperature after incubation with Cd^{2+} for 48 h. After 5 min in the EDTA solution, the supernatant was collected by centrifugation at 2000*g* for 20 min. None of Cd^{2+} taken up in the living cells was released by washing with EDTA. This indicates that the cells incorporated Cd^{2+} intracellularly.

3.9. Potential for bioremediation

The effectiveness in reversing Cd^{2+}-inhibited plant growth was determined. At concentrations of 5 mg/l, Cd^{2+} inhibited hypocotyl and shoot growth of rice seedlings. The shoot growth of rice seedlings was restricted to 60% in the presence of Cd^{2+} when compared with control rice seedlings. The shoot growth of rice inhibited by Cd^{2+} increased with *Chlorella* cell concentration. As little as 0.25 g (wet weight) of *Chlorella* cells prevented Cd^{2+} toxicity. Smaller amounts, 0.03–0.1 g (wet weight), of *Chlorella* cells had no effect on the growth inhibition by Cd^{2+} (Fig. 5). These results suggest that the isolated *Chlorella* cells had the potential for bioremediation of rice husks by preventing Cd^{2+} accumulation near the plants.

4. Discussion

Cadmium was an important environmental pollutant and a potent toxicant to bacteria, algae and fungi. Mechanisms

of Cd^{2+} toxicity and resistance varied depending on the organism. However, the form of the metal and the environment it is studied in play an important role in how Cd^{2+} exerted its effects and how organisms respond. A wide range of Cd^{2+} concentrations have been used to designate resistance in organisms. To date, however, no particular concentration has been specified as applicable to all species under standardized conditions. Cadmium exerted its toxic effects over a wide range of concentrations. In most cases, algae and cyanobacteria were the most sensitive organisms, while bacteria and fungi appear to be more resistant. In some bacteria, plasmid-encoded resistance could lead to reduced Cd^{2+} uptake, although some Gram-negative bacteria without plasmids were just as resistant as bacteria containing plasmids encoding Cd^{2+} resistance.

Insufficient information is available on the genetics of Cd^{2+} uptake and resistance in cyanobacteria and algae, and mechanisms remain largely unknown. Cd^{2+} was toxic to these organisms, causing severe inhibition of physiological processes, such as growth, photosynthesis and nitrogen fixation, at concentrations of less than 2 ppm, and often in the ppb range (Perfus-Barbeoch et al., 2002). Cd^{2+} also caused pronounced morphological aberrations in these organisms, and these were probably related to deleterious effects on cell division. Such effects may be direct or indirect, perhaps as a result of Cd^{2+} effects on protein synthesis and cellular organelles such as mitochondria and chloroplasts. Cd^{2+} accumulated internally in algae (Perez-Rama et al., 2002) as a result of a two-phase uptake process. The first phase involved rapid physicochemical adsorption of Cd^{2+} onto cell wall binding sites, which were probably proteins and/or polysaccharides. This was followed by a lag period and a steady intracellular uptake. This latter phase was energy dependent and may involve the transport systems used to accumulate other divalent cations, such as Mn^{2+} and Ca^{2+}. Some data indicated that Cd^{2+} resistance and possibly uptake in algae and cyanobacteria were controlled by plasmid-encoded genes (Ren et al., 1998). Although considerable information is available on Cd^{2+}toxicity to and uptake in fungi, further work was clearly needed in several areas. There was little information regarding Cd^{2+} uptake by microalgae, filamentous fungi, or even in yeast, while information on specificity, kinetics, and mechanisms of Cd^{2+} uptake was limited.

Chlorella sp. is a unicellular green alga having worldwide distribution in all aquatic environments, soil (Koelewijn et al., 2001) and tree bark (Cho et al., 2002). It is also found as a symbiote in animals such as *Hydra viridis* (Habetha and Bosch, 2005). *Chlorella* only reproduces asexually, with the mature cell dividing mitotically to produce autospores. *Chlorella* is a very small alga with a single chloroplast, and it does not possess an eyespot or flagella. Algae of genus *Chlorella* can grow photosynthetically, and certain strains among these are known to grow in the dark by utilizing organic carbon sources (Rehman and Shakoori, 2001). It has been reported that the growth rate of algae was generally much slower than bacteria and yeast, and dark heterotrophic growth was even slower. However, if strains of algae could be found that possess higher growth rates under heterotrophic conditions, then production of algal proteins by industrial means may become more practical. *C. sorokiniana* ANA9, which had a much higher growth rate under heterotrophic conditions than other reported strains, was successfully isolated from soil. This isolated alga possessed a high growth rate (50 g/l) in batch culture.

The heavy metal binding potential of *Chlorella* sp. was first discovered in the mining industry, and metal-resistant algae have since been reported in a number of studies. Matsunaga et al. (1999) screened marine microalgae for efficient Cd^{2+} removal with the aim of onsite heavy metal removal from the marine environment. They reported a maximum uptake in *Chlorella* of 39.4 μg Cd^{2+}/mg dry cells, which was higher than that of any other previously characterized microalgae. The maximum amounts of Cd^{2+}, Zn^{2+}, and Cu^{2+} taken up by the present isolate were 43.0, 42.0, and 46.4 μg, respectively, per milligram (dry weight). Thus, the isolated *C. sorokiniana* ANA9 had the highest metal adsorption ever reported for algae.

Agricultural soils were primarily contaminated with Cd^{2+} due to the excessive use of phosphate fertilizers, dispersal of sewage sludge and atmospheric deposition. Cd^{2+} was readily taken up by numerous crops including cereals, potatoes, rice and fruits (Ingwersen and Streck, 2005). Consumption of rice grown in paddy soils contaminated with Cd^{2+}, Cr^{6+} or Zn^{2+} may pose a serious risk to human health, because 22–24% of the total metal content in rice biomass was concentrated in the rice grains (Wang et al., 2003). Thus, contamination by Cd^{2+} is increasing in both human food and overall in the agricultural environment. Plants readily take up Cd^{2+} from the soil. However, exposure to high levels of Cd^{2+} resulted in reduced rates of photosynthesis, chlorosis, growth inhibition, browning of root tips, decreased water and nutrient uptake, and ultimately death (Marcano et al., 2002). Phytoremediation technologies are becoming recognized as cost-effective methods for remediating sites contaminated with toxic metals at a fraction of the cost of conventional technologies, which include soil replacement, solidification and washing strategies. Phytoremediation is defined as the use of plants for environmental cleanup, and in terms of phytoremediation of heavy metals, is divided into three categories; (1) phytoextraction, in which metal-accumulating plants are used to transport and concentrate metals from the soil in harvestable parts of roots and above-ground shoots; (2) rhizofiltration, in which plant roots absorb, precipitate and concentrate toxic metals from polluted effluents; and (3) phytostabilization, in which heavy-metal-tolerant plants are used to reduce the mobility of heavy metals, thereby reducing the risk of further environmental degradation by leaching into ground water or by airborne spread. Here, we identified the potential for metal-accumulating *Chlorella* to reduce the mobility of heavy metals in soil. As shown in Fig. 5, our results indicated that the isolated *C. sorokiniana* ANA9 may be useful in preventing Cd^{2+} diffusion in the soil environment.

Further investigation of the effects of other factors on adsorption, such as ions in water and pH, will contribute to further optimizing the adsorption efficiency of this *Chlorella* strain.

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